RNA binding and R-loop formation by the herpes simplex virus type-1 single-stranded DNA-binding protein (ICP8)

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ABSTRACT

In an effort to decipher the molecular mechanisms of homologous recombination during herpes simplex virus type-1 replication, we recently demonstrated that the virus-encoded single-stranded (ss) DNA-binding protein (ICP8) promotes the salt-dependent assimilation of ssDNA into a homologous plasmid, resulting in the formation of a displacement loop. In this paper, the results presented show for the first time a direct interaction between ICP8 and RNA. ICP8 binds to RNA with positive cooperativity but with ~5-fold lower affinity than to ssDNA. In addition, competition experiments indicate that the dissociation rate of ICP8 from RNA is faster than from ssDNA, although it is also dependent on the nature of the challenger. Importantly, ICP8 can promote the salt-dependent assimilation of RNA into a homologous acceptor plasmid to generate a joint molecule in which the RNA is stably paired with the complementary strand of the acceptor DNA, indicative of an R-loop. These findings have important implications on the role of ICP8 in mediating recombination reactions using viral transcripts. The RNA-binding activity of ICP8 also provides a molecular basis for its role in the regulation of viral gene expression.

INTRODUCTION

The ~152 kb linear genome of herpes simplex virus type-1 (HSV-1) is known to undergo high frequency recombination [reviewed in (1)]. Recombination of the viral genome may serve several important functions during the viral life cycle including the following.

- (i) As a mechanism to replicate the 3' ends of the linear genome similar to that in bacteriophage T4 [reviewed in (2)]. This would generate highly branched replication intermediates that are prevalent during viral replication (3,4).
- (ii) To repair DNA breaks that may arise at sites of oxidative DNA damage that is induced during viral replication (5,6).

- (iii) During isomerization of the viral genome at viral *a* sequences (7,8).
- (iv) During circularization of the genome as a preface to entering the latent state (9,10).

To better understand the molecular mechanisms that underlie homologous recombination in HSV-1, we previously examined the ability of the virus-encoded single-stranded (ss) DNA-binding protein (SSB), ICP8, to promote recombination reactions. In cooperation with the viral replicative helicase, ICP8 was found to promote strand exchange (11). The annealing activity of ICP8 in conjunction with exonucleolytic processing, either by the viral UL12 nuclease or by other exonucleases, was also seen to form joint molecules [(12,13); A.V Nimonkar and P.E. Boehmer, unpublished data]. More recently, ICP8 was shown to promote the salt-dependent assimilation of an ssDNA donor into a homologous plasmid, resulting in the formation of a displacement loop (D-loop) (14). This process occurs by means of a single-strand annealing mechanism, distinct to strand invasion promoted by the RecA-type recombinases (15). We also showed that D-loops formed by the action of ICP8 are utilized by the viral replisome for long-chain DNA synthesis (16,17). This led us to propose a model for recombination-dependent DNA replication in HSV-1 that may be active during the replication of genomic termini and the repair of DNA breaks (16).

This paper examines the possibility that ICP8 interacts with RNA and promotes recombination reactions with RNA as donor. This would provide a molecular basis for the role of ICP8 in the regulation of viral gene expression (18). It would also support the notion that viral transcripts may be used to initiate replication by R-loop formation, both as an alternative strategy to initiate replication and as a DNA repair mechanism. In this scenario, assimilation of a transcript into the viral genome provides a platform for the assembly of a replisome in which the invading RNA primes leading strand synthesis, similar to what occurs during recombination-dependent initiation of replication in T4 or *oriC*-independent initiation in *Escherichia coli*, and during recombination-dependent repair of DNA breaks (19–22). Accordingly, the present data show that ICP8 can indeed bind to RNA and promote the formation of R-loops with homologous acceptor plasmid.

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MATERIALS AND METHODS

Enzymes and reagents

ICP8 was purified as described previously (23). Its concentration, expressed in moles of monomeric protein, was determined using an extinction coefficient of $82\,720\text{ M}^{-1}\text{ cm}^{-1}$ at 280 nm, calculated from its predicted amino acid sequence (24,25). *E. coli* SSB (E-SSB) and T4 polynucleotide kinase were purchased from USB Corporation. E-SSB concentrations are in moles of tetramer. PCR was performed using the iTaq DNA polymerase and reagents from Bio-Rad Laboratories. The T7 RNA polymerase and recombinant RNasin ribonuclease inhibitor were purchased from Promega Corporation. *E. coli* RNase H and RNase I were obtained from Epicentre Technologies and New England Biolabs, respectively. Proteinase K and ATP (disodium salt) were purchased from Roche Molecular Biochemicals and Sigma, respectively. CTP, GTP and UTP (diphosphate salts) were from Amersham Biosciences. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4500 Ci/mmol) and $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (800 Ci/mmol) were purchased from MP Biomedicals.

Nucleic acids

M13 mp18 ssDNA was purchased from New England Biolabs. pUC18 form I DNA was prepared from *E. coli* JM109 with the Promega Wizard *Plus* DNA purification system followed by ethanol precipitation. Plasmid DNA concentrations are expressed in moles of molecules. Oligodeoxyribonucleotide PB11 (100mer), complementary to residues 379–478 of the minus strand of pUC18, was as described previously (26). PB11 was 5'- ^{32}P -labeled with T4 polynucleotide kinase and purified using Sephadex G-25 (fine) Quick spin columns (Roche Molecular Biochemicals). Its concentration is expressed in moles of molecules. Oligodeoxyribonucleotides PB86 (20mer, 5' GAAACAGCTATGACCATGAT) and PB190 (40mer, 5' AATTTAATACGACTCACTATAGG-TAAAACGACGGCCAGTG) were synthesized by QIAGEN. The underlined sequence of PB190 corresponds to the T7 RNA polymerase promoter, while the position in italics corresponds to the transcription start site. PB86 and PB190 (0.5 μM molecules) were used as primers to amplify a 122 bp fragment in a 50 μl reaction containing 10 ng of M13 mp18 template, 1.25 U iTaq, 1.5 mM MgCl_2 and 200 μM dNTP, using the following parameters: 20 cycles of 30 s at 95°C, 30 s at 56°C and 30 s at 72°C. The 122 bp PCR product was used as template in an *in vitro* run-off transcription reaction to generate a ^{32}P -labeled 101 nt RNA that is complementary (with the exception of the 5' terminal guanine residue) to residues 379–478 of the minus strand of pUC18. The *in vitro* transcription reaction (30 μl) contained 9 μl PCR product, 38 U T7 RNA polymerase, 500 μM ATP, CTP, GTP, UTP, 100 μCi $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (800 Ci/mmol) and 40 U RNasin. After 2 h of incubation at 37°C, the products were purified by chromatography using Sephadex G-25 (fine) Quick spin columns (Roche Molecular Biochemicals). Alternatively, transcription products obtained with unlabeled NTP were treated successively for 15 min at 37°C with 1 U each of shrimp alkaline phosphatase (USB Corporation) and RQ1 RNase-free DNase (Promega Corporation). RNA was purified by extraction with phenol:chloroform:isoamyl alcohol (99:24:1, pH 4.7) and ethanol precipitation. The purified RNA was then

5'- ^{32}P -labeled with T4 polynucleotide kinase and purified using Sephadex G-25 (fine) Quick spin columns (Roche Molecular Biochemicals). The purity of the *in vitro* transcribed RNA was analyzed by denaturing PAGE followed by storage phosphor analysis using a Molecular Dynamics Storm 820. Of the product, $\geq 90\%$ migrated as a 101mer (data not shown). The concentration of the RNA was determined spectroscopically at 260 nm (1 absorbance unit equals 30 $\mu\text{g}/\text{ml}$) and is expressed in moles of molecules.

Nucleoprotein filament and R-loop formation

To form nucleoprotein (NP) filaments, ICP8 or E-SSB were incubated with 101mer RNA or PB11 at concentrations indicated in the figure legends for 8 min on ice in 25 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 1 mM DTT, 100 $\mu\text{g}/\text{ml}$ BSA and 4 U of RNasin. The reactions were supplemented with loading buffer (final concentration: 40 mM Tris-acetate pH 7.6, 1 mM EDTA, 10% glycerol, 0.1% bromophenol blue and 0.1% xylene cyanol) and immediately subjected to electrophoresis through 0.75% agarose in 40 mM Tris-acetate pH 7.6, 1 mM EDTA for 3 h at 4.5 V/cm and 4°C. For R-loop and D-loop formation, NP filament reactions were supplemented with pUC18 form I DNA (3 nM) and incubation was continued for 30 min at 30°C. The reactions were quenched by the addition of termination buffer (final concentration: 25 mM EDTA, 1.7% SDS, 10% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol and 0.7 mg/ml proteinase K) followed by incubation for 15 min at 30°C. Reaction products were resolved by agarose gel electrophoresis as stated above except that electrophoresis was at room temperature. Following electrophoresis, the gels were dried onto DE81 chromatography paper (Whatman), analyzed and quantitated by storage phosphor analysis with a Molecular Dynamics Storm 820. NP filament formation was calculated as a percentage of the bound probe over total radioactivity. The apparent loss of signal upon incubation with ICP8 is an artifact due to the spread of the label between multiple complexes instead of a single unbound species. R-loop formation was calculated as a percentage of the donor RNA assimilated in the acceptor plasmid and expressed with respect to the concentration of plasmid after subtracting the protein-independent reaction from that catalyzed by ICP8. In the figures, product formation is reported as a fraction of the maximum signal obtained in a particular experiment.

RESULTS

RNA binding and R-loop formation by ICP8

We had previously demonstrated the ability of ICP8 to promote assimilation of an ssDNA 100mer into a homologous form I acceptor plasmid (D-loop formation) (14). In that reaction, ICP8 acts by a salt-dependent strand annealing mechanism. The active species in this reaction appears to be an ssDNA:ICP8 NP filament (15). To test whether ICP8 can also bind to RNA, its interaction with an *in vitro*-transcribed ^{32}P -labeled 101mer RNA was examined. Figure 1A shows the result of a gel-mobility shift assay in which increasing concentrations of ICP8 were incubated with the RNA probe. The data show formation of ICP8:RNA complexes with increasing

Figure 1. RNA binding and R-loop formation by ICP8. Reactions were performed as described in Materials and Methods with 25 nM uniformly ^{32}P -labeled RNA and increasing concentrations of ICP8 as indicated. (A and B) storage phosphor images showing NP filament and R-loop formation, respectively. Lanes 1–10, reactions with 0, 15.625, 31.25, 62.5, 125, 250, 500, 1000, 2000 and 4000 nM ICP8 respectively. (C) Quantitation of NP filament (closed circles) and R-loop (open circles) formation. Values are expressed as a fraction of maximal activity (75% RNA bound at 4 μM ICP8 and 6.6% R-loops at 1 μM ICP8). The positions of ICP8-RNA complex, R-loops and ssRNA are as indicated.

concentrations of ICP8. This observation is consistent with the formation of an NP filament in which ICP8 coats the RNA, as is the case for ICP8 binding to ssDNA (15,27,28).

To examine whether ICP8 can also promote assimilation of the RNA into a homologous form I acceptor plasmid, leading to the formation of an R-loop, one half of the above-described binding reactions were supplemented with pUC18 form I DNA. Figure 1B shows that ICP8 could indeed promote the pairing of the donor RNA with homologous plasmid DNA (i.e. R-loop formation). The correlation between RNA binding and R-loop formation is shown in Figure 1C. The data show that R-loop formation was linear at ICP8 concentrations up to 250 nM, with a maximum at 1 μM protein at which $\sim 7\%$ of the form I acceptor DNA participated in R-loop formation (Figure 1C). The peak of R-loop formation corresponds to a concentration of ICP8 at which only $\sim 75\%$ of maximum RNA binding (NP filaments) was observed (Figure 1C). Moreover,

half-maximal R-loop formation occurred at 200 nM ICP8 at which $<5\%$ RNA binding (NP filaments) was observed (Figure 1C). These findings are in contrast to what we had earlier observed for D-loop formation where NP filament and D-loop formation were coincident, leading us to conclude that the active species during D-loop formation was an NP filament (15). At concentrations $>1 \mu\text{M}$ ICP8, there was a decrease in R-loop formation (Figure 1C). This is similar to the decrease in D-loop formation observed with supersaturating concentrations of ICP8 at which the helix-destabilizing activity of ICP8 'unwinds' the D-loops (15). However, ICP8, even at supersaturating concentrations (up to 3 μM), did not exhibit any helix-destabilizing activity with purified R-loops (data not shown).

To compare the binding properties of ICP8 to RNA and ssDNA of comparable length and sequence, ICP8 was titrated with fixed concentrations of either 5'- ^{32}P -labeled 101mer

Figure 2. Comparison of the RNA and ssDNA binding activities of ICP8. 5'-³²P-labeled RNA or PB11 (10 nM) were incubated with increasing concentrations of ICP8 as indicated. (A) Storage phosphor image showing NP filament formation. Lanes 1–10 and 11–20, reactions with 0, 15.625, 31.25, 62.5, 125, 250, 500, 1000, 2000 and 4000 nM ICP8 and PB11 or RNA, respectively. (B) Quantitation of RNA (closed circles) and ssDNA (open circles) binding. Values are expressed as a fraction of maximal activity (82% RNA and 99% PB11 bound at 4 μ M ICP8). The positions of ssRNA or PB11 (free), NP filaments (bound) and intermediates are as indicated.

RNA or 100mer PB11 ssDNA and NP filament formation analyzed by a gel-mobility shift assay (Figure 2A). Previous studies have established that ICP8 binds to ssDNA with moderate affinity and cooperativity (15,27–30). Consistent with these reports, the current data show that binding of ICP8 to an ssDNA polymer with multiple binding sites (i.e. 100mer) led to the formation of intermediates at subsaturating protein concentrations and distinct complexes (depending on the degree of occupancy) at coating concentrations of ICP8 (Figure 2A, lanes 1–10). Similar to the data in Figure 1A, binding of ICP8 to the 101mer RNA apparently required higher protein concentrations and also led to the formation of NP filaments with an electrophoretic mobility comparable to those formed between ICP8 and the ssDNA 100mer, without significant intermediates (Figure 2A, lanes 11–20). Quantitation of ICP8 binding to the 101mer RNA shows a sigmoidal binding isotherm, indicative of positive cooperativity (Figures 1C and 2B). The concentration of ICP8 required for half-maximal RNA binding (apparent K_d) was $\sim$ 500 nM. Similar values were obtained in experiments performed at

RNA concentrations of 50 and 125 nM (data not shown). This K_d is $\sim$ 5-fold higher than that for binding the ssDNA 100mer ($\sim$ 100 nM) (Figure 2B).

Characterization of R-loop formation by ICP8

To further characterize the formation of R-loops by ICP8, the requirement for $MgCl_2$ was examined. Figure 3 shows that R-loop formation by ICP8 was $MgCl_2$ -dependent, with a maximum at 15 mM. Higher concentrations of $MgCl_2$ (>15 mM) inhibited R-loop formation, probably due to stabilization of duplex DNA, thereby essentially preventing intermolecular pairing. The salt dependency of R-loop formation is identical to what we had observed previously for D-loop formation (15). Figure 4 shows a linear increase in R-loop formation with time of incubation until the maximum extent of product formation was reached, further supporting that this is an ICP8-mediated reaction.

The assimilation of the RNA donor into an acceptor plasmid by ICP8 was further characterized as shown in Figure 5. The

Figure 3. R-loop formation as a function of MgCl_2 concentration. R-loop reactions were performed as described in Materials and Methods with 45 nM uniformly ^{32}P -labeled RNA, 1 μM ICP8 and increasing concentrations of MgCl_2 as indicated. Values are expressed as a fraction of maximal activity (9% R-loops at 15 mM MgCl_2).

Figure 4. Time course of R-loop formation. R-loop reactions were performed as described in Materials and Methods with 45 nM uniformly ^{32}P -labeled RNA, 1 μM ICP8 and incubated for the times indicated. Values are expressed as a fraction of maximal activity (5% R-loops at 45 min).

data show that R-loop formation was dependent on the presence of the homologous form I acceptor plasmid since no products were formed in the absence of plasmid or with heterologous pUC19 DNA (Figure 5A, lanes 1–3). To examine whether ICP8 promotes complete and stable assimilation of the RNA donor into the acceptor plasmid, R-loop reactions were treated with RNase I, an ssRNA nuclease (31). The data show that R-loops were resistant to RNase I treatment, indicating that the RNA is fully assimilated in the plasmid and protected from nucleolytic degradation (Figure 5A, compare lane 2 to lanes 4 and 5). As a control, Figure 5B shows that 1 U of RNase I was sufficient to completely degrade the input RNA (compare lanes 1 and 6). Similar to the R-loops, the RNA within the NP filament was also resistant to RNase I treatment as compared to free RNA (Figure 5B, compare lanes 2 and 3 to lane 6). Assimilation of the RNA donor into the homologous

plasmid should create a double-stranded RNA:DNA hybrid that is a substrate for RNase H. Figure 5A shows that R-loops formed by the action of ICP8 were sensitive to RNase H (compare lane 2 to lanes 6 and 7). This finding indicates that, as predicted for the structure of an R-loop, the RNA donor is engaged in Watson–Crick hydrogen bonding with the complementary strand of the acceptor plasmid and that the non-complementary strand is excluded and does not adopt a triple helix. This finding is similar to what we had previously observed for ICP8-mediated D-loop formation, in which the donor DNA also forms Watson–Crick base pairs with the complementary strand of the acceptor plasmid (14). As expected, neither the NP filament nor the free RNA was sensitive to RNase H (Figure 5B, compare lane 1 to lanes 4, 5 and 7).

Stability of the ICP8:RNA complex

The data in Figure 2 show that ICP8 has a ~ 5 -fold lower affinity (apparent K_d) for RNA than for ssDNA. To determine if this is due to differences in dissociation rate, competition experiments were performed. Figure 6A shows the results of an experiment in which complexes of ICP8 with either $5'$ - ^{32}P -labeled 100mer PB11 ssDNA or 101mer RNA were challenged with excess concentrations of unlabeled ssDNA or RNA probes. If the dissociation rates of ICP8 from ssDNA and RNA were similar, comparable levels of competition should be observed. The data show that ssDNA was a much more potent challenger for the ICP8:RNA complex than for the ICP8:ssDNA complex. Specifically, a 50-fold molar excess of the challenger caused only marginal disruption of the ICP8:ssDNA complex while it resulted in complete disruption of the ICP8:RNA complex (Figure 6A, compare lanes 2 and 5 and lanes 10 and 13). This finding suggests that the dissociation rate of ICP8 from RNA is significantly faster than from DNA. If this were the case, similar levels of competition should also be observed with RNA challenger. However, when the ssDNA or RNA NP filaments were challenged with up to a 50-fold molar excess of RNA competitor, there was no effect on the stability of the ICP8:ssDNA complexes, and only a marginal disruption of those formed with RNA (Figure 6A, compare lanes 2 and 8 and lanes 10 and 16). This observation indicates that the nature of the challenger, i.e. ssDNA versus RNA, affects the stability of the complexes.

To further examine the effect of the ligand on the dissociation of the ICP8:RNA complex, a time course of competition with either the 100mer ssDNA or the 101mer RNA was performed. The data show that a 50-fold molar excess of the ssDNA competitor resulted in complete destabilization of the NP filaments within 2 min of challenge (Figure 6B, compare lanes 2 and 3). In contrast, the ICP8:RNA complex was affected only marginally, even after 60 min, by the same excess of RNA competitor (Figure 6B, compare lane 2 to lanes 6–8 and 10). These results show that the half-life of the ICP8:RNA complex under these conditions is much shorter when challenged with ssDNA (< 2 min) than when challenged with RNA (> 60 min).

Collectively, these data suggest that the dissociation rate of ICP8 from RNA may be faster than from ssDNA. However, this conclusion is complicated by the observation that the challenger appears to be an active component in the

Figure 5. Characterization of R-loops and NP filaments. R-loop reactions were performed as described in Materials and Methods with 45 nM uniformly ^{32}P -labeled RNA and 1 μM ICP8 except that incubation was continued for an additional 30 min at 30°C with the indicated components. (A) Storage phosphor image of R-loop reactions. Lane 1, reaction in the absence of acceptor DNA. Lanes 2 and 3, reactions with homologous (pUC18, complete reaction) and heterologous (pUC19) acceptor DNA, respectively. Lanes 4 and 5, complete reactions with 0.1 and 1 U of RNase I, respectively. Lanes 6 and 7, complete reactions with 0.1 and 1 U of RNase H, respectively. (B) Storage phosphor image of NP filaments. Lane 1, complete reaction. Lanes 2 and 3, complete reactions with 0.1 and 1 U of RNase I, respectively. Lanes 4 and 5, complete reactions with 0.1 and 1 U of RNase H, respectively. Lanes 6 and 7, incubation of free RNA with 1 U of RNase I and RNase H, respectively. The positions of R-loops, ssRNA and degraded RNA are as indicated.

dissociation of the complexes. This may be due to the fact that ICP8 interacts cooperatively with nucleic acid ligands with multiple binding sites. In this regard, ssDNA was much more potent than RNA, possibly due to the fact that ICP8 binds to it with higher affinity.

Specificity of RNA binding and R-loop formation

The ability of SSBs to bind RNA has been investigated in several cases including E-SSB and in the case of T4 (gp32) (32,33). Figure 7A compares the ssDNA and RNA binding activities of ICP8 to an equivalent concentration of E-SSB in terms of DNA binding site size equivalents based on site sizes of 10 ± 1 nt per ICP8 monomer and 40 nt per E-SSB tetramer (30,34). Under these stoichiometries, 100% of the ssDNA 100mer was bound by both SSBs (Figure 7A, lanes 1 and 3). In addition, both SSBs also bound the 101mer RNA, although not all of the probes were retained (Figure 7A, lanes 2 and 4).

Importantly, while ICP8 could promote the formation of both D-loops and R-loops, neither product was formed with E-SSB under these conditions of ionic strength and Mg^{2+} (Figure 7B, compare lanes 1 and 3 to lanes 2 and 4). This finding indicates that the ability to bind ss nucleic acid alone is not sufficient for its assimilation into a homologous plasmid. This property requires inherent pairing activity which, in this case, is true of ICP8 but not of E-SSB.

DISCUSSION

As described in the introduction, the HSV-1 genome undergoes high-frequency recombination during its replicative cycle that may serve several important purposes including replication of genomic termini, repair of DNA breaks, genome circularization and isomerization of the U_L and U_S segments with respect to each other. From a mechanistic perspective, these recombination reactions appear to be dependent on the virus-encoded DNA replication factors (35) and occur in the absence of a viral RecA-type recombinase (1). Our previous studies have established that ICP8 is capable of promoting recombination reactions (both strand exchange and D-loop formation) by means of a strand annealing mechanism (11,14,15), suggesting that it is the primary virus-encoded factor that is active in recombination reactions.

In this study, the ability of ICP8 to bind RNA and to utilize it as a donor in recombination reactions was examined. The rationale for this was several fold. (i) Polyadenylic acid had previously been found to act as a competitor for the ssDNA binding activity of ICP8 (27). (ii) The observed role of ICP8 in late viral gene expression (18) may be mediated through a direct interaction with transcripts. (iii) It is possible that viral transcripts are used in recombinational repair of double-strand DNA breaks. (iv) Finally, it is possible that transcripts are used to initiate recombination-dependent replication. This may occur at later times during the viral life cycle but may also represent an alternative strategy to initiate replication independent of the mode that involves UL9 -dependent recognition of the origins of replication. Such a strategy would explain the residual replication that is observed in UL9 null mutants (36). One transcript that may function in this capacity is oriSRNA_1 , a short (~ 165 nt) RNA that initiates within the ICP22/47 genes and terminates ~ 285 bases from the middle of the oriS palindrome at the ICP22/47 transcription initiation sites (37). This transcript is expressed with the same kinetics as the viral immediate-early genes and exists in the absence of viral protein synthesis (37). oriSRNA_1 is also expressed in the highly homologous HSV-2 and lacks an open reading frame. Consequently, this RNA may be involved in R-loop formation in the vicinity of the origin to initiate DNA replication.

The findings presented here address some of the above scenarios. Thus, ICP8 was seen to bind RNA with apparent positive cooperativity albeit with lower affinity than it exhibits for ssDNA as indicated by a ~ 5 -fold higher K_d . The weaker interaction of ICP8 with RNA compared to ssDNA was confirmed by the results of competition experiments which showed that the ICP8:RNA NP filament was more susceptible to challenge with ssDNA competitor than the ICP8:ssDNA NP filaments, indicative of a faster dissociation rate. Interestingly, the dissociation rate was affected by the

Figure 6. Stability of the RNA:ICP8 NP filament. (A) NP filaments formed with 5'-³²P-labeled PB11 or RNA (10 nM) and 500 nM and 2 μM ICP8, respectively, were challenged with 100, 250 or 500 nM unlabeled polymer (100mer ssDNA or 101mer RNA) for 5 min at 30°C as indicated, followed by immediate electrophoresis. (B) ICP8 (2 μM) was incubated with 10 nM 5'-³²P-labeled RNA as described in Materials and Methods and challenged with 500 nM unlabeled 100mer ssDNA or 101mer RNA at 30°C for the times indicated, followed by immediate electrophoresis, giving rise to the staggered pattern of the bands. The positions of ssRNA or PB11 (free) and NP filaments (bound) are as indicated.

nature of the competitor. Thus, ssDNA was more effective than RNA, possibly because ICP8 binds to it with higher affinity and cooperativity. These findings are consistent with the earlier observation that polyadenylic acid only weakly competed with ssDNA for binding to ICP8 (27). Further quantitative equilibrium binding studies along the lines previously performed on the interaction of ICP8 with ssDNA (30), are required to characterize the affinity, cooperativity and RNA-binding site size. Regarding the role of ICP8 in the regulation of the late viral gene expression, it has been postulated that this is due to its interactions with RNA polymerase II and the virus-encoded immediate-early protein ICP27, which is implicated in mRNA stability, transport, processing and splicing (18).

However, based on the current data, a direct interaction with viral transcripts may also be a contributing factor.

Importantly, ICP8 was found to promote assimilation of RNA into a homologous acceptor plasmid in a dose-, time- and Mg²⁺-dependent manner. The resistance of the assimilated RNA to the nucleolytic action of RNase I and its sensitivity to RNase H indicate that the RNA is stably hydrogen-bonded to its complementary region in the acceptor DNA, while the non-complementary strand of DNA is displaced, consistent with the formation of an R-loop. The efficiency of this reaction (up to 10% of the acceptor plasmid participated in R-loop formation) was comparable to what we previously observed for ICP8-mediated D-loop formation, and is presumably limited

Figure 7. Specificity of NP filament formation and strand assimilation. NP filament (A) and D-loop/R-loop reactions (B) were performed as described in Materials and Methods with 11 nM PB11 DNA and 33 nM uniformly 32 P-labeled RNA and either 1 μ M ICP8 or 375 nM E-SSB as indicated. The positions of donor PB11 or ssRNA (free), NP filaments (bound), D-loops and R-loops are as indicated.

by the availability of denatured homology in the acceptor, owing to the fact that it proceeds by a single-strand annealing mechanism (15). We had previously concluded that the active species in D-loop formation was an ICP8:ssDNA NP filament based on the observation that complex formation and the pairing reaction were coincident and that ICP8 was apparently not required to interact with the acceptor DNA (15). This scenario does not translate to the current findings where ICP8:RNA complex and R-loop formation did not coincide. In fact, R-loops formed at much lower concentrations of ICP8 than those required for efficient NP filament formation (i.e. $<K_d$), but comparable to those required for optimal D-loop formation (15). This finding challenges our earlier conclusion since efficient NP filament formation does not appear to be a prerequisite for pairing. Since the pairing reaction is limited by the availability of denatured acceptor DNA, and given the apparent cooperativity in DNA and RNA binding, it is possible that both D-loop and R-loop formation may occur when NP filament formation is equivalent to the number of available targets. This would predict that the pairing reaction is thermodynamically highly favorable and efficient. Further work is

required to investigate the exact mechanism by which ICP8 promotes R-loop (and D-loop) formation, in terms of its interaction with both donor and acceptor species. In this regard, it is noteworthy that ICP8 did not destroy preformed R-loops by virtue of its helix-destabilizing activity.

R-loop formation is a novel activity of ICP8 that has only recently been observed with the prototypical recombinase RecA (21,22). In the case of RecA, it occurs by an 'inverse' reaction in which the RNA adopts the role of the homologous acceptor, while the donor is a RecA-coated duplex NP filament (22). R-loop formation by RecA has been implicated in origin- and DnaA-independent initiation of replication as well as in recombinational repair of DNA breaks in *E.coli* (21,22). Similarly, it is well established that recombination-mediated formation of R-loops is a mechanism of replication initiation at later times of the T4 replicative cycle [reviewed in (19)]. As postulated above, the results presented herein indicate that ICP8 may act in a similar capacity. In this regard, preliminary data show that the viral replication proteins (ICP8, helicase-primase and DNA polymerase holoenzyme) could utilize RNA-primed M13 ssDNA as a template for long-chain DNA synthesis, indicating that RNA primers are efficiently recognized by the viral replisome (P. E. Boehmer, unpublished data).

In summary, the results presented in this paper show for the first time the interaction of ICP8 with RNA and its ability to assimilate RNA into a homologous plasmid acceptor. These activities may provide a molecular basis for the role of ICP8 in the regulation of viral gene expression and indicate that viral transcripts may be used in recombination reactions. RNA binding is not unique to ICP8 since other SSBs, including T4 gp32 and E-SSB also exhibit this property, albeit with lower binding affinity than to ssDNA (32,33). However, RNA-binding activity alone is not sufficient to account for R-loop formation, since this reaction is not promoted by E-SSB which lacks inherent pairing activity (38).

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