Avian Retrovirus pp32 DNA Binding Protein. Preferential Binding to the Promoter Region of Long Terminal Repeat DNA†

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ABSTRACT: The avian retrovirus pp32 protein possesses DNA endonuclease activity and unique DNA binding properties. An improved purification procedure was developed for pp32, resulting in a severalfold increase in the yield of this virion protein. By use of the nitrocellulose filter binding assay, the protein retains approximately 2-fold more supercoiled (form I) DNA molecules than equivalent linear duplex DNA molecules. Single-stranded DNA is only slightly preferred over double-stranded DNA for pp32 binding. The pp32 DNA binding sites on form I pBR322 DNA which contained an insert of avian retrovirus long terminal repeat (LTR) DNA were determined. A preformed protein–DNA complex was digested with one of several different multicut restriction enzymes and filtered through nitrocellulose filters. Fragments containing viral LTR DNA sequences and plasmid DNA containing promoter sequences for the ampicillin and tetracycline genes, sequences for the "left-end" inverted repeat of transposon 3, and sequences encompassing the carboxyl terminus of the β-lactamase gene were preferentially retained on the filter by pp32. Partial mapping of pp32 DNA binding sites on LTR DNA was accomplished by generation of deletions in LTR DNA sequences. The pp32 protein preferentially bound viral DNA fragments which contain the viral promoter (TATTTAA) and the adjacent "R" repeat sequences. Computer analysis revealed that three of the four plasmid DNA fragments retained by pp32 contained LTR DNA promoter-like sequences (one mismatch only) which were part of statistically significant and thermodynamically stable hairpin structures.

The avian retrovirus pp32 protein possesses a DNA nicking activity which prefers form I[1] DNA as substrate (Grandgenett et al., 1978). The partially phosphorylated 32 000-dalton protein termed pp32 shares peptide sequences with the β subunit, but not the α subunit, of the avian retrovirus reverse transcriptase (Schiff & Grandgenett, 1978, 1980). This protein is derived from the carboxyl terminus of the β subunit in vivo (Eisenman et al., 1980; Copeland et al., 1980), and peptide fragments similar in size and activity to pp32 can also be generated by proteolysis of the β subunit in vitro (Grandgenett et al., 1980).

The pp32 protein selectively binds to the most conserved region of avian retrovirus LTR DNA (Misra et al., 1982). Because this protein recognizes these specific LTR DNA sequences, we have suggested that this protein may be involved in either replication, integration, or possibly transcription of viral DNA. Although it is not known whether form I or form III DNA is the immediate precursor to integrated viral DNA, the conserved LTR DNA sequences of retrovirus DNA are apparently involved in the integration process (Sutcliff et al., 1980; Shoemaker et al., 1980; Shimotohno et al., 1980; Dahr et al., 1980; Van Beveren et al., 1980; Swanstrom et al., 1981). We wanted to further define, using the nitrocellulose filter binding assay, if pp32 could preferentially bind to DNA fragments containing these conserved LTR DNA sequences and to define more precisely what LTR DNA sequences or possible DNA secondary structures promote pp32 binding. Form I ^{32}P-labeled pPvuII-DG, a plasmid containing a 1670 bp avian retrovirus DNA insert possessing tandem copies of LTR DNA, was complexed with pp32, digested with one of several different multicut restriction enzymes, and filtered through nitrocellulose. The pp32–DNA fragments retained on the filter were examined on 5% polyacrylamide gels. By using several viral LTR DNA deletion mutants derived from pPvuII-DG, we found that pp32 preferentially retains viral LTR DNA fragments containing promoter and adjacent "R" sequences. The protein did not preferentially retain DNA fragments containing viral *gag* or *src* specific sequences. However, pp32 was also able to preferentially retain four plasmid DNA fragments. Computer analysis permitted identification of LTR DNA promoter-like sequences on pp32-bound plasmid fragments derived from intercistronic regions of pBR322 (Sutcliffe, 1978a,b; Müller & Fitch, 1982; Brosius et al., 1982) and an evaluation of primary and secondary DNA structures involved in pp32 DNA binding.

Materials and Methods

Restriction enzymes were obtained from New England Biolabs or Bethesda Research Laboratory and were used as directed. Nitrocellulose filters were obtained from Schleicher & Schuell (BA85) or Millipore (HAWP). Polyuridylate–Sepharose 4B was obtained from Pharmacia.

DNA Preparation. The plasmid designated pPvuII-DG (Swanstrom et al., 1981), containing a 1670 bp avian retrovirus

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[1] Abbreviations: form I, covalently closed supercoiled duplex DNA; form II, relaxed duplex DNA containing one or more phosphodiester bond discontinuities; form III, full-length linear duplex DNA; DDT, dithiothreitol; NP-40, Nonidet P-40; BSA, bovine serum albumin; LTR, long terminal repeat; SRA, Schmidt–Ruppin strain A; AMV, avian myeloblastosis virus; RSV, Rous sarcoma virus; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; bp, base pairs.

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SRA DNA fragment inserted at the *Pvu*II site of pBR322, and the plasmid (pPvuII-E) containing only SRA *src* specific sequences were generous gifts from J. M. Bishop and coworkers. The plasmids were labeled by growing the bacteria in a low phosphate-containing medium with [^{32}P]orthophosphoric acid. Form I plasmid DNA was purified by two successive CsCl buoyant density gradient sedimentation steps and by velocity sedimentation through sucrose (Grandgenett et al., 1978). The specific activity of the purified form I DNA was varied from 35 000 to 100 000 cpm/μg of DNA. In experiments which required labeled form I DNA, the preparations of DNA were always greater than 94% form I. Form II was obtained by permitting ^{32}P-labeled form I DNA to decay for 2 weeks and subsequently purifying form II from form I by velocity sedimentation in sucrose gradients. The pPvuII-DG plasmid was cut with *Sac*I to obtain form III DNA. Preparation of ^{3}H-labeled adenovirus 2 DNA (5700 cpm/μg) and alkali denaturation of adenovirus and form III pPvuII-DG DNA have been described (Golomb & Grandgenett, 1979).

Generation of LTR DNA Deletion Mutants. pPvuII-DG DNA was cut with the single-cut *Sac*I restriction enzyme (see Figure 2), and the DNA was then digested with *Bal*31 nuclease. The ligated DNA was transfected into *Escherichia coli* HB101, and various deletion mutants in the LTR DNA region were identified.

DNA Sequencing. DNA sequence analysis was preformed as previously described (Misra et al., 1982) by using the Maxam–Gilbert sequencing technique (Maxam & Gilbert, 1980).

Nitrocellulose Filter Binding Assay. Nitrocellulose filters were treated in 0.4 M potassium hydroxide for 20 min at 21 °C and then neutralized with 0.1 M Tris-HCl (pH 7.5) (Lin & Riggs, 1972; Strauss et al., 1981). The filters were stored at 4 °C in sterile water and prior to use were washed with 6 mL of buffer A consisting of 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 3 mM DTT, and 0.2 mM EDTA. The DNA binding reaction (100 μL) contained the above buffer supplemented with 50 μg of BSA/mL. The reaction was initiated by adding the pp32 protein to the mixture (prewarmed at 37 °C) containing the appropriate DNA and was further incubated at 37 °C for 5 min. The reaction mixture was diluted with 1 mL of buffer A (25 °C) and filtered at a flow rate of approximtely 2 mL/min. The filters were washed with an additional 1 mL of buffer A, dried, and counted by using an organic scintillation fluid. Background counts (<1% of the input DNA) due to DNA retained on the filter in the absence of pp32 were subtracted.

Form I, form II, or form III pPvuII-DG were also complexed to pp32 as described above prior to digestion of this protein–DNA complex with either *Hae*III, *Hin*fI, or *Hpa*II restriction enzymes. The concentration of restriction enzyme used was usually 4–8-fold that amount recommended by the supplier. The restriction enzyme was added to the DNA binding reaction, and the sample was diluted to the NaCl concentration close to the optimum for the enzyme used. The Mg^{2+} concentration was maintained at 1 mM. The restriction enzyme reaction was terminated after a 5–10-min incubation by adding EDTA to a final concentration of 4 mM. The mixture was immediately diluted with 1 mL of buffer A, filtered, and washed as described above. DNA retained on the filter was eluted and analyzed on 5% polyacrylamide gels as previously described (Misra et al., 1982).

Purification of the AMV or RSV pp32 Proteins. The pp32 DNA binding protein was purified from AMV or RSV as previously described (Grandgenett et al., 1978) except that the virus was lysed with 2% NP-40 and precipitated by 70% solid $(NH_4)_2SO_4$. The precipitated protein was centrifuged and the dissolved protein dialyzed overnight in 50 mM potassium phosphate (pH 7.1), 0.2 mM EDTA, 3 mM DTT, and 10% glycerol. The dialyzed sample was purified by phosphocellulose and poly(U)-Sepharose 4B chromatography. No NP-40 was present in the chromatography elution buffers as previously described (Grandgenett et al., 1978). The resulting purified protein was stored in 20 mM Tris-HCl (pH 7.4), NaCl (0.5–0.8 M), 17% glycerol, 3 mM DTT, and 0.2 mM EDTA at −70 °C.

Purification of the AMV $\alpha\beta$ DNA Polymerase. The $\alpha\beta$ DNA polymerase was purified through heparin–Sepharose (Golomb & Grandgenett, 1979).

Glycerol Gradient Centrifugation. Sedimentation of poly(U)–Sepharose purified AMV or RSV pp32 on 20–40% glycerol gradients was previously described except that no NP-40 was present in the gradient buffer (Grandgenett et al., 1978).

Computer Analysis. A computer program described previously (Fitch, 1966) was slightly modified to search the pBR322 genome (Müller & Fitch, 1982) and the SRA viral insert DNA for the presence of TTAAATA and TATTTAA sequences, allowing 0–1 mismatch. Potential hairpinlike secondary structures with uninterrupted perfectly base paired stems containing at least six base pairs (no G·T pairing allowed) and loop sizes of at least 3 but not exceeding 20 nucleotides were identified by a computer program (Fitch, 1983). The statistical significance of such structures has already been demonstrated (Müller & Fitch, 1982).

Results

Improved Purification of AMV pp32. The purification of pp32 was modified to increase the yield of this protein from nonionic detergent-lysed virions (Grandgenett et al., 1978). Instead of absorbing the virus onto phosphocellulose immediately after lysis, the lysed virions were first precipitated with 70% $(NH_4)_2SO_4$. The precipitated protein was dissolved in a 50 mM potassium phosphate buffer, dialyzed overnight in the same buffer, and absorbed to phosphocellulose. The pp32 protein was eluted from the column with a potassium phosphate buffer gradient. This precipitation procedure resulted in the elution of pp32 from phosphocellulose in a more distinct peak of DNA binding activity and also resulted in an average 2-fold increase in the amount of pp32 DNA binding activity recovered from the same amount of input virion protein (unpublished results). This enhanced yield may have been partially due to increased in vitro proteolytic processing of the presumed precursor of pp32, the β DNA polymerase subunit, during overnight dialysis. The pp32 protein was greater than 95% pure after phosphocellulose and poly(U)–Sepharose 4B chromatography as analyzed by SDS–polyacrylamide gel electrophoresis (unpublished results). The DNA binding properties of pp32 are stable for at least 6 months when stored at −70 °C in high salt (0.5 M NaCl) at concentrations greater than 50 μg/mL.

Binding of pp32 to Form I and Form III DNA. The quantitative binding of pp32 to form I or form III pPvuII-DG using the nitrocellulose filter assay was investigated. The pp32 protein is able to retain form I DNA better than form III DNA at various protein/DNA ratios either in the presence or in the absence of Mg^{2+} under these standard assay conditions (Figure 1). The high protein to DNA ratios required to retain either DNA suggests that more than one pp32 dimer molecule is bound per DNA molecule. It is likely that only a fraction of the pp32 molecules present in solution are active

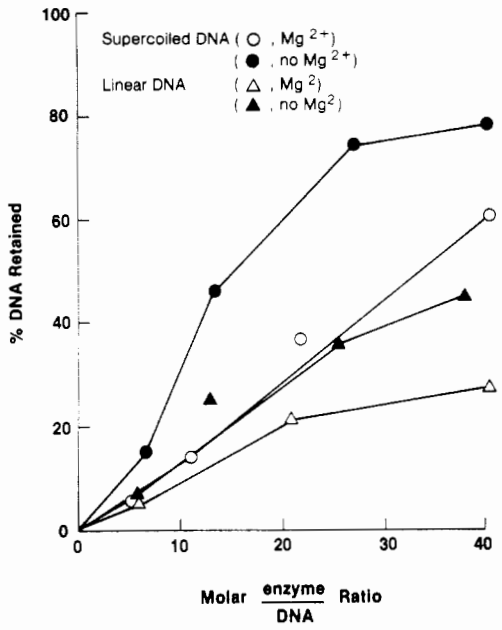

FIGURE 1: Binding of pp32 to form I and form III DNA. The pp32 protein was incubated at 37 °C for 5 min with the various indicated ^{32}P-labeled DNAs under standard binding conditions described under Materials and Methods. The specific activity of the ^{32}P-labeled DNA was 34 000 cpm/μg. Form III DNA was obtained by digestion of this pPvuII-DG plasmid with *Sac*I. The Mg^{2+} concentration was 5 mM. The samples were filtered through nitrocellulose filters, and the percent DNA retained was calculated. The molar ratio of the pp32 to DNA is indicated on the figure. The molecular weight of pp32 was taken as 64 000 because the enzyme exists as a dimer in vitro. In this figure, the actual concentration of pp32 at a 40 to 1 ratio of protein to DNA was 3.3 μg/mL. Similar results were obtained if the concentration of pp32 was varied from 0.06 to 3.3 μg/mL.

for binding so the actual stoichiometry is as yet undefined. There is also greater retention of form I pPvuII-DG by pp32 in the absence of Mg^{2+} than in its presence (Figure 1). Under these DNA binding conditions (protein/DNA ratios and time of incubation) in the presence of Mg^{2+}, the conversion of form I to form II DNA by the DNA nicking activity associated with pp32 would be less than 5% (Grandgenett et al., 1978).

Competition experiments were performed by using equivalent native and denatured DNAs to determine if pp32 preferred to bind to single-stranded or double-stranded DNA. ^{32}P-Labeled form I pPvuII-DG (0.33 μg) and varying amounts of native or alkali-denatured form III pPvuII-DG (0.1–2.1 μg) in the same reaction mixture were complexed to pp32 (0.1 or 0.2 μg) in the presence of 0.1 M NaCl at 37 °C for 5 min and filtered. In all cases, pp32 is not saturating with respect to labeled substrate (see Figure 1). Both native and denatured DNA effectively competed for the binding of pp32 to form I DNA, although single-stranded DNA was slightly more effective. The binding of pp32 to ^{32}P-labeled form I DNA was decreased by 50% when 0.4 μg of native DNA or 0.2 μg of denatured form III DNA were used as competitors. Similar results were obtained if ^{32}P-labeled form III pPvuII-DG was used instead of pPvuII-DG form I DNA as the labeled substrate. In addition, competition studies using unlabeled native and alkali-denatured adenovirus DNA with ^{3}H-labeled adenovirus DNA as substrate gave comparable results to those obtained above.

The formation of pp32–form I DNA complexes is rapid at both 37 and 25 °C in the presence of 0.1 M NaCl. The same amount of input DNA is retained by pp32 on the filter at 1, 5, or 7 min. The half-life of pp32–DNA complexes at 37 °C under standard assay conditions (0.1 M NaCl and 5-min preincubation) in the absence of Mg^{2+} for form I pPvuII-DG is 28 min. The same results were obtained if the protein to DNA ratio was either 16 to 1 or 32 to 1, respectively. The formation of these complexes with either DNA is highly sensitive to the salt concentrations in the initial reaction mixture. Increasing the salt concentration to 0.2 or 0.3 M NaCl in the reaction mixture and in the subsequent washing step decreased the amount of DNA bound by pp32 to approximately 40% and 15%, respectively, relative to the amount of DNA bound in 0.1 M NaCl.

To define the dissociation of pp32–DNA complexes, pp32 and labeled form I pPvuII-DG at ratios of 20 to 1 or 40 to 1, respectively, were incubated for 5 min to reach equilibrium. A 50-fold excess of unlabeled form I pPvuII-DG was then added and at selected times, the samples were filtered. The amount of pp32–DNA complex remaining bound was calculated as the percent of DNA retained on the filters compared to samples without the addition of unlabeled pPvuII-DG. The half-life for the pp32–DNA complexes are 5.0 and 5.8 min, respectively. Since pp32 appears to bind to six separate sites on pPvuII-DG (see below), a calculated dissociation rate constant (Riggs et al., 1970) would be an average of multiple unequal parameters of this DNA binding reaction.

Determination of pp32 Binding Sites on Form I pPvuII-DG. Our laboratory (Misra et al., 1982; V. Parsons and M. Golomb, unpublished results) has previously demonstrated that binding of pp32 to form I pPvuII-DG, followed by *Hae*III restriction enzyme digestion, resulted in the preferential retention of six DNA fragments by pp32 on nitrocellulose (Figure 3). This particular experiment was repeated here for comparison of data presented in this report on binding of pp32 to various LTR DNA deletion mutants and computer analysis of DNA sequences involved in pp32 recognition. *Hae*III fragments numbered 1, 4, 6, 8, 9, and 12 are selectively retained by pp32 in comparison to other similar size DNA fragments (Figure 3). Fragments 1 and 12 contain the promoters for ampicillin and tetracycline genes in pBR322, respectively (Sutcliffe, 1978a,b; Brosius et al., 1982) (Figure 2). Fragment 4 contains the "left-end" inverted repeat of transposon 3. Fragment 9 is AT rich and includes the carboxyl terminus of the β-lactamase gene. Fragments 6 and 8 contain viral sequences which consist almost entirely of LTR DNA sequences. Quantitation of these data either by counting the uniformly labeled DNA fragments or by densitometer scans of exposed X-ray film demonstrated that fragments preferentially bound by pp32 are retained 2–3-fold better (molar basis) than the other fragments of similar size (unpublished results). As illustrated in Figure 2 and evident in Figure 3, pp32 selectively binds to six fragments over a wide range of protein to DNA ratios (lanes B, C, D, E, and F at 12/1, 24/1, 36/1, 48/1, and 60/1, respectively). Increasing the amount of pp32 in the binding reaction also results in increased amounts of undigested and partially digested DNA appearing at the top of the gel (Figure 3). This increase in partial digestion does not appear to affect the overall results. The pp32 protein, rather than the *Hae*III restriction enzyme, is responsible for retention of these fragments because little or no DNA is retained on the filter by the restriction enzyme in the absence of pp32 (Figure 3, lane A). Glycerol gradient purified AMV pp32 possesses the same DNA binding properties as poly(U)–Sepharose-purified enzyme. Heating of pp32 preparations at 60 °C for 10 min completely eliminated the ability of the enzyme to retain DNA on nitrocellulose filters.

The AMV pp32 protein also retained these same six *Hae*III fragments (Figure 3) if the pPvuII-DG substrate was present as form II or form III DNA (unpublished results). These

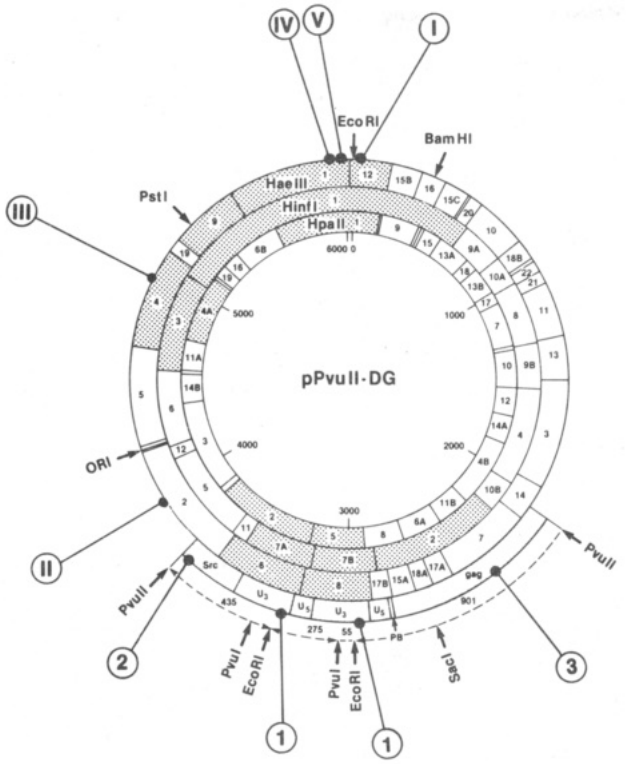

FIGURE 2: Location of preferred pp32 DNA binding sites on pPvuII-DG. The restriction enzyme cleavage maps of *Hae*III, *Hin*fI, and *Hpa*II for pPvuII-DG are shown on the circular diagram. The maps were generated from published data (see text) and data from J. M. Bishop (personal communication). The fragments generated by digestion with a particular enzyme are numbered by size. Unique enzyme sites are indicated by arrows around the perimeter. The important features of the viral insert are indicated in the semicircle bound by the *Pvu*II sites. The terminal repeat consists of two identical copies (330 bp in length), each of which contains the region unique to the 3′ terminus (U_3) and 5′ terminus (U_5) of the viral genome. Only part of the *src* and *gag* sequences are present in this subclone. The abbreviation, PB, is the primer binding site. Fragments marked with heavy dots are preferentially bound by pp32. Viral DNA fragment sizes generated by cutting with different restriction enzymes for end labeling with [γ-^{32}P]ATP are shown in the outer most semicircle (see Discussion for DNase I footprinting experiments utilizing these end-labeled fragments. The outer circled numbers refer to the position of the viral promoter-like sequences found in pPvuII-DG (see Figure 9 and Table I for sequence and potential secondary structures accompanying each circled number).

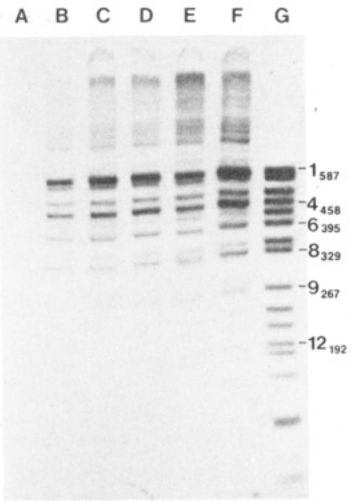

FIGURE 3: Preferential retention of six *Hae*III fragments of pPvuII-DG by pp32. The pp32 protein was bound to 0.5 μg of form I pPvuII-DG under standard assay conditions. The pp32–DNA complex was digested with *Hae*III before filtration on nitrocellulose as described under Materials and Methods. The filter-bound DNA was eluted and analyzed on 5% polyacrylamide gels. The concentrations of pp32 were as follows: lane A, 0 μg; lane B, 0.1 μg; lane C, 0.2 μg; lane D, 0.3 μg; lane E, 0.4 μg; lane F, 0.5 μg; lane G, no pp32 but 0.1 μg of *Hae*III-digested DNA which was not filtered. Not all of lane E DNA was recovered. See Figure 2 for location of *Hae*III fragments on pPvuII-DG.

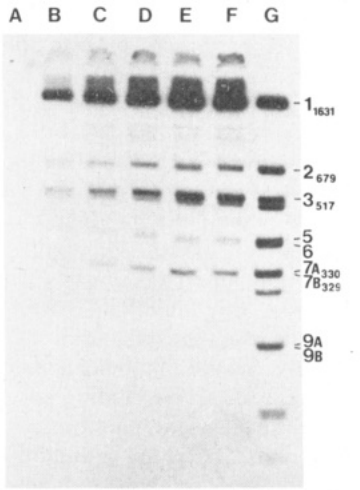

FIGURE 4: Preferential retention of five *Hin*fI fragments of pPvuII-DG by pp32. The assay conditions were exactly the same as Figure 3 except *Hin*fI was used instead of *Hae*III. The concentrations of pp32 used were as follows: lane A, 0 μg; lane B, 0.1 μg; lane C, 0.2 μg; lane D, 0.3 μg; lane E, 0.4 μg; lane F, 0.5 μg; lane G, no pp32 but 0.1 μg of *Hin*fI-digested DNA which was not filtered. See Figure 2 for location of *Hin*fI fragments on pPvuII-DG.

results suggest that secondary structures may not play a primary role in pp32 recognition of DNA although this possibility is feasible (see Discussion).

To confirm that pp32 could selectively bind DNA fragments containing the same DNA sequences found in *Hae*III fragments (Figure 3), the pp32–DNA complexes were digested with other restriction enzymes. Digestion of pp32–form I pPvuII-DG complexes by *Hin*fI resulted in the preferential retention of fragments numbered 1, 2, 3, 7A, and 7B (Figure 4). Comparison of pp32-bound *Hin*fI and *Hae*III fragments clearly established that pp32 preferentially binds to certain regions on this plasmid (Figure 2). *Hin*fI fragment 1 (Figure 4) contains all the sequence information of three *Hae*III fragments (Figure 2) preferentially retained by pp32. The relatively large *Hin*fI fragment 2 (679 bp) contains most of viral *Hae*III fragment 7 (351 bp) (Figure 2), which is not preferentially retained by pp32 (Figure 3). The viral LTR sequences of *Hin*fI fragment 2 represents only 7% of the total sequences in this fragment. Therefore, the retention of *Hin*fI fragment 2 by pp32 may not be a true representation of LTR DNA sequences recognized by this protein (see next section).

The retention of *Hin*fI fragment 2 suggests that pp32 recognizes sequence information in the U_5 region of LTR DNA or the immediate adjacent area of the primer binding site (PB) (Figure 2). Comparison of pp32-retained *Hin*fI fragment 4 (506 bp) with HinfI fragment 2 or 3 demonstrates the preference of pp32 for the latter two fragments (Figure 4). The same analogy can be made for the preferential retention of *Hin*fI fragments 7A and 7B which contain LTR DNA sequences, in comparison to *Hin*fI fragments 5 (400 bp), 6 (396 bp), 8 (298 bp), 9A (221 bp), or 9B (220 bp). We could not distinguish whether pp32 retained fragment 7A or 7B, or both, because these fragments were not separated in this gel system. The very large size distribution of these *Hin*fI fragments makes it difficult to quantitate the stoichiometric relationship of

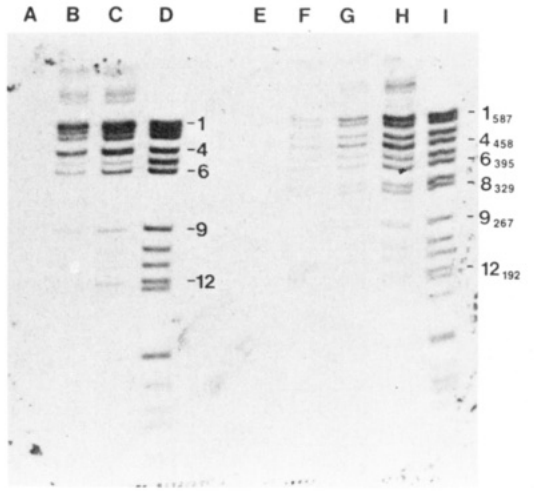

FIGURE 5: Binding of pp32 to pPvuII-TM1 deletion mutant and $\alpha\beta$ DNA polymerase to pPvuII-DG. The pp32 protein was bound to 0.3 μg of form I pPvuII-TM1 under standard assay conditions as described in Figure 3 and digested with *Hae*III restriction enzyme. Lane A, 0 μg pp32; lane B, 0.07 μg; lane C, 0.15 μg; lane D, no pp32 but 0.09 μg of *Hae*III-digested pPvuII-TM1. $\alpha\beta$ DNA polymerase was complexed to form I pPvuII-DG (0.3 μg) and digested with *Hae*III. Lane E, 0 μg; lane F, 0.025 μg; lane G, 0.050 μg; lane H, 0.1 μg; lane I, no $\alpha\beta$ but 0.09 μg of *Hae*III-digested pPvuII-DG. The molar ratio of $\alpha\beta$ to pPvuII-DG was 3.3, 6.6, and 13.2 to 1 in lanes F, G, and H, respectively.

pp32-bound fragments, particularly if pp32 possibly has only one preferential binding site on a large fragment (fragment 2) or if pp32 has at least three binding sites on a larger fragment (fragment 1) (Figures 2–4).

Further confirmation of pp32 selective binding to the previously described regions on pPvuII-DG was also obtained by digestion of pp32–form I pPvuII-DG complexes with *Hpa*II (Figure 2). *Hpa*II fragments 1, 2, 4A, and 5 were preferentially retained. We assumed that 4B was not retained as previously demonstrated (Figure 2) permitting us to score 4A as positive. We were not able to determine which of the several *Hpa*II fragments (6A, 6B, 7, and 8; Figure 2) pp32 preferentially retained because of the similar sizes of these fragments.

As state previously, the pp32 protein and not the restriction enzyme or other possible DNA binding proteins in the restriction enzyme preparation is responsible for retention of the DNA fragments on nitrocellulose. For additional controls, AMV $\alpha\beta$ DNA polymerase was complexed to form I pPvuII-DG as described previously for pp32 and then digested with *Hae*III (Figure 5, lanes E–I). Under these conditions, the $\alpha\beta$ DNA polymerase does not appear to preferentially retain viral LTR DNA containing fragments 6 or 8 in comparison to pp32 (Figure 3), consistent with results obtained previously by using the DNase I footprinting procedure (Misra et al., 1982). However, $\alpha\beta$ DNA polymerase is able to bind better to plasmid fragments 1 and 4 and possibly 9 and 12, than the rest of the *Hae*III fragments. We have not excluded the possibility that under different assay conditions (salt, pH, divalent metal ion, etc.), the $\alpha\beta$ DNA polymerase or the β_2 DNA polymerase (Hizi & Joklik, 1977) may also selectively retain viral DNA fragments containing LTR DNA sequences. Lastly, AMV pp32 did not preferentially retain *Hae*III or *Hin*fI DNA fragments containing only SRA *src* specific sequences present in pPvuII-E by using the filter binding assay (unpublished results). The viral *src* insert was derived from the unintegrated SRA genome (Czernilofsky et al., 1980), and the DNA was inserted into pBR322 at the *Pvu*II site.

Mapping of pp32 DNA Binding Sites on LTR DNA Using Deletion Mutants. By the use of deletion mutants, we have partially defined which viral LTR DNA sequences are necessary for pp32 recognition. By use of the filter binding assay, the results obtained confirmed and extended previous mapping data of pp32 binding to the conserved sequences on LTR DNA by using the DNase I footprinting procedure (Misra et al., 1982). The pp32 protein was bound to form I DNA which possessed a deletion in the viral insert. The deletion mutant (designated pPvuII-TMI) was generated by cutting of the plasmid at the single SacI site (Figure 2 and Figure 6), followed by digestion with *Bal*31 nuclease. To the left of the *Sac*I site, the only viral *Hae*III fragment intact was fragment 6 which contains all of U_3 and 44 bp of the adjacent R and U_5 regions (Figure 5, lane D, and Figure 6). This intact fragment contains the viral LTR promoter and other regulatory signals (Swanstrom et al., 1981). The pp32 protein was able to preferentially retain *Hae*III fragment 6 in this deletion mutant (Figure 5, lanes B and C) as well as plasmid *Hae*III fragments 1, 4, 9, and 12 as previously shown. To the right of the *Sac*I site, all viral fragments including the viral-plasmid fusion fragment (fragment 14, Figure 6) were deleted (Figure 5, lane D). As demonstrated by restriction enzyme (Figure 6) and DNA sequencing analysis (unpublished results), the new fusion fragment generated (Figure 5, lane D, third band) contains approximately half of the 3′ terminus of the LTR U_5 region and 51 bp of the adjacent U_3 region which is fused to pBR322, 412 nucleotides from the right most shown pBR322 *Hae*III site (Figure 6). This new *Hae*III fragment is not preferentially retained by pp32 (Figure 5, lanes B and C, third band) (unpublished data). This indicates that these viral LTR joint region sequences alone are not responsible for the preferential

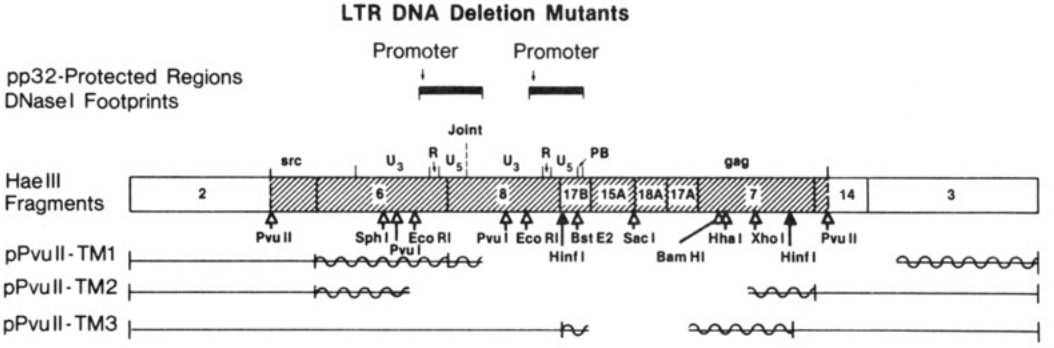

FIGURE 6: Mapping of LTR deletion mutants and illustration of pp32-protected LTR sequences from partial DNase I digestion. The illustration depicts the *Hae*III fragments of the viral insert (shaded area) in pBR322. All of the enzyme restriction sites shown were demonstrated to be present in the appropriate deletion mutants. The waved lines define the fragment of interest (pPvuII-TM1, fragments 3 and 6 in Figure 5; pPvuII-TM2, fragment 3a in Figure 7; pPvuII-TM3, fragment 6a in Figure 8). The open region defines the boundaries of the deletion. The exact boundaries of the deletions were defined by DNA sequencing (see text). See Figure 2 for explanation of viral DNA abbreviations. The top two dark lines designate the region of LTR DNA which pp32 binds as analyzed by DNase I footprinting (see text).

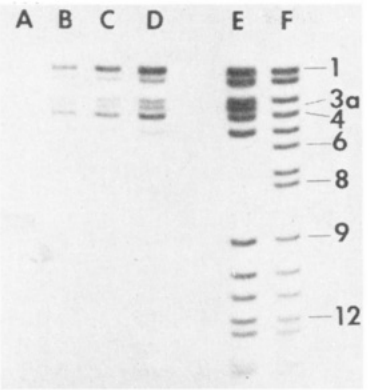

FIGURE 7: Binding of pp32 to deletion mutant pPvuII-TM2. Form I pPvuII-TM2 (0.3 μg) was complexed to various concentrations of pp32, digested with *Hae*III, and filtered as described in Figure 3. Lane A, 0 μg of pp32; lane B, 0.05 μg; lane C, 0.1 μg; lane D, 0.15 μg; lane E, 0.1 μg of *Hae*III-digested pPvuII-TM2; lane F, 0.1 μg of *Hae*III-digested pPvuII-DG. The numbered fragments are the same as described in Figure 3 except 3a which is the same as *Hae*III fragment 6 minus the promoter and adjacent regions (see Figure 6).

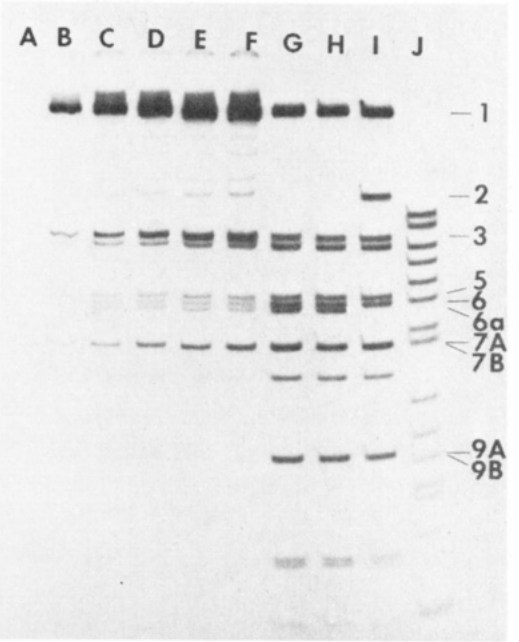

FIGURE 8: Binding of pp32 to deletion mutant pPvuII-TM3. Form I pPvuII-TM3 (0.3 μg) was complexed with various concentrations of pp32, digested with *Hin*fI, and filtered as described in Figure 3. Lane A, 0 μg pp32; lane B, 0.05 μg; lane C, 0.1 μg; lane D, 0.15 μg; lane E, 0.20 μg; lane F, 0.25 μg; lanes G and H, 0.1 μg of pPvuII-TM3 digested with *Hin*fI; lanes I and J, 0.1 μg each of pPvuII-DG digested with *Hin*fI and *Hae*III, respectively. The *Hin*fI fragments are numbered the same as in Figures 2 and 4 except 6a which is the same as *Hin*fI fragment 2 but lacks sequences to the right of the PB site (see Figure 6).

retention of natural *Hae*III fragment 8 nor are they necessary for retention of the neighboring *Hae*III fragment 6.

To determine that the U_3 promoter region adjacent to U_5 was essential for pp32 binding, another deletion mutant was isolated which lacked the *Eco*RI site contained in *Hae*III fragment 6 (Figure 6). This deletion mutant, deisgnated pPvuII-TM2 (Figure 6), lacked the *Eco*RI site but contained the *Pvu*I site (Figure 7, lane E, fragment 3a). The pPvuII-TM2 plasmid lacks viral *Hae*III fragments 6, 7, and 8 while the plasmid-viral fusion *Hae*III fragment 14 (Figure 6) was still intact (Figure 7). The new *Hae*III fragment 3a (499 bp) (Figure 7) contains mostly *src*-U_3 sequences fused to *gag* sequences found in *Hae*III fragment 7. From size and restriction enzyme analysis, the deletion boundary maps between the *Pvu*I and *Eco*RI sites on the left side and 21 bp to the left of the *Xho*I site (Figure 6). *Hae*III fragment 3a also contains the single-cut restriction enzyme sites *Sph*I and *Xho*I, which confirms the identification of this fragment. DNA sequence analysis established that the LTR U_3 sequences terminate 46 bp to the right of the *Pvu*I site (unpublished results). Since the *Eco*RI site is absent, the new *Hae*III fragment 3a does not contain promoter sequences (23 bp to the right of the *Eco*RI site) and the adjacent R sequences which are direct repeats found on both the 5′ and 3′ ends of the viral RNA (Swanstrom et al., 1981). As is evident in Figure 7, pp32 does not preferentially retain fragment 3a in comparison to positive binding control *Hae*III fragments 1 and 4. The binding of pp32 to fragment 3a is comparable to negative binding control *Hae*III fragment 2, 3, and 5 (Figures 2, 3, and 7). The above data from both deletion mutants demonstrate that LTR sequences between the *Eco*RI site in U_3 and the left most region of U_5 (Figure 6) appear essential for preferential binding of pp32 to LTR DNA.

The binding of pp32 to the large viral *Hin*fI fragment 2 (Figure 4) suggested that the enzyme may recognize U_5 sequences and possibly primer-binding (PB) site sequences. A deletion mutant (pPvuII-TM3) which contained both U_5 sequences and the *Bst*E2 restriction site (Figure 6) was isolated. DNA sequence analysis revealed that on the left boundary of pPvuII-TM3, the *Bst*E2 site is intact, and the DNA extends for an additional 8 bp to the right of the PB site. On the right boundary, the U_5–PB site region is fused near the *Hae*III site between fragments 17A and 7, 199 bp to the left of the *Xho*I site (Figure 6). The new fused *Hin*fI fragment was designated 6a (390 bp in length) (Figure 8, lane G or H, sixth band from the top). At various concentrations of pp32, there appears to be no preferential retention of *Hin*fI fragment 6a (Figure 8, lanes B–F). The binding of pp32 to this *Hin*fI fragment 6a was comparable to negative binding control *Hin*fI fragments 4, 5, and 6 (Figures 2 and 4). Preferential binding of pp32 to *Hin*fI fragments 1, 3, 7A, and 7B (Figure 8) are evident as previously shown (Figure 4). These data again demonstrate that U5 sequences whether to the right of the *Hae*III site located between fragments 6 and 8 or in *Hae*III fragment 17B with the PB site do not appear to enhance pp32 binding to DNA (Figure 6).

Binding of RSV pp32 to Viral LTR DNA. Studies were undertaken to determine if pp32 purified from RSV, Prague strain C, had similar DNA binding properties as AMV pp32. The AMV pp32 protein is derived from the leukosis virus helper, MAV-1 and MAV-2, which together constitute ~95% of the AMV complex (Duesberg et al., 1980). The RSV pp32 protein had previously been shown to possess DNA endonuclease activity and appears structurally similar to AMV pp32 (Schiff & Grandgenett, 1980). The properties of poly(U)–Sepharose or glycerol gradient purified RSV pp32 are (i) the RSV protein at the same protein to DNA ratios established for AMV pp32 retains form I pPvuII-DG 2–3-fold better than form III DNA, (ii) RSV pp32 preferentially retained *Hae*III fragments 1, 4, 6, 8, 9, and 12 of pPvuII-DG as previously shown for AMV pp32, and (iii) the RSV protein did not preferentially retain *Hae*III fragment 3a of deletion mutant pPvuII-TM2 which lacked the promoter and R region (unpublished results). From these studies, the RSV pp32 protein appears to have DNA binding properties similar to AMV pp32.

Computer Search for Primary–Secondary Structure Combinations Recognized by pp32. The sequence TATTTAA has been implicated to act as a viral promoter in vivo (G. Gilmartin

Table I: Location of LTR DNA Promoter-like Sequences on the Genome of pBR322[a]

nucleotide position on pBR322	viral promoter TATTTAA	complement of promoter TTAAATA	assigned no. of promoter groups	located on pBR322 *Hae*III fragment
56		TTAAATT	I	9
2255		TTAACTA	II	2
2307		TGAAATA	II	2
3232		TTAAATT	III	4
3237		TTAAAAA	III	4
3251		TTAAATC	III	4
4143		TTGAATA	IV	1
4166		TTCAATA	IV	1
4174	TATTGAA		IV	1
4217	TATTTGA		V	1
4227	TATTTAG		V	1

[a] The first nucleotide of the viral promoter-like sequence in pBR322 and the location of this sequence on a particular pBR322 *Hae*III restriction fragment are identified *according* to Sutcliffe's mapping units (see text). The underlined letter identifies the single mismatched nucleotide. The fourth nucleotide remains unchanged. See Figures 2 and 9 for correlation of promoter-like sequences and palidromic structures capable of forming potential stable hairpins on pPvuII-DG.

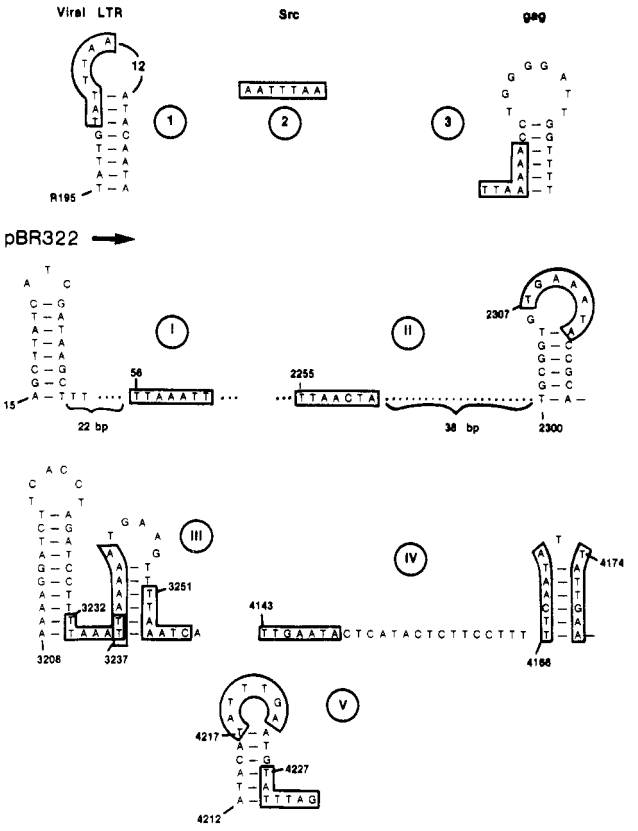

FIGURE 9: TATTTAA-like sequences and potential secondary structures on pBR322. The viral insert in pPvuII-DG and pBR322 DNA were searched for TATTTAA and its complementary sequence allowing zero and one mismatch. One perfect match was found per LTR DNA copy (see sequence 1), 1 "near matches" each in *gag* and *src* (see sequence 2 and 3), and 11 "near matches" in pBR322 (all boxed in). See Table I for classification of pBR322 TATTTAA-like sequences into groups (I–V). See Swanstrom et al. (1981) for identification for LTR DNA numbering (sequence 1) and Figure 2 for relative position of these sequences on the circular map of pPvuII-DG.

and J. T. Parsons, unpublished results) and in vitro (Yamamoto et al., 1980; Mitsialis et al., 1981, 1983). Since the pp32 protein appears to bind to the potential viral promoter region on the LTR DNA as well as several regions on the pBR322 genome, we searched both DNAs for the presence of these heptanucleotide sequences as well as its complementary sequence. Only one exact match was found per copy of the LTR DNA, but not exact matches were found on pBR322. When one mismatch in any of the seven nucleotide positions was allowed for, 11 such sequences were identified in pBR322 and 2 in the cloned retrovirus sequence (Figures 2 and 9). These "near matches" were not randomly distributed in the pBR322 genome but appear to be clustered in five groups (I–V), all of which are located in intercistronic (nontranslated) regions of the pBR322 genome (Table I and Figure 2), known to contain important regulatory elements (Sutcliffe, 1978a,b; Brosuis et al., 1982). Moreover, the location of four of these groups (I, III, IV, and V) containing 9 of the 11 near matches coincide with regions of the pBR322 genome shown to have elevated affinity for the pp32 protein. The two near matches found in the cloned retrovirus DNA were located in the *gag* gene and the *src* gene (Figures 2 and 9). The lack of significant retention of *Hae*III restriction fragment 2 in the filter binding assay, which contains pBR322 group II and *src* promoter-like sequence 2 (Figures 2 and 3), or *Hae*III fragment 6a of pPvuII-TM2 containing *gag* promoter-like sequence 3 (Figures 2, 6, and 8) suggests that the heptanucleotide sequence alone is not sufficient for binding of the pp32 protein, and additional factors seem to contribute to the affinity of a given DNA sequence for this protein.

The finding that the pp32 protein apparently binds with a higher affinity to form I DNA rather than form III DNA suggests that the secondary structure may be one of these contributing factors. Since we have previously shown that intercistronic regions in pBR322 DNA contain an elevated potential for statistically significant hairpin (cruciform) structures (perfectly based paired stems, no G·T pairs, at least 6 bp in the stem, 3–20 nucleotides in the loop) (Müller & Fitch, 1982), we have searched both the pBR322 genome and the cloned retrovirus sequence for potential overlaps of such structures with heptanucleotides shown in Table I. The only perfect match (LTR DNA sequence 1), the near match in the *gag* gene (sequence 3), and all near matches in the pBR322 genome either overlap or are in close proximity to sequences which have the potential to form statistically significant hairpin structures (Figure 9). Since there are only 3 such hairpin structures (Müller & Fitch, 1982) per viral LTR, 7 in the *gag* sequence, and 27 in the pBR322 genome, the TATTTAA sequence–structure overlap appears to be more than coincidental.

Other Studies on the AMV pp32 DNA Endonuclease Activity. The pp32 DNA endonuclease prefers form I DNA as substrate, and Mn^2 is much preferred over Mg^{2+} as required divalent metal ion (Grandgenett et al., 1978). We have further characterized other properties of the DNA endonuclease activity. The pp32 protein lacks detectable 5′ or 3′ DNA exonuclease activities with highly end-labeled substrates. pPvuII-DG was cut with *Eco*RI and the 901, 435, and 330 bp viral DNA restriction fragments (Figure 2) were labeled on the 5′ end with [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase or on the 3′ end with α-^{32}P-labeled deoxynucleotides (2400 Ci/mmol) and *E. coli* DNA polymerase I. The latter labeling procedure resulted in blunt-end molecules. After extended periods of incubation at 37 °C using varying concentrations of pp32 with these DEAE-cellulose purified end-labeled fragments, no acid-soluble counts could be detected.

The pp32 protein in the presence of Mg^{2+} or Mn^{2+} generates 3′-OH groups upon nicking of unlabeled pBR322 or SV-40

form I DNA. The protein/DNA ratios were varied so that 15% or 60% of form I DNA was converted to form II DNA. The DNA was then treated with phenol and extracted with ether. Following ethanol precipitation, both samples were nick-translated at 14 °C by *E. coli* DNA polymerase I. After 10 min of incubation with ^{32}P-labeled dCTP and unlabeled dGTP, dATP, and dTTP, both pp32-nicked DNA species acted as primers for *E. coli* DNA polymerase I, confirming that 3′-OH groups were generated by pp32. The incorporation of labeled triphosphate was 2-fold and 5-fold over background with the conversion of form I DNA to 15% and 60% form II DNA, respectively. The DNA endonuclease of $\alpha\beta$ DNA polymerase also generates 3′-OH groups (unpublished results), in the presence of Mn^{2+} (Golomb & Grandgenett, 1979; Nissen-Meyer et al., 1981) or when the polymerase was subjected to partial proteolytic cleavage resulting in a Mg^{2+}-dependent endonuclease activity (Grandgenett et al., 1980).

In the presence of Mg^{2+}, pp32 DNA endonuclease prefers form I DNA as substrate in comparison to equivalent linear single-stranded or form III DNA. Under our assay conditions AMV pp32 does not nick form I pPvuII-DG, SV-40, or pBR322 DNA at specific sites although only form II DNA is produced (Grandgenett et al., 1978; see Discussion) (unpublished data).

Discussion

The apparent preferential retention of form I DNA on nitrocellulose by pp32 and the preference of these molecules as substrates for pp32 DNA nicking activity suggest, but do not prove, that secondary structures in DNA may partially determine the binding of this viral protein in vitro. The nicking of form I DNA by pp32 (Grandgenett et al., 1978) or by its presumed parental molecule $\alpha\beta$ (Golomb & Grandgenett, 1979) under limited enzyme concentrations results in circular molecules containing only one nicked strand. This mode of action is similar to the first step of S1 nuclease nicking of form I DNA molecules. That is, nicking by S1 is limited to one of two strands preferentially at cruciform structures on certain form I DNAs (Beard et al., 1973; Panayotatos & Wells, 1981). In contrast to S1 activity, the pp32-nicked circular DNA is not cleaved by pp32 on the opposite strand at or near nicks to yield a form III molecule (Grandgenett et al., 1978). Interestingly, the β_2 form of polymerase from RSV or AMV can cleave both DNA strands resulting in the formation of form III DNA (Hizi et al., 1982; Leis et al., 1983), which the $\alpha\beta$ enzyme cannot do in the presence of Mg^{2+} (Golomb & Grandgenett, 1979; Nissen-Meyer et al., 1981; Hizi et al., 1982; Leis et al., 1983).

Alkaline sucrose gradient centrifugation analysis of ColE_1 DNA, which was nicked by pp32 in the presence of Mg^{2+} and subsequently digested with *Eco*RI, suggested a limited number of preferred regions for pp32 endonuclease activity on this DNA (Grandgenett et al., 1978). Using more stringent analytical methods, i.e., agarose or polyacrylamide gel electrophoresis, we have found that the pp32 DNA endonuclease apparently nicks SV-40, pBR322, and pPvuII-DG at random sites. In all cases, no specific size fragments were generated by pp32 when the nicked DNA was subjected to single-cut restriction enzymes, which would order the pp32-nicked sites on form I molecules. It is difficult to assess the specificity of the DNA endonuclease activity of pp32 on form I pPvuII-DG because there are at least six preferential binding sites on this molecule for pp32, thereby resulting in at least six possible nicked regions. Different assay substrates and conditions not previously used may permit pp32 to specifically generate a staggered nick at the region of covalently closed LTR ends on circular retrovirus DNA, thereby suggesting an integrative role for this protein.

We have previously demonstrated that AMV pp32 protects a specific region of avian retrovirus LTR DNA from partial DNase I digestion (Misra et al., 1982) as analyzed by the DNase I footprinting procedure of Galas & Schmidt (1978). The protected region of this complex on the 275 bp end-labeled restriction fragment (see Figure 2) was a stretch of 170 ± 10 nucleotides that encompasses the viral promoter (23 bp to the right of the *Eco*RI site) region in U_3 extending through U_5 and proceeds past the U_5–U_3 joint for ~34 bp (see Figure 6 for illustrated region protected by pp32 using this assay). Partial DNase I treatment of the 275 bp fragment complexed with pp32 (possibly tetramic structures are formed) gave upon electrophoresis in denaturing gels a pattern of protected and enhanced regions similar to that observed with *E. coli* DNA gyrase–DNA complexes (Kirkegaard & Wang, 1981) or eucaryotic nucleosome structures (Lutter, 1979; Rhodes & Klug, 1980). While, the viral promoter and its surrounding regions (17–20 nucleotides total) were almost completely protected by pp32 from DNase I digestion (Misra et al., 1982), the adjacent fragments (435 and 55 bp; Figure 2) were not protected from DNase I digestion by pp32. Possibly, the initial binding site for formation of the pp32–LTR DNA complexes involves the viral promoter region or the immediately adjacent R sequences, or both, because (i) changing of sequences (insertion of 21 bp of foreign DNA) at the U_5–U_3 joint prevented pp32 from protecting the surrounding viral DNA sequences from DNase I digestion but did not affect pp32 protection of the viral promoter region and its adjacent downstream sequences (Misra et al., 1982), (ii) there was no apparent preferential binding of pp32 to pPvuII-TM3 deletion mutant *Hin*fI fragment 6a which contained the 3′ sequences of U_5 and the PB site (Figure 8), (iii) pp32 specifically recognized and retained *Hae*III fragment 6 (containing promoter region and R sequences) which lacks the 3′ terminus of U_5 and the corresponding U_5–U_3 joint (Figure 4), and (iv) deletion of the viral promoter region and adjacent R sequences eliminated the ability of pp32 to preferentially retain fragment 3a in deletion mutant pPvuII-TM2 (Figure 7). Like the SV-40 large T antigen which has strong and weak binding sites on a control region of SV-40 DNA thereby modulating two essential viral activities, replication and transcription (Tegtmeyer et al., 1975; Alwine et al., 1977; Myers et al., 1981; Tegtmeyer et al., 1983), the pp32 protein may have dual functions by binding to the conserved region of LTR DNA.

Other highly specific DNA binding proteins likewise bind to DNA at random locations besides at unique sequences (Schmitz & Galas, 1980; Strauss et al., 1981; Berg et al., 1981; Montenark & Henning, 1982; Hamilton et al., 1981). This also appears to be the case with the partially phosphorylated pp32 protein. Assuming that pp32 preferentially recognizes viral promoter or promoter-like sequences, the surrounding nonpromoter DNA may facilitate pp32 binding to promoter regions by a translocation mechanism similar to that proposed for *E. coli lac* repressor–operator or *E. coli* RNA polymerase–promoter (Hamilton et al., 1981; Winter & von Hippel, 1981) interactions. Because of initial nonspecific binding of protein to DNA, the search process for a specific target site is reduced resulting in an increased apparent rate of binding to specific target locations. We have no definitive evidence at this time that pp32 is capable of translocation. However, consistent with a translocation mechanism, the pp32 protein appears to favor form I, form II, or form III pPvuII-DG DNA molecules as substrates for retention of the specific six *Hae*III

DNA fragments to filters (Figure 3) rather than the more numerous and smaller *Hae*III fragments (a total of 26) of the same DNA as substrate for binding to pp32.

The preferential retention of form I supercoiled pPvuII-DG over the linearlized form of this DNA by pp32 is at best 2–3-fold (Figure 1). The selection of the six pPvuII-DG *Hae*III fragments by pp32 (Figure 3) whether the DNA substrate is form I, form II, or form III DNA suggests that primary DNA sequence is one of the major parameters in defining pp32-binding properties. The presence of any of these three forms of DNA in the filter binding assay followed by restriction enzyme cleavage did not markedly enhance the ability of pp32 to select the six *Hae*III fragments.

Presently, the precise mechanisms involved in the recognition of specific bacterial plasmid DNA sequences and the viral LTR DNA sequences by pp32 are unknwon. Sequence information, however, appears to play a major role in defining pp32 binding to viral and nonviral DNAs. We have demonstrated that pp32 selectively binds to viral fragments containing LTR sequences and not to fragments containing *gag* information or to fragments comprising almost the entire SRA *src* gene (this report; Misra et al., 1982) (unpublished data). Computer analysis of sequence homologies between LTR DNA and pBR322 involving viral promoter sequences as well as structural features (hairpins and loops) suggests that pp32 may recognize these viral promoter-like sequences in pBR322 (Table I). However, there are several exceptions. On the basis of the filter binding assay, pp32 recognizes *Hae*III fragment 9 (Figure 2) which lacks viral promoter-like sequences or significant secondary structures. Second, even though pPvuII-DG *Hae*III fragment 2 contains two viral promoter-like sequences (Figure 2), this fragment appears not to be preferentially recognized by pp32. Clearly, not all of the parameters for pp32 binding to a specific set of viral LTR DNA sequences or corresponding pBR322 fragments are defined. Other factors such as A-T richness may be of influence.

The biological function(s) of the pp32 protein or its corresponding moiety located at the carboxyl terminus of the β subunit of the avian retrovirus polymerase is (are) undefined. The 62 000-dalton α subunit, derived from the amino terminus of the 92 000-dalton β polypeptide, contains DNA polymerase and RNase H activities whose functions appear distinct in viral DNA synthesis (Gerard & Grandgenett, 1980). A nonconditional RSV mutant isolated from RSV-transformed quail cells and containing a large deletion in the carboxyl terminus of the polymerase gene produces noninfectious virus particles and encodes a *gag-pol* polyprotein containing only α subunit but not pp32 determinants (Mason et al., 1979, 1982). Generation of a variety of small deletions (4 to ~50 bp) at several unique sites in the carboxyl-terminal region of the polymerase gene in cloned infectious RSV Prague A DNA eliminates the ability of this DNA to produce infectious virions upon transfection of this DNA into chick embryo fibroblast cells (P. Hippenmeyer and D. Grandgenett, unpublished results). These studies suggest that the pp32 DNA binding protein or the pp32 moiety of the β subunit, or both, is required in the life cycle of avian retroviruses (Golomb et al., 1981).

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Structural Studies of Apolipoprotein A-I/Phosphatidylcholine Recombinants by High-Field Proton NMR, Nondenaturing Gradient Gel Electrophoresis, and Electron Microscopy†

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ABSTRACT: Complexes formed between apolipoprotein A-I (apo A-I) and dimyristoylphosphatidylcholine (DMPC) or egg phosphatidylcholine have been studied by high-field ^{1}H NMR, nondenaturing gradient gel electrophoresis, electron microscopy, and gel filtration chromatography. Emphasis has been placed on an analysis of the particle size distribution within the micellar complexes produced at lipid/protein molar ratios of 40-700. As determined by electron microscopy and gel filtration of DMPC/apo A-I complexes, the size of the discoidal micelles produced appears to increase uniformly with an increasing lipid/protein ratio. By electron microscopy, the diameters of isolated DMPC/apo A-I discoidal micelles range from approximately 89 Å at a 40 molar ratio to 205 Å at a 700 molar ratio. Analysis of the micellar complexes by ^{1}H NMR shows that concomitant with the increase in size is the progressive downfield shift of the choline *N*-methyl proton resonance of the complex which is observed from 3.245 to 3.267 ppm over the above molar ratio range. The relationship between chemical shift and micelle size is most simply interpreted as arising from a weighted averaging of two lipid environments—lipid–lipid and lipid–protein. In contrast to the above interpretation of the gel filtration experiments on DMPC/apo A-I complexes, nondenaturing gradient gel electrophoresis analysis of particle size distribution leads to an unexpected observation: as the DMPC/apo A-I ratio increases, discrete complexes of increasing size are formed in an apparently quantized manner. A mechanism is proposed for this incremental increase in size that involves a combination of conservative changes in the stoichiometry and conformation of the lipid-associating domains (amphipathic helices) of apo A-I.

The lipid-associating properties of apolipoprotein A-I, the major protein of high-density lipoprotein (HDL),[1] have been widely studied both as a specific model for assessing the structure and function of HDL and as a general model for investigating protein–lipid interactions. A widely studied model system is apolipoprotein A-I/DMPC (Middelhoff et al., 1976; Pownall et al., 1978; Swaney & Chang, 1980; Morrisett et al., 1977; Jonas et al., 1977; Tall et al., 1977). In this system, apo A-I interacts spontaneously with aqueous dispersions of DMPC near the gel to liquid-crystalline phase transition to form small micellar complexes. The ability of apo A-I to spontaneously associate and form stable complexes with phospholipid is a phenomenon observed with exchangeable

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[1] Abbreviations: apo, apolipoprotein; DMPC, dimyristoylphosphatidylcholine; EM, electron microscopy; HDL, high-density lipoprotein; ^{1}H NMR, proton nuclear magnetic resonance; LCAT, lecithincholesterol acyltransferase; MLV, multilamellar vesicle(s); PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; SUV, small unilamellar vesicle(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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