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Vaccinia virion protein VP8, the 25 kDa product of the L4R gene, binds single-stranded DNA and RNA with similar affinity

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ABSTRACT

Vaccinia virus protein VP8 is a 25 kDa product of the L4R gene and is an abundant virion protein that binds single-stranded (ss) and double-stranded (ds) DNA. Binding of ssDNA is preferred at high salt concentrations. Using a recombinant 25 kDa L4R (rL4R) protein and a gel mobility shift assay with radiolabelled oligonucleotides, the K_d for a 45mer oligonucleotide was determined to be 2 nM. The K_d was unaltered by 50 mM KCl but was reduced 35-fold by 100 mM KCl. Multiple rL4R molecules bound to a single 45mer oligonucleotide, and using oligonucleotides of different lengths it was calculated that one rL4R molecule bound every 17 nt. Binding to ssDNA was competed by both deoxyribo- and ribopolynucleotides. RNA binding was observed for both rL4R and native VP8, purified from virions, using a gel mobility shift with a radiolabelled ssRNA of 130 nt. The K_d of rL4R for this ssRNA substrate was 3 nM in the absence of salt and binding was positively cooperative. The potential roles of L4R protein in vaccinia virus early transcription are discussed.

INTRODUCTION

Vaccinia virus (VV) replication involves the co-ordinated expression of ~200 genes and the packaging of a 192 kb covalently closed dsDNA molecule into a brick-shaped virion with dimensions of 350 by 270 nm (for review see 1). Viral replication occurs in the cytoplasm of infected cells. Two consequences of this mode of replication are that VV encodes all the enzymes required for transcription of the viral genome [except for a host-derived intermediate transcription factor (2)] and that the entire early transcriptional apparatus is packaged into virus particles. Virions are complex structures containing at least 100 different proteins arranged into a core particle surrounded by two (intracellular mature virus) or three (extracellular enveloped virus) lipid membranes (3–5). The major structural proteins are 4a (encoded by gene A10L), 4b (gene A3L), VP11b (gene F17R) and VP8 (gene L4R) which together constitute ~70% of the mass of the core particle. Enzymes packaged into virions include an eight subunit DNA-dependent RNA polymerase (6,7), a heterodimeric early transcription factor (8), a heterodimeric capping enzyme/termination factor (9), a heterodimeric polyA polymerase (10), a DNA helicase (gene A18R) (11) and a helicase with both DNA and RNA helicase activity (gene I8R) (12,13). Core particles, virions from which the membranes have been removed, are fully competent for specific transcription of early viral genes and produce translatable mRNA molecules (14).

VP8 is encoded by VV gene, L4R (15,16). It is synthesised as a 28 kDa protein at late times during infection and is proteolytically cleaved during virion assembly to a 25 kDa protein with an alanine at the N-terminus (16). Cleavage occurs at an Ala–Gly–Ala site (16) and is possibly performed by the putative proteinase encoded by VV gene G1L (17). A 25 kDa virion protein, which may have been VP8, was found to bind DNA but not RNA by adsorption of nucleic acid to proteins immobilised on nitrocellulose (18). Subsequently, VP8 (henceforth referred to as the L4R protein) was purified from vaccinia virions and was demonstrated to bind both single-stranded (ss) and double-stranded (ds) DNA (19). Binding to ssDNA was unaffected by the presence of 100 mM NaCl whereas binding to dsDNA was greatly reduced. It was estimated from sedimentation of radiolabelled DNA and protein through density gradients that 10.2 nt of ssDNA were bound per monomer of L4R protein. It was suggested that the L4R protein could maintain a large proportion (30%) of the viral genome as ssDNA.

The function of the L4R protein in VV replication has also been characterised genetically. A mutant VV was constructed in which expression of the L4R gene was controlled by an IPTG-inducible late VV promoter (20). Repression of L4R synthesis inhibited production of plaques but did not prevent synthesis of morphologically normal, but non-infectious, viral particles (20). These virions bound to cells normally and contained wild-type amounts of most viral proteins (including the proteins known to be involved in early virus transcription) except for the 25 kDa L4R protein (21). Despite this, virions lacking L4R protein were defective for transcription of early viral genes in both *in vitro* transcription reactions using intact viral cores and after infection of cells (21). It was hypothesised that the DNA binding activity of the L4R protein is required for early transcription from virions. Proposed functions of the L4R protein included a role in packaging and maintaining the VV DNA genome in a

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transcribable configuration, binding of ssDNA during transcription initiation and cooperation with the I8R protein to unwind early promoter regions (21). This latter idea was consistent with the observation that binding of ssDNA by the L4R protein stimulated the DNA helicase activity of the I8R protein (22), another vaccinia virion protein whose mutation results in production of non-infectious virions defective in early virion-derived transcription (23). The demonstration in this report that the L4R protein also binds RNA suggests a further function of the L4R protein in either transcription elongation or release of mRNA molecules from viral particles.

MATERIALS AND METHODS

Materials

Purified recombinant 25 kDa L4R protein (rL4R) and fractions containing native L4R protein were identical to those described previously (22). The following oligonucleotides were used: 68mer 5′-GTTATTGCATGAAAGCCCGGCTGACTCTAGAGGATCCCCGGGTACGTTATTGCATGAAAGCCCGGCTG, 51mer 5′-CCCGAATTCTTAGAGAGCGAGGCTCTTCTTCGGTAGCAATTTATGGAACTT, 48mer 5′-TAGTAGGGATCCGATGACGATGACAAAATGGATGGACTTCCAATGGGA, 45mer 5′-TGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCG, 35mer 5′-TGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAG, 33mer 5′-GGACGAAAAAGTAAGGTTATATTACAAAGTGAA, 29mer 5′-TGCAGGTCGACTCTAGAGGATCCCCGGGT, 25mer 5′-TGCAGGTCGACTCTAGAGGATCCCC, 20mer 5′-CAGCCGGGCTTTCATGCAAT. The 45, 35, 29 and 25mer oligonucleotides had identical sequences at their 5′ termini whilst the other oligonucleotides differed in sequence. Homopolymers were obtained from Sigma (St. Louis, MA, USA), T7 RNA polymerase from Promega (Heidelberg, Germany) and T4 polynucleotide kinase (PNK) from New England Biolabs (Beverley, MA, USA).

Preparation of radiolabelled nucleic acids

Oligonucleotides were labelled in reactions containing [γ-^{32}P]ATP and T4 PNK. Labelling reactions were extracted with an equal volume of phenol, chloroform (1:1, pH 8) and oligonucleotides were separated from unincorporated nucleotides by centrifugation through G50 sephadex columns at 1000 r.p.m. in a Beckman GPR centrifuge. The percentage incorporation of [γ-^{32}P]ATP and recovery of radiolabelled oligonucleotides were assessed using DE81 filters and liquid scintillation counting (24).

The ssRNA was prepared from plasmid pEC10B10 which contained a clone of segment 10 of bluetongue virus. This plasmid was cut with *Sty*I and transcribed with T7 RNA polymerase in the presence of [α-^{32}P]GTP. This produced a 130 nt RNA transcript which was purified as described previously (13). The percentage incorporation of [α-^{32}P]GTP and recovery of radiolabelled ssRNA were assessed as for oligonucleotides (above).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed in 20 µl reactions containing 20 mM Tris–HCl pH 7.5, 5 mM DTT and 10% [v/v] glycerol (1× reaction buffer). Nucleic acids and proteins were added at the concentrations indicated in the figure legends and were diluted in either water (nucleic acid) or 0.25× reaction buffer (proteins). Reactions were incubated for 15 min at 37°C and then loaded directly onto 6% native polyacrylamide gels. Dyes were not added to samples as preliminary experiments indicated that they inhibited binding. Gels were electrophoresed for ~2 h at 70 V in 0.5× Tris-borate–EDTA buffer at 4°C. After electrophoresis, gels were dried and the radioactivity was detected and quantified using PhosphorImager screens, a Storm machine and ImageQuant software (Molecular Dynamics). In some cases radioactivity was also visualised by autoradiography.

The fraction of free nucleic acid (Figs 1B and 6B) was determined by dividing the radioactivity in the free nucleic acid band of a particular reaction by that of a reaction lacking protein. For DNA, but not RNA, the fraction of free nucleic acid was adjusted for (i) the total radioactivity present in the whole lane relative to the total radioactivity in the whole control lane and (ii) background which was taken as the fraction of free DNA in a reaction with 100 nM rL4R protein under which conditions binding was found to have reached completion. Hill coefficients, a measure of the co-operativity of binding, were derived from plots of log[bound/free] against log[protein].

The amount of binding in reactions containing radiolabelled DNA and cold competitor nucleic acids (Figs 3 and 4) was determined relative to control reactions with no competitor. The percentage of bound nucleic acid in a reaction was calculated by dividing the radioactivity in the bands representing bound nucleic acid by the total radioactivity present in the whole lane. A background value, the percentage bound in a reaction lacking protein, was subtracted from this reading and then the reading was divided by the percentage bound in a control reaction with no competitor.

RESULTS

Recently it was shown that VP8, the 25 kDa L4R gene product, stimulates the DNA helicase activity of the I8R protein (22). Although, VP8 had previously been described to bind both ss and dsDNA non-specifically (19), its affinity for DNA had not been defined. To further characterise the nucleic acid binding activity of this protein, an electrophoretic gel mobility shift assay was used.

Two forms of L4R protein were examined. One was purified from VV particles and represents native VP8 (L4R). The second was a purified recombinant protein (rL4R) that was produced in *Escherichia coli* (22) and which differs from native L4R protein by two additional amino acids (histidine and methionine) at the N-terminus. Native L4R and rL4R exhibited indistinguishable properties in both gel mobility shift assays (data not shown and Fig. 5B) and in the stimulation of DNA helicase activity (22). Therefore rL4R, which was available in higher abundance than native L4R, was used to assess the affinity and specificity of the 25 kDa L4R protein for ssDNA.

Affinity of DNA binding by the rL4R protein

To measure the affinity of rL4R for ssDNA a gel mobility shift assay was used. A radiolabelled 45mer deoxyribo-oligonucleotide was incubated with increasing concentrations of rL4R protein and complexes were resolved from free DNA by gel electrophoresis and quantitated using a PhosphorImager (Fig. 1A). At low protein concentrations (≤0.5 nM) no complex was formed but as the concentration increased single (2.5 and 5 nM) or multiple

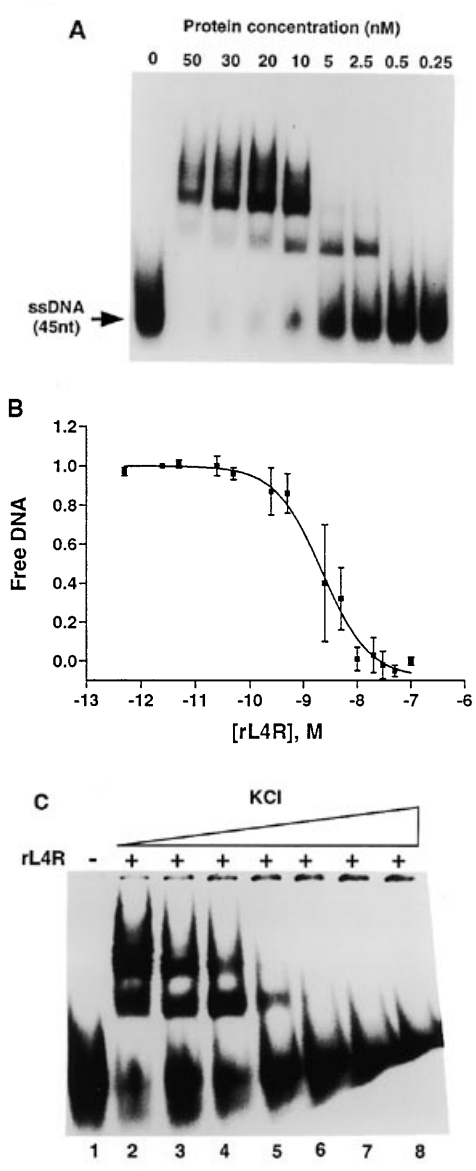

Figure 1. Binding of rL4R protein to ssDNA. (**A**) Autoradiograph of an electrophoretic mobility shift assay. Assays were performed with the indicated concentrations of rL4R protein and 2 fmol of [^{32}P]45mer oligonucleotide (0.1 nM) in a 20 µl reaction volume. Reactions were incubated at 37°C for 15 min and then electrophoresed on a 6% native acrylamide gel. (**B**) Quantification of electrophoretic mobility shift assays. Assays similar to (A) were quantitated using a PhosphorImager. The fraction of free oligonucleotide was determined as an average of between 3 and 12 assays and plotted (with standard deviations) against the protein concentration. The equilibrium binding constant was determined as the protein concentration at half-maximal binding. The solid line represents a sigmoid curve which was fitted to the data using the sigmaplot program, Prism. (**C**) Effect of KCl on DNA binding by the rL4R protein. Electrophoretic mobility shift assays were performed using 10 nM rL4R protein, 1 nM [^{32}P]45mer oligonucleotide and KCl at 0, 25, 50, 100, 150, 200 and 300 mM (lanes 2–8, respectively). The control without protein (lane 1) was incubated in the absence of KCl. A PhophorImager scan of the gel is shown.

(≥10 nM) complexes appeared. The decrease in total radioactivity observed with 30 and 50 nM rL4R (Fig. 1A) was not reproducible and no evidence of nuclease contamination was found upon incubation of rL4R with other ssDNA substrates. Several experiments similar to that shown in Figure 1A were performed and quantified. Two values were calculated; first, the percentage of DNA bound, and second, the amount of free DNA relative to a control lacking protein. These values were separately plotted against protein concentration and both methods produced similar results. Figure 1B shows free DNA plotted against rL4R concentration. These assays were performed at a low concentration of DNA (0.1 nM) such that [protein free] = [protein total] and so at half-maximal saturation there was a >10-fold excess of protein relative to nucleic acid (25,26). The equilibrium binding constant, K_d can therefore be derived as the total protein concentration at half-maximal saturation. This value was obtained from a sigmoid curve which was fitted to the data using the sigmaplot program, Prism (Fig. 1B). The K_d for rL4R protein binding to a 45mer oligonucleotide in the absence of salt was 2 nM (Fig. 1B). The cooperativity of binding was examined by plotting the data on a Hill plot (data not shown). A Hill coefficient of 0.8 was obtained, indicating that binding of this substrate was non-cooperative.

Significant binding of L4R protein to ssDNA in the presence of 100 mM NaCl was reported (19) and, therefore, the effect of KCl on rL4R binding to ssDNA was examined (Fig. 1C). Binding was reduced partially by 25 and 50 mM KCl and drastically by 100 mM or higher concentrations of KCl. Similar results were observed with NaCl. The effect of KCl on the affinity of binding was measured by performing gel mobility shifts with constant DNA concentration and increasing rL4R concentration as described above in the presence of 50 or 100 mM KCl (data not shown). The K_d for rL4R binding the 45mer oligonucleotide in the presence of 50 or 100 mM KCl were ~2 and 74 nM, respectively. The latter figure was estimated from titrations in which complete binding of the oligonucleotide was not reached.

Stoichiometry of rL4R binding to ssDNA

Previously it was estimated that the L4R protein bound 10 nt of ssDNA per monomer (19). However, a different stoichiometry was suggested by the observation that the L4R protein formed only two complexes in gel mobility shifts with a 45mer oligonucleotide (Fig. 1A). One possible explanation of the two complexes is that the L4R protein is binding as a dimer. However, rL4R protein migrated as a monomer during fractionation on an FPLC Superdex 75 gel filtration column in 50 mM KCl (data not shown), indicating that the protein does not form a stable dimer in solution. The stoichiometry was, therefore, further analysed by counting the number of complexes formed in mobility shift assays with rL4R protein and oligonucleotides of different lengths. The number of complexes was proportional to the length of the oligonucleotide such that no complexes were observed with a 20mer, one with 25, 29 and 33mers, two with 35, 45 and 48mers, three with a 51mer and four with a 68mer. These results are presented in Figure 2 in comparison to a theoretical line which represents the number of complexes expected for a protein binding exactly 17 nt per monomer. This analysis suggested that the L4R protein saturates DNA at a density of 17 nt per monomer.

Surprisingly, no complexes were observed in gel mobility shifts with rL4R protein and a 20mer oligonucleotide. Preliminary results indicated that the K_d for rL4R binding to a 25mer was >10 nM (data not shown). This result suggests that binding becomes progressively weaker as the size of the oligonucleotide approaches the DNA binding site size of the protein. This might

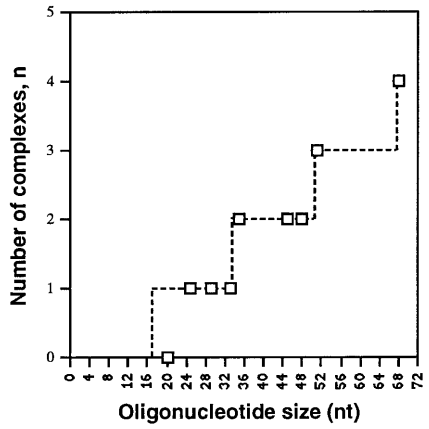

Figure 2. Stoichiometry of DNA binding by L4R protein. Electrophoretic mobility shifts were performed with ^{32}P-labelled oligonucleotides (0.25 nM) of different lengths (20, 25, 29, 33, 35, 45, 48, 51 and 68 nt) and three concentrations of rL4R protein (2, 20 and 100 nM). Samples were analysed on 6% native acrylamide gels and the number of complexes were counted. The number of complexes is plotted against the length of oligonucleotide and compared to a theoretical value (dotted line) assuming 17 nt per complex.

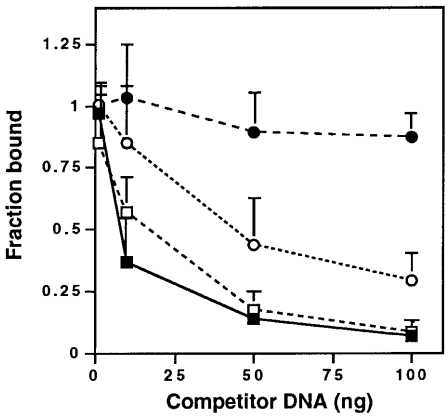

Figure 3. Competition analysis with ss and dsDNA. Electrophoretic mobility shift assays were performed using 10 nM rL4R protein, 1 nM (0.3 ng) [^{32}P]45mer oligonucleotide, various amounts of competitor DNA (1, 10, 50 or 100 ng) and, where indicated, 50 mM KCl. Competitor DNAs were added to the reaction mix before the rL4R protein. Reactions were incubated at 37°C for 15 min, electrophoresed on 6% native acrylamide gels and then quantified using a PhosphorImager. Results, an average of at least two experiments, were calculated as percentage bound and then expressed as a fraction of the percentage bound in a reaction lacking competitor. Circles, ds-pGEM DNA; squares, ssM13mp18 DNA; filled symbols, plus 50 mM KCl.

explain why no binding to a 20mer oligonucleotide was detected in a gel mobility shift assay.

Specificity of nucleic acid binding by rL4R

It has been shown that the L4R protein binds both ss and dsDNA with an equal affinity in low salt (25 mM) but with a higher affinity for ssDNA in high salt (100 mM) (19). To further characterise the specificity of nucleic acid binding by the L4R protein, gel mobility shift assays were performed in which binding of the rL4R protein to a radiolabelled 45mer oligonucleotide was competed with various unlabelled nucleic acids. Binding was competed effectively by ssM13 DNA in both the presence and absence of 50 mM KCl (Fig. 3). Double-stranded plasmid DNA (pGEM) competed binding in the absence but not in the presence of KCl, although the competition was slightly less effective than with ssM13 DNA (Fig. 3). Similar results were observed with another dsDNA plasmid preparation both before and after linearisation (data not shown). The effect of competition by various homopolynucleotides was then tested. Poly(dT) and poly(dC) competed binding in both the presence and absence of KCl (Fig. 4A and C). Poly(dC) was a much poorer competitor than poly(dT) which competed as effectively as ssM13 DNA. Poly(dI:dC) competed binding in the absence but not in the presence of KCl and poly(dA) did not compete binding under either condition (Fig. 4A and C). These results broadly agree with the previously described analysis (19). However, surprisingly the majority of ribo-polynucleotides also competed binding both in the presence and absence of KCl (Fig. 4B and D). In the absence of KCl, all of the ribo-polynucleotides competed binding as effectively as ssM13 DNA except for poly(A) which was slightly less effective and poly(C) which was a weak competitor. In the presence of KCl, poly(C) did not compete binding whilst the other ribo-polynucleotides competed binding with the following effectiveness relative to ssM13 DNA, poly(A)<poly(U), poly(I:C) and ssM13 DNA<poly(I) and poly(G). These results demonstrated that the L4R protein binds both ss and dsRNA. However, only synthetic ribo-polynucleotides had been used and therefore a non-synthetic RNA, a 130 nt T7 RNA polymerase transcript, was also used to compete DNA binding of the L4R protein. This RNA molecule competed binding more effectively than ssM13 DNA both in the presence and absence of 50 mM KCl (data not shown).

RNA binding by the L4R protein

The competition experiments showed that the rL4R protein bound RNA. To demonstrate that native L4R protein also binds RNA, gel mobility shifts were performed with a radiolabelled T7 RNA transcript and FPLC column fractions that contained the L4R protein (Fig. 5). These fractions were identical with those shown in ref. 22, which were obtained during purification of the A18R DNA helicase from vaccinia virions, and contained varying amounts of >95% pure L4R protein. A slowly migrating RNA–protein complex was observed in gel mobility shifts with either rL4R protein or with fractions (51, 53 and 55) that contained the highest concentration of virion-derived L4R protein (Fig. 5A). These fractions also caused the formation of complexes in gel mobility shifts with a radiolabelled 45mer deoxyribo-oligonucleotide (Fig. 5B). The sizes of the RNA–protein and DNA–protein complexes were indistinguishable for rL4R and virion-derived L4R. Fractions 47 and 57 that contained lower concentrations of L4R protein caused some retardation of RNA but no apparent retardation of DNA (Fig. 5). When these fractions were used at a higher concentration, the two DNA–protein complexes were observed (data not shown). Fractions 41 and 68, which contained no L4R protein, did not affect the mobility of either RNA or DNA (Fig. 5). These results demonstrated that the native L4R protein bound ssRNA.

The affinity of the L4R protein for ssRNA was measured by titrating rL4R into assays containing a constant amount of ssRNA, 0.05 nM, (Fig. 6A). A single large RNA–protein complex was observed with high concentrations (10–100 nM) of protein. With

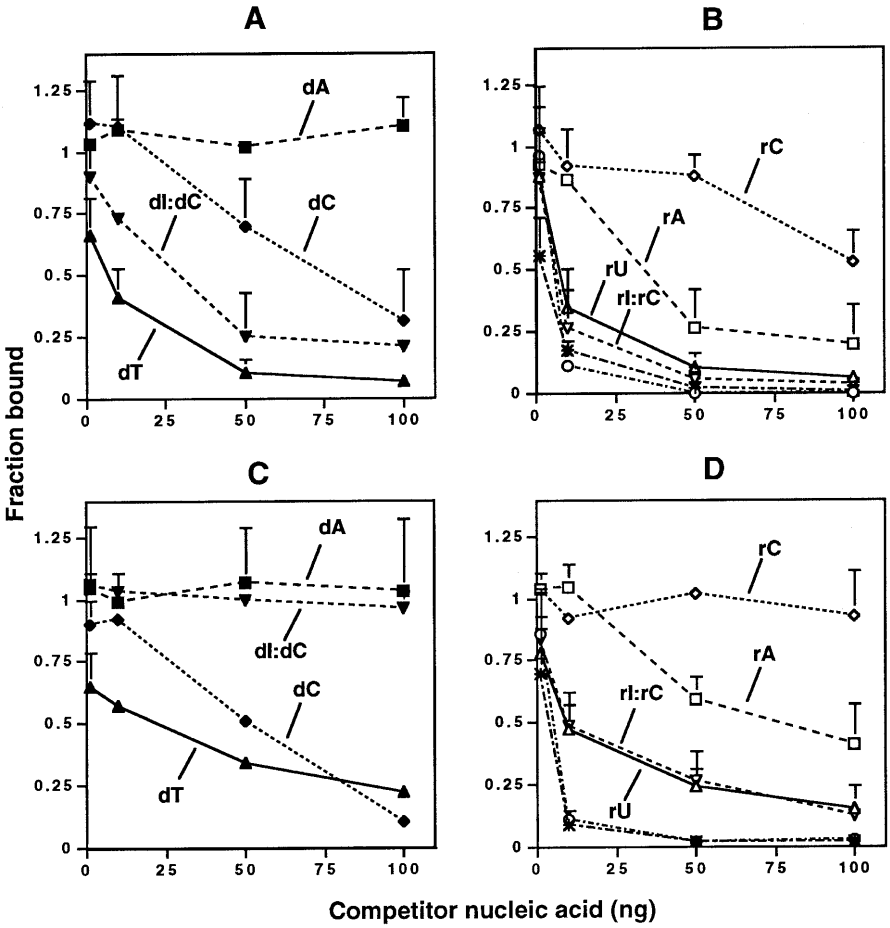

Figure 4. Competition analysis with synthetic polynucleotides. Assays were performed and quantitated as described in Figure 3. (**A**) Competition with deoxyribo-polynucleotides in the absence of salt. Filled square, poly(dA); filled diamond, poly(dC); filled triangle, poly(dT); filled inverted triangle, poly(dI:dC). (**B**) Competition with ribo-polynucleotides in the absence of salt. Square, poly(A); diamond, poly(C); triangle, poly(U); circle, poly(G); star, poly(I); inverted triangle, poly(I:C). (**C**) Competition with deoxyribo-polynucleotides in the presence of 50 mM KCl. Symbols as for (A). (**D**) Competition with ribo-polynucleotides in the presence of 50 mM KCl. Symbols as for (B).

lower protein concentrations (1–5 nM) several complexes were observed, but no complexes were observed with concentrations of rL4R <0.1 nM. Further titrations indicated that the large complex consisted of at least six bound monomers of rL4R protein. The data from four gels were plotted in the same way as for the DNA binding data and a value for half-maximal binding was obtained from a sigmoid curve which was fitted to the data using the sigmaplot program, Prism (Fig. 6B). The K_d for the rL4R protein binding to ssRNA in the absence of salt was 3 nM. The steeper slope of the RNA rather than DNA binding curve (compare Figs 1B and 6B) suggested that there was a degree of cooperativity in the binding of the rL4R protein to the RNA substrate. It was determined that the Hill coefficient for binding the RNA substrate was 1.7 (data not shown) which indicates positively cooperative binding.

The effect of KCl on the binding of the L4R protein to ssRNA was examined in assays containing a constant amount of protein and ssRNA (Fig. 6C). Under these conditions a single large complex was observed and the formation of this complex was unaffected by 25 or 50 mM KCl (lanes 3 and 4, respectively). In the presence of 100–200 mM KCl (lanes 5–7) the large complex was not observed and only smaller complexes were seen. RNA binding was almost completely inhibited by 300 mM KCl (lane 8). A similar effect was found with NaCl (data not shown). The effect of 50 mM KCl on the binding of the L4R protein to ssRNA was examined further, and a small increase in affinity (K_d 1 nM) was observed (data not shown).

DISCUSSION

The 25 kDa product of VV gene L4R is found in the cores of vaccinia virions and is essential for formation of transcriptionally competent viral particles. This protein, otherwise known as VP8, had previously been shown to bind both ss and dsDNA. It is demonstrated here that this protein also binds ss and dsRNA.

The affinity of the 25 kDa L4R protein for nucleic acid was investigated using a recombinant protein (rL4R). The equilibrium binding (K_d) constant of rL4R for ssDNA (a 45mer oligonucleotide) was 2 nM and for ssRNA (a 130 nt T7 transcript) was 3 nM, indicating that the L4R protein has similar affinities for DNA and RNA. Other ssDNA binding proteins, such as the T4 gene 32 protein, also bind RNA but usually with a lower affinity than for ssDNA (27). This may indicate that RNA binding by the L4R protein has functional significance. The shapes of the DNA and RNA binding curves differed with the RNA binding curve displaying a steeper slope. This suggested that the L4R protein

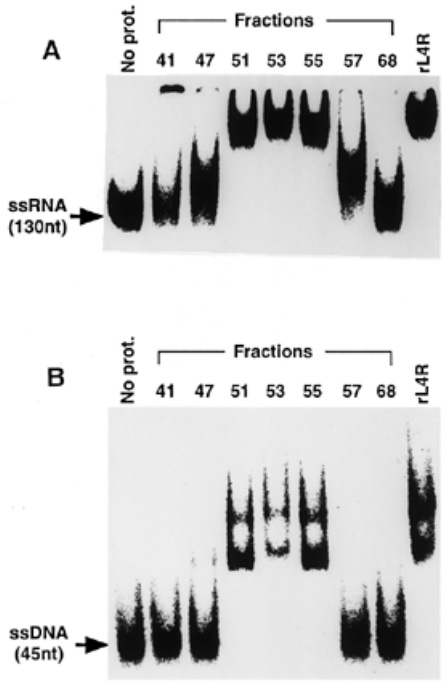

Figure 5. Binding of native L4R and rL4R protein to ssRNA. Electrophoretic gel mobility shift assays were carried out using either 2 µl of the indicated FPLC fractions, which contained different amounts of the native L4R protein purified from vaccinia virions (see text), or 20 nM of the rL4R protein. (**A**) RNA binding. Assays contained 10 fmol (0.5 nM) of a 130 nt [^{32}P]RNA that had been purified by elution from an acrylamide gel. (**B**) DNA binding. Assays contained 20 fmol (1 nM) of [^{32}P]45mer oligonucleotide. The images shown were generated by scanning gels with a PhosphorImager.

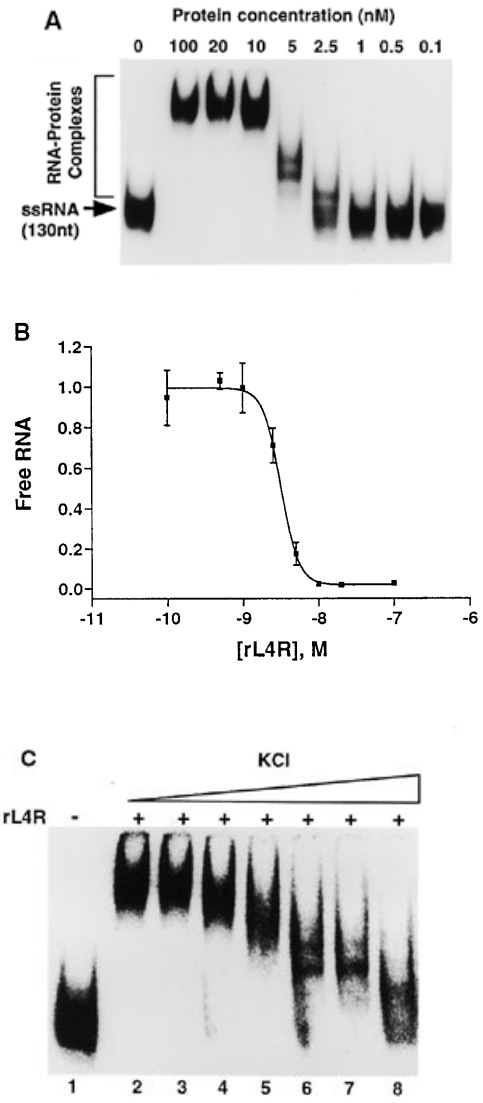

Figure 6. Estimation of the binding affinity of the rL4R protein for ssRNA. (**A**) Autoradiograph of an electrophoretic mobility shift assay. Assays were performed with the indicated concentrations of rL4R protein and 1 fmol of a 130 nt [^{32}P]RNA (0.05 nM) in a 20 µl reaction volume. Reactions were incubated at 37°C for 15 min and then electrophoresed on 6% native acrylamide gels. (**B**) Quantification of ssRNA binding. Assays were analysed as described in Figure 1B. Each point represents the average of four experiments. The solid line represents a sigmoid curve which was fitted to the data using the sigmaplot program, Prism. (**C**) Effect of KCl on RNA binding by the rL4R protein. Electrophoretic mobility shift assays were performed using 10 nM rL4R protein, 0.05 nM [^{32}P]RNA (a 130 nt T7 RNA transcript) and KCl at 0, 25, 50, 100, 150, 200 and 300 mM (lanes 2–8, respectively). The control without protein (lane 1) was incubated in the absence of 50 mM KCl. A PhophorImager scan of the gel is shown.

bound RNA cooperatively and Hill coefficients demonstrated that the rL4R protein bound the DNA substrate non-cooperatively and the RNA substrate with positive cooperativity. However, the two substrates were of different lengths and it is possible that the lack of cooperativity observed with the DNA substrate was due to the short length of the oligonucleotide used in the assay. The 45mer deoxyribo-oligonucleotide bound only two monomers of L4R protein which implies a maximum of only two protein–protein interactions during binding of this substrate by the L4R protein. In contrast, the RNA substrate had seven potential binding sites (assuming 17 nt per monomer) and at least six complexes were observed. This substrate had, therefore, the potential for numerous interactions between monomers of L4R protein. Thus the positive cooperativity observed with the RNA substrate suggests that binding of L4R protein to nucleic acid involves cooperative interactions between protein subunits.

Some nucleic acid binding proteins are able to destabilise short nucleic acid duplexes (28,29). Previously, both native L4R and rL4R proteins were found not to destabilise DNA–DNA duplexes (either a 20mer or a 45mer annealed to ssM13) (22). This assay was repeated under the conditions described for the gel mobility shift assay using rL4R and either a DNA–DNA duplex, a 68mer (Fig. 2) annealed to ssM13 (this substrate had a 23 bp duplex region), or an RNA–RNA duplex, this substrate consisted of annealed 43 and 600 nt *in vitro* transcribed RNA transcripts. No unwinding was observed (data not shown).

The number of complexes formed between the L4R protein and deoxyribo-oligonucleotides of varying lengths suggested a stoichiometry of 17 nt of DNA per monomer of L4R protein. This stoichiometry is higher than that observed previously (19) which

was calculated as 10.2 nt per monomer. This value was based on co-sedimentation of radiolabelled DNA and protein in sucrose gradients which may have overestimated the number of L4R molecules actually bound to DNA. Previously, it was described that the L4R protein stimulates the DNA helicase activity of the I8R protein (22). Full stimulation of the helicase was observed when there were <13 nt of DNA per monomer of L4R protein and partial stimulation was found at between 15 and 18 nt of DNA per monomer of L4R. It was inferred that full stimulation was occurring close to the point when all the DNA was bound by the L4R protein. However, a DNA binding site size of 17 nt per monomer of L4R suggests that the DNA helicase was only partially stimulated when all the ssDNA was bound by the L4R protein and that full stimulation was not found until there was an excess of L4R protein over ssDNA. This conclusion supports the hypothesis that the L4R protein is required to bind the ssDNA as it is released from the DNA duplex by the helicase.

Repression of L4R synthesis during a VV infection results in production of morphologically normal, but non-infectious, viral particles which are defective for early VV transcription (20,21). These particles contain normal amounts of VV transcriptional proteins and virion extracts are fully competent for transcription from early VV promoters on plasmids. Four specific defects have been noted, namely a morphological difference in the immature particles, increased sensitivity of the virions to extraction with sodium deoxycholate, decreased rates of early transcription and reduced rates of RNA extrusion from virions (21). A number of hypotheses for the function of the L4R protein in VV replication have been proposed based on the phenotype of this mutant (21). In particular, it has been proposed that the L4R stimulated-I8R DNA helicase activity is required for initiation of early virion-derived transcription (22). The relatively high affinity of the L4R protein for ssDNA supports this hypothesis.

The demonstration of RNA binding by the L4R protein suggests two possible further functions for the L4R protein. The I8R protein also has RNA helicase activity (12) and it has been hypothesised that this activity is essential for virion-derived VV early transcription (23). Thus, it can be proposed that RNA binding by the L4R protein stimulates the I8R RNA helicase activity and is required for full activity of the I8R protein in vaccinia virion transcription. A second proposal is that the L4R protein may be directly involved in extrusion of viral RNA from intact virions. Characterisation of the effect of the L4R protein on I8R RNA helicase activity and creation of mutants that perturb nucleic acid binding by the L4R protein will be required to further elucidate the functions of the L4R protein in formation of VV virions competent for transcription.

In summary, the VV L4R protein has been demonstrated to bind both ssDNA and ssRNA with a nanomolar affinity and to bind RNA cooperatively. This high affinity and mode of binding indicate that nucleic acid binding by the L4R protein is an important part of its function in the vaccinia virion.

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