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Avian Retrovirus pp32 DNA-Binding Protein
I. Recognition of Specific Sequences on Retrovirus DNA Terminal Repeats

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The avian retrovirus pp32 protein possesses a DNA-nicking activity which prefers supercoiled DNA as substrate. We have investigated the binding of pp32 to avian retrovirus long terminal repeat (LTR) DNA present in both supercoiled and linear forms. The cloned viral DNA was derived from unintegrated Schmidt-Ruppin A (SRA) DNA. A subclone of the viral DNA in pBR322 (termed pPvuII-DG) contains some *src* sequences, tandem copies of LTR sequences, and partial *gag* sequences in the order *src*-U_3 U_5:U_3 U_5-*gag*. Binding of pp32 to supercoiled pPvuII-DG DNA followed by digestion of this complex with a multicut restriction enzyme (28 fragments total) permitted pp32 to preferentially retain on nitrocellulose filters two viral DNA fragments containing only LTR DNA sequences. In addition, pp32 also preferentially retained four plasmid DNA fragments containing either potential promoters or Tn*3* "left-end" inverted repeat sequences. Mapping of the pp32 binding sites on viral LTR DNA was accomplished by using the DNase I footprinting technique. The pp32 protein, but not the avian retrovirus αβ DNA polymerase, is able to form a unique protein-DNA complex with selected regions of either SRA or Prague A LTR DNAs. Partial DNase I digestion of a 275-base pair SRA DNA fragment complexed with pp32 gives upon electrophoresis in denaturing gels a unique ladder pattern, with regions of diminished DNase I susceptibility from 6 to 10 nucleotides in length, in comparison with control digests in the absence of protein. The binding of pp32 to this fragment also yields enhanced DNase I-susceptible sites that are spaced between the areas protected from DNase I digestion. The protected region of this unique complex was a stretch of 170 ± 10 nucleotides that encompasses the presumed viral promoter site in U_3, which is adjacent to the *src* region, extends through U_5, and proceeds past the joint into U_3 for about 34 base pairs. No specific protection or DNase I enhancement by pp32 was observed in experiments with a 435-base pair SRA DNA fragment derived from a part of U_3 and the adjacent *src* region or a 55-base pair DNA fragment derived from another part of U_3. The DNA sequence of Prague A DNA at the fused LTRs differs from that of SRA DNA. The alteration in the sequence at the juncture of the LTRs prevented pp32 from forming a stable complex in this region of the LTR. Our results are relevant to two aspects of the interaction between pp32 and LTR DNA. First, the pp32 protein in the presence of selected viral DNA restriction fragments possibly forms a higher order oligomer analogous to *Escherichia coli* DNA gyrase-DNA complexes or eucaryotic nucleosome structures. Second, the specificity of the binding suggests a role for pp32 and the protected DNA sequences in the retrovirus life cycle. The preferred sequences to which pp32 binds include two adjacent 15-base pair inverted terminal repeats at the joint between U_5 and U_3 in SRA DNA. This region is involved in circularization of linear DNA and is perhaps the site that directs integration into cellular DNA.

The analysis of avian cells infected with avian retroviruses has shown that replication of viral DNA commences in the cytoplasm of the cell within 1 h after infection and proceeds with the synthesis of a linear duplex DNA followed by the formation of a covalently closed circular form found exclusively in the nucleus (26, 30, 40). Linear cytoplasmic avian retrovirus DNA is

slightly larger than unit length because it contains two long direct repeats (330 base pairs [bp]), one at each terminus (12, 29). Each long terminal repeat (LTR) is composed of nucleotide sequences unique to the 5′ and 3′ ends (U_5 and U_3) of the viral RNA genome. Possible mechanisms involved in the biosynthesis of linear viral DNA have been suggested by Gilboa et al. (4). The nucleus contains linear viral DNA as well as two classes of covalently closed circular viral DNA. The circular DNAs differ only in the number of copies of the LTR. The smaller species contains a single copy of the LTR, whereas the larger species contains a tandem arrangement of LTRs joined together in the arrangement *src*-U_3 U_5:U_3 U_5-*gag*. Which of the three viral DNA forms is the immediate precursor to the integrated provirus still remains to be clarified.

Retroviruses involved in the natural infectious process integrate their DNA such that the integrated viral DNA is colinear with unintegrated linear viral DNA (12, 26, 29). The cellular site for virus integration is apparently not specific, although direct sequencing of the cellular DNA around the integrated retrovirus genome is necessary to rule out regional specificity (2, 32, 33, 39).

Molecular cloning of retrovirus DNA and subsequent DNA sequencing studies have permitted a direct comparison of the structure of the retrovirus terminal repeats with the structures of eucaryotic and procaryotic transposable elements (1). The most striking revelation is the close structural relationship which exists between retrovirus DNA and transposable elements (2, 32, 33, 37, 39). Transposable elements and retrovirus DNA share the following properties, although there are some variations within each system. Both systems apparently utilize nonhomologous integration mechanisms; their DNAs are flanked by either long inverted or direct repeat elements that have short terminal inverted repeat structures; and upon integration of transposon DNA or of retrovirus DNA there is a loss of several base pairs from their termini, and a small number of base pairs at the recipient site are reiterated at both ends of the inserted DNA.

Presently, no retrovirus proteins have been directly implicated in integration of retrovirus DNA. Our biochemical studies reported here suggest that retroviruses encode a protein which might participate in viral DNA circularization or integration because of its preferential binding to selected regions of retrovirus LTR DNA. The partially phosphorylated protein, termed pp32, is apparently derived from the β subunit of the avian retrovirus RNA-directed DNA polymerase by a proteolytic cleavage event in vivo (6, 27). It possesses a Mg^{2+}-dependent DNA endonuclease activity which introduces nicks in supercoiled DNA, its preferred substrate, generating only unit-length DNA (5, 8, 27). A presumed virus-coded DNA endonuclease biochemically similar to pp32 isolated from Friend (22) and Rauscher (16) murine leukemia viruses has recently been identified. We exploited the recent advancement of DNase I footprinting (3) to study protein-DNA complexes and the nitrocellulose filter binding assay (14) to examine the binding of pp32 to retrovirus LTR DNA and other nonviral DNA. DNase I footprinting identifies regions of DNA that are protected from partial DNase I digestion by bound protein. We demonstrated by the footprinting technique that avian myeloblastosis virus (AMV) pp32 appears to form a unique protein-nucleic acid complex with selected regions of restriction enzyme fragments derived from cloned Schmidt-Ruppin A (SRA) or Prague A (PrA) viral DNA containing LTR sequences. Using the nitrocellulose filter binding assay, we also showed that pp32 is able to preferentially bind to several regions on a supercoiled subclone of the viral DNA in pBR322 (pPvuII-DG) containing tandem copies of LTR DNA sequences (38). Digestion of preformed pp32-supercoiled pPvuII-DG complexes by a multicut restriction enzyme followed by filtration on nitrocellulose filters permitted the identification of specific pp32-bound DNA fragments. In addition to two DNA fragments containing viral LTR DNA sequences, pp32 also preferentially retained four pBR322 DNA fragments containing either promoters or Tn*3* "left-end" inverted repeat sequences.

MATERIALS AND METHODS

DNAs. A subclone of SRA, pPvuII-DG, which contained two complete copies of the LTR DNA in tandem (38), was generously supplied by J. M. Bishop (University of San Francisco, San Francisco, Calif.) and colleagues. A clone containing the complete PrA Rous sarcoma virus (RSV) genome (pS1-102) (10) and a subclone (pXBm-102) containing the *src* gene and tandem copies of LTR DNA were constructed in the plasmid vector pBR322 (Gilmartin and Parsons, unpublished data).

Preparation of labeled DNAs. The SRA viral DNA, inserted into pBR322 DNA at the *Pvu*II position, was cut with *Eco*RI. The fragments were end labeled with [γ-^{32}P]ATP (13) by T4 polynucleotide kinase. The labeled DNA was digested with *Pvu*II, yielding three different labeled viral DNA restriction fragments of 330, 435, and 901 bp in length (see Fig. 1). The fragments were separated on 5% acrylamide gels, eluted, and purified by DEAE-cellulose chromatography. The double-labeled 330-bp fragment was subsequently cleaved with *Pvu*I, generating 55- and 275-bp fragments. To generate the 275-bp DNA fragment labeled on the 5′ end of the minus viral DNA strand, cloned DNA was first cleaved with *Pvu*I, labeled with

[γ-^{32}P]ATP by T4 kinase, and digested with *Eco*RI. Restriction fragments from the PrA RSV clones were prepared in a manner similar to that described above, except that an additional *Hin*dIII site, which is located at the joint of the terminal repeats, was also labeled (10).

The pPvuII-DG plasmid was uniformly labeled by growing the bacteria in a low-phosphate-containing medium with $^{32}P_i$. The plasmid DNA was purified by two successive CsCl buoyant density gradient sedimentation steps and by velocity sedimentation through sucrose (8). The specific activity of the initially purified supercoiled DNA varied from 20,000 to 35,000 cpm per μg of DNA. The preparations of supercoiled DNA utilized were always greater than 95% supercoiled.

Nitrocellulose filter binding assay. Nitrocellulose filters were treated in 0.4 M potassium hydroxide for 20 min at 21°C, washed extensively with distilled water, and neutralized with 0.1 M Tris-hydrochloride (pH 7.4) (17, 34). The filters were stored in sterile distilled water, and each was washed with 6 ml of the appropriate wash buffer before use.

The standard reaction mixture for DNA binding contained 20 mM Tris-hydrochloride (pH 7.5 at 25°C), 0.1 M NaCl, 3 mM dithiothreitol, 0.2 mM EDTA, and bovine serum albumin at 50 μg per ml in a volume of 100 μl. The appropriate DNA was added, and the mixture was equilibrated at 37°C. The binding reaction was initiated by the addition of the pp32 protein (1 to 5 μl). After incubation (usually 3 to 5 min), the reaction mixture was diluted with 1 ml of 20 mM Tris-hydrochloride (pH 7.4)–0.1 M NaCl–3 mM dithiothreitol–0.2 mM EDTA (wash buffer) and filtered at a flow rate of 1 to 2 ml per minute. The filters were then washed with an additional 1 ml of 0.1 M NaCl buffer, dried, and counted with an organic scintillation fluid. Background counts due to DNA retained on the filter in the absence of pp32 were always subtracted.

The DNA retained by pp32 on the nitrocellulose filters or the filtrates was also examined by polyacrylamide gel electrophoresis. ^{32}P-labeled DNA in the filtrates was quantitatively coprecipitated with yeast tRNA (7.5 μg) and the DNA retained by pp32 on the filters was eluted at 37°C for 3 h with 0.3 ml of 0.2% sodium dodecyl sulfate–0.02 M Tris-hydrochloride (pH 7.5)–0.3 M sodium acetate containing yeast tRNA (4 μg). After ethanol precipitation, the pellets were suspended in 50 μl of buffer and subjected to electrophoresis on 5% polyacrylamide gels. The gels were dried and exposed to Kodak XR-5 X-ray film in the presence or absence of DuPont Lightning Plus intensifying screens at −70°C.

The binding of pp32 to supercoiled pPvuII-DG DNA before digestion of this complex by *Hae*III or other restriction enzymes was similar to the standard binding reaction. The pp32 protein was complexed to ^{32}P-labeled DNA at 37°C for 5 min in the absence of divalent metal ion followed by addition of a 5- to 10-fold excess of the *Hae*III restriction enzyme as recommended by the supplier (New England Biolabs) to digest the DNA (~0.3 μg). The divalent metal ion, Mg^{2+} (100 μl of a 2 mM solution), was immediately added, and the reaction mixture was incubated for an additional 5 to 10 min. The restriction enzyme digestion was stopped by the addition of 5 mM EDTA. The sample was diluted with 1 ml of 0.1 M NaCl wash buffer, filtered, and washed with an additional 1 ml of buffer. The DNA retained on the filter was eluted as described above. As a control for nonspecific binding of the restriction enzyme to DNA, the enzyme was incubated with the DNA as described above in the absence of pp32.

DNase I footprinting. The partial DNase I digestion procedures used were similar to those described for the phage λ integrase (24). Approximately 5 to 15 ng of end-labeled DNA fragment was incubated with various amounts of pp32 DNA-binding protein (2.75 to 12 μg/ml) in 40 μl of reaction buffer containing 10 mM Tris-hydrochloride (pH 7.5), 1 mM $MgCl_2$, 5 mM dithiothreitol, 500 μg of bovine serum albumin per ml, and 5% glycerol. The DNA samples were preincubated at 20°C for several minutes followed by addition of pp32. After an additional 3 min to allow for pp32 binding to DNA, the DNA was digested with pancreatic DNase I (Worthington Diagnostics) at a concentration of 0.25 μg/ml for either 2 or 2.5 min at 20°C. The reaction was quenched with 3.4 μg of sonicated calf thymus DNA in the presence of 10 μg of *Escherichia coli* tRNA and 10 mM EDTA and was followed by phenol extraction and ethanol precipitation. The amount of end-labeled fragments layered on a particular gel varied only by approximately 10% in any lane. In some experiments, the binding of pp32 to DNA was challenged by the addition of heparin (2- to 5-fold molar excess over pp32) for 2 min before DNase I digestion.

Electrophoresis conditions. The chemical sequence markers for the various restriction fragments were prepared as described by Maxam and Gilbert (20). The DNA sequencing gels were 8, 10, or 15% polyacrylamide–8 M urea (0.5 mm thick, 34 cm long). The electrophoresis buffer used was 90 mM Tris-borate (pH 8.0)–2 mM EDTA. The DNA samples were denatured with 7 M urea in the electrophoresis buffer at 90°C for 5 min. Electrophoresis was carried out at 1,200 V, and the gels were exposed at −70°C to X-ray film, with or without intensifying screens.

Purification of AMV pp32. The pp32 protein w[illegible] purified from AMV as described previously (8).

Purification of AMV αβ DNA polymerase. The AMV αβ DNA polymerase was purified by phosphocellulose and heparin-Sepharose chromatography (7).

RESULTS

Retention of DNA restriction enzyme fragments by AMV pp32 on nitrocellulose filters. To determine whether AMV pp32 could selectively retain avian retrovirus LTR DNA fragments over plasmid DNA fragments on nitrocellulose filters, pPvuII-DG was digested with *Hae*III restriction enzyme. The pBR322 DNA contains a 1,670-bp viral insert (38) consisting of some *src* sequences, tandem copies of LTR DNA, and *gag* sequences (Fig. 1). The *Hae*III-digested fragments were incubated with various amounts of pp32 under standard assay conditions (see above) and filtered on nitrocellulose (Fig. 2). At low protein-to-DNA ratios (Fig. 2; lanes B, C, and D), pp32 appears to retain six fragments (numbered 1, 4, 6, 8, 9, and 12) on the filter slightly better than other corresponding DNA

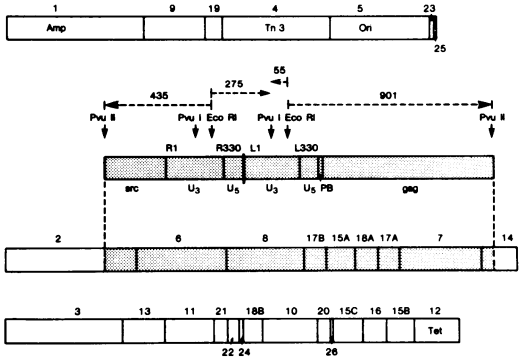

FIG. 1. *Hae*III restriction fragments of pPvuII-DG DNA. pPvuII-DG was digested with *Hae*III, and the combined SRA- and pBR322-generated fragments are illustrated on the blocked lines 1, 3, and 4 (35, 36, 38; J. M. Bishop, personal communication). Fragments are ordered by size. Fragment 1 contains the proposed promoter for ampicillin (Amp) and proceeds through the Tn*3*-region, the origin of replication (Ori), and the viral insert and terminates at the tetracycline gene (Tet). The shaded region in line 3 indicates viral DNA sequences inserted at the *Pvu*II position of pBR322. The second blocked line illustrates the fragments used in our DNase I footprinting experiments. The 1,670-bp viral DNA subclone has two direct tandem repeat copies designated R and L. The numbers R1 and R330 refer to the terminal repeat sequences adjacent to the *src* region on the right end of the linear map of unintegrated avian retrovirus DNA, whereas the numbers L1 and L330 refer to the terminal repeat adjacent to the *gag* region on the left end of the linear map. Only part of the *src* and *gag* sequences are present in this subclone. The joint identifies the probable blunt end joining of linear DNA in vivo (38). 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 the 5′ terminus (U_5) of the viral genome. The *Eco*RI, *Pvu*I, and *Pvu*II restriction sites of this subcloned DNA are shown. End-labeled fragments identified as 901, 55, 275, and 435 bp were obtained by cutting the inserted viral DNA with *Eco*RI, followed by dephosphorylation, end labeling, and cutting with the appropriate restriction enzymes as shown in the diagram. The 275- and 901-bp fragments were labeled on the plus viral DNA strand, and the others were labeled on the minus viral DNA strand. The dashed lines with arrows indicate the direction for reading of sequencing gels.

fragments similar in size. Fragments 6 and 8 retained by pp32 contain viral LTR DNA sequences (Fig. 1). The other four pBR322 fragments preferentially retained by pp32 contain promoter sequences for either ampicillin (fragment 1) or tetracycline (fragment 12), sequences for the left-end inverted repeat of Tn*3* (fragment 4), and sequences encoding the carboxyl terminal end of the β-lactamase gene (fragment 9) (35, 36). The slight preferential retention of these six fragments over the other fragments is less evident at higher protein-to-DNA ratios (Fig. 2; lanes E and F). In the absence of pp32, little or no DNA is retained on the filter (Fig. 2; lane A).

Specificity of pp32 binding sites on supercoiled pPvuII-DG. The topography of the DNA, i.e., supercoiling, may enhance pp32 recognition of sequences contained in the six fragments described above relative to the larger set of fragments (a total of 28). Supercoiled DNA is the preferred substrate for the pp32-associated DNA endonuclease activity (8). The pp32 protein was bound to supercoiled pPvuII-DG at several different protein-to-DNA ratios in the absence of Mg^{2+} (Fig. 3). The quantity of pp32

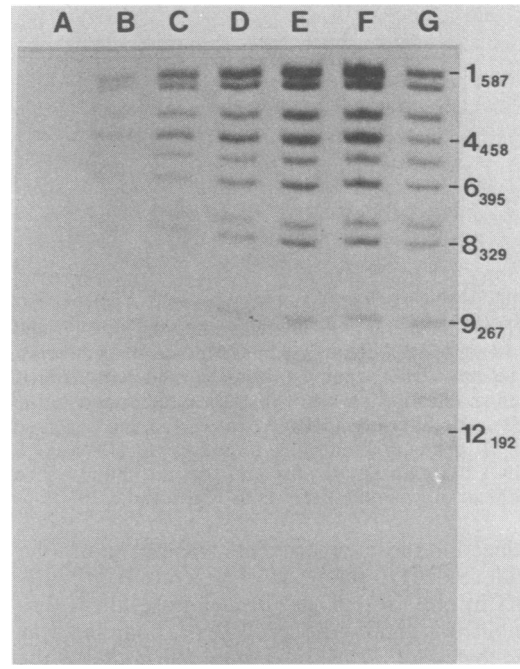

FIG. 2. Binding of pp32 to *Hae*III restriction fragments of pPvuII-DG DNA. Various amounts of pp32 protein were incubated with 0.5 μg of *Hae*III fragments derived from pPvuII-DG DNA under standard assay condition (see text), except these filtered samples were each washed with 3 ml of 0.1 M NaCl buffer instead of the usual 1 ml buffer wash. The pp32-retained DNA was eluted and analyzed on 5% polyacrylamide gels. The numbers on the right identify the six fragments preferentially retained by pp32 and are numbered according to size. The numbered fragments are 587, 458, 395, 329, 267, and 192 bp in length, respectively (indicated as subscripts to the fragment number). The complete *Hae*III digestion pattern of pPvuII-DG DNA is shown in Fig. 1. The molecular weights of these fragments were compared to other known molecular-weight standards. Lane A, 0 μg of pp32; lane B, 0.13 μg; lane C, 0.24 μg; lane D, 0.36 μg; lane E, 0.50 μg; lane F, 0.63 μg. Lane G contains 0.09 μg of control *Hae*III-digested fragments which were not filtered.

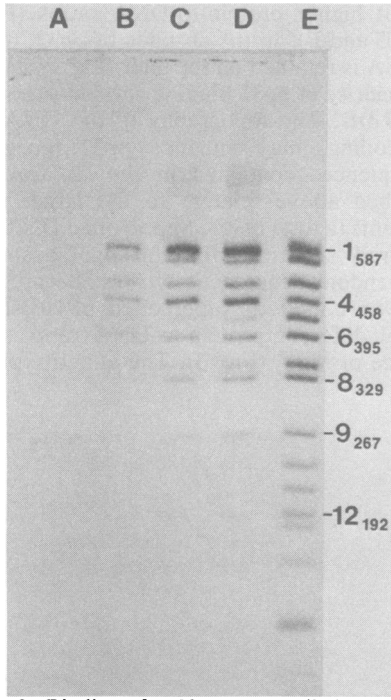

FIG. 3. Binding of pp32 to supercoiled pPvuII-DG followed by *Hae*III digestion. Supercoiled pPvuII-DG DNA (0.32 μg) was incubated with 0, 0.056, 0.11, and 0.18 μg of pp32 (lanes A, B, C, and D, respectively). The pp32-DNA complex was digested with *Hae*III before filtration on nitrocellulose as described in the text. The filter-bound DNA was eluted and analyzed on 5% polyacrylamide gels. Lane E is *Hae*III-digested DNA (0.09 μg DNA) only and was not filtered. The nomenclature is the same as in Fig. 1 and 2.

utilized in these experiments was not saturating with respect to retention of supercoiled pPvuII-DG by the protein on nitrocellulose filters (unpublished data). The pp32-DNA complex was incubated at 37°C for 5 min followed by the simultaneous addition of *Hae*III restriction enzyme and Mg^{2+}. After 10 min of further incubation, the samples were filtered on nitrocellulose and washed. The DNA retained on the filters was extracted and analyzed on 5% polyacrylamide gels. The *Hae*III enzyme retained little DNA on the filter by itself (Fig. 3; lane A). Binding of pp32 to supercoiled DNA followed by digestion with a multicut restriction enzyme resulted in enhanced retention of fragments 1, 4, 6, 8, 9, and 12 by pp32 relative to the other fragments (Fig. 3; lanes B, C, and D). The preferential retention of these fragments by pp32 appears similar at several different protein-to-DNA ratios (10:1, 20:1, and 30:1) (Fig. 3; lanes B, C, and D, respectively). No fragments smaller than the fragment 12 specifically retained by pp32 were evident upon prolonged exposure of the gel. Quantitative analysis of gel scans in Fig. 3 or actual counting of the labeled DNA fragments revealed that pp32 binds to sequences in these six fragments approximately two- to three-fold better (molar basis) than the other nonselectively retained fragments. The selectivity of pp32 binding with the above filter assay to these six *Hae*III fragments confirms data which will be presented in greater detail elsewhere (V. Parsons and M. Golomb, submitted for publication).

Like numerous other highly specific nucleic acid binding proteins which bind to DNA at random locations (9, 17, 28), pp32 retains some of the other 22 *Hae*III DNA fragments on nitrocellulose filters, but at a much decreased capacity as compared to the 6 fragments described above (Fig. 2 and 3). This apparent nonspecific binding to DNA by pp32 is more evident at high protein-to-DNA ratios in the presence of *Hae*III digestion fragments (Fig. 2; lanes E, F).

Mapping of pp32 binding sites on SRA terminal repeats. With the nitrocellulose filter binding assay, pp32 retained only two viral DNA fragments derived from pPvuII-DG; both fragments contained LTR DNA sequences (Fig. 1 through 3). The two fragments (fragments 6 and 8) retained by pp32 contained mostly U_3 and U_5 DNA sequences (Fig. 1). We wanted to map the location of pp32 binding sites within these LTR DNA sequences by the DNase I footprinting method (3).

We first investigated the interaction of pp32 with the 275-bp restriction enzyme fragment derived from the internal region of the tandem LTR DNA copies (Fig. 1). This figure depicts both copies of the SRA terminal repeat fused together as found in circular DNA obtained from infected cells (38). Each purified end-labeled DNA restriction fragment was incubated with or without purified pp32 protein and then analyzed by the DNase I footprinting procedure (3). The binding of pp32 to this 275-bp fragment generates a unique pattern of enhanced and reduced susceptibilities to DNase I nicking along the DNA (Fig. 4). A group of specific DNA fragments is produced in the presence of pp32 which, on electrophoresis in denaturing gels, gives rise to a ladder pattern with protected regions from 6 to 10 nucleotides in length. Protection from DNase I digestion by pp32 is indicated by the absence of bands or by their diminished intensities in the presence of pp32 as compared with control digests (for example, compare lanes C, G, and H from R252 to R260, R284 to R291, etc., in Fig. 4). In addition to the observed effect of reduced sensitivity to DNase I in selected areas on this 275-bp fragment, the association of pp32 with this DNA results, in most cases, in enhanced DNase I cleavage between regions of diminished DNase I susceptibility. When similar

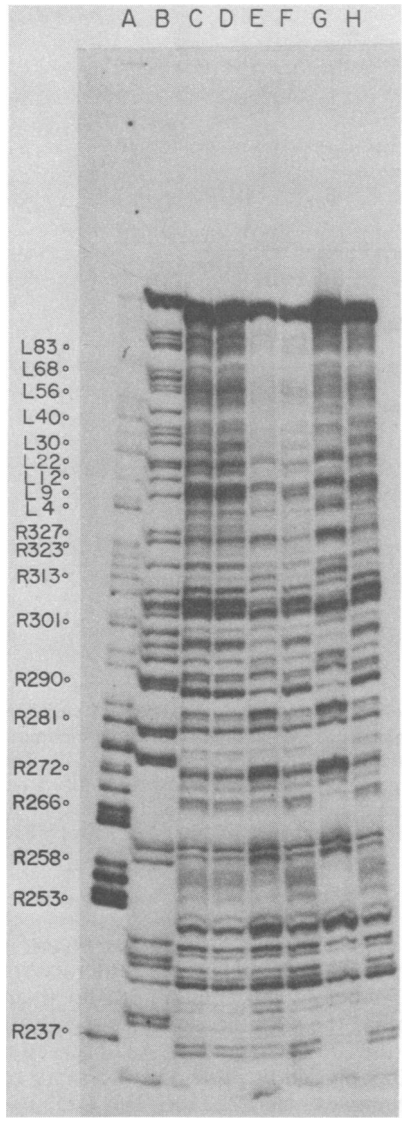

FIG. 4. DNase I footprints of AMV pp32 binding to the SRA 275-bp restriction fragment. The 275-bp fragment (Fig. 1, line 2) was subjected to various treatments (described below) and then analyzed on an 8% polyacrylamide DNA sequencing gel. Lane A, chemical G (20); lane B, chemical C (20); lane C, approximately 10 ng of end-labeled DNA in binding buffer (see text) was digested by DNase I. The reaction was terminated, and samples were analyzed on polyacrylamide gels. Lane D was treated the same as lane C except that the sample was treated with heparin (4.25 μg/ml, 2 min exposure) before DNase I digestion. Lane E, was treated the same as lane C except that the DNA was incubated with pp32 (2.7 μg/ml) for 3 min before DNase I digestion. Lane F, was treated the same as lane E except heparin was added after pp32 was incubated with DNA. Lane G, was treated the same as lane E except that the concentration of pp32 was 5.5 μg/ml. Lane H was treated the same as lane F except that the concentration of pp32 was 5.5 μg/ml. The numbers on the photograph identify specific nucleotides (see Fig. 5 for sequence).

samples (as described in Fig. 4) were subjected to electrophoresis in a 15% polyacrylamide gel, regions protected by pp32 between R190 and L4 were also characterized (not shown; see Fig. 12). Figure 5 contains the nucleotide sequence information of the cloned SRA LTR DNA (38). The regions protected by pp32 are indicated by discontinuous underlining, with enhanced DNase I nicking indicated by arrows. Starting in the U_3 region adjacent to *src*, the protected region extends from the presumed promoter region (R194, Fig. 5) (38) in U_3, through the U_5 region, and then terminates approximately 34 bp past the fused terminal repeats, for a total of 170 ± 10 bp. The exact nucleotides are not defined in some areas that are protected by pp32 from DNase I digestion, because DNase I does not nick DNA uniformly. Also, DNase I digestion yields fragments with 3′-OH termini which migrate slightly slower than chemical sequence fragments that terminate in a 3′-PO_4 (24).

The observed changes in the DNase I digestion pattern of the 275-bp fragment in the presence of pp32 were reversed by the addition of the polyanion, heparin, before the addition of DNase I (Fig. 4; compare lanes E and F or lanes G and H). Under these conditions, heparin does not appreciably affect DNase I digestion as compared to control digests (Fig. 4; lanes C and D), but it causes the apparent detachment of pp32 from the restriction fragment. Heparin has been used to help distinguish bacterial and phage attachment sites for phage λ integrase (11).

The concentration of pp32 needed to generate this unique DNA-protein complex on the 275-bp fragment was defined (Fig. 4). For example, in lane E of Fig. 4, the concentration of pp32 was 2.7 μg per ml. There appears to be partial protection of specific regions (see R252 to R260 and other regions) in the 275-bp fragment at this concentration; also present are the readily identifiable areas of enhanced DNase I cleavage. Increasing the concentration of pp32 by twofold results in almost complete protection of most of the specified areas (Fig. 4; lane G). If the concentration of pp32 is increased to 12 μg per ml, no apparent changes in the observed protection or DNase I enhancement patterns were observed (not shown). The near total absence of some bands in the digests containing pp32 suggests that the pp32 binding site is nearly saturated. The same protection patterns were obtained with six different preparations of purified pp32 which were greater than 95% homogeneous (8). Heat-inactivation studies demonstrated that the

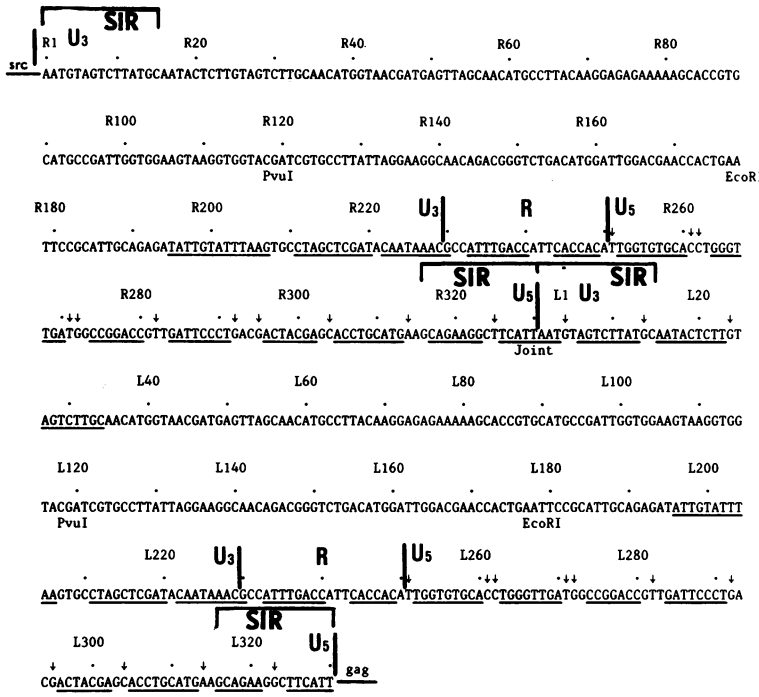

FIG. 5. Nucleotide sequence of SRA LTR DNA. The sequence information (plus strand of viral DNA) is presented for two copies of LTR DNA fused together as found in in vivo circular DNA (38) and are numbered as designated in Fig. 1. The short inverted repeats at the ends of the LTR DNA are designated as SIR. The bold letter R identifies the terminal repeat present at both ends of the genomic RNA. The brackets identify U_3 and U_5 sequences. The regions protected by pp32 are indicated by discontinuous underlining, and the arrows indicate areas of enhanced DNase I cleavage. The boundaries of protection and enhanced DNase I cleavage induced by pp32 were derived from analysis of numerous gels.

half-life for pp32 DNA-binding activity with the DNase I footprinting assay was ~4 min at 60°C, comparable to that observed with pp32 DNA endonuclease activity or pp32 binding to supercoiled DNA (D. Grandgenett, T. Misra, and P. Hippenmeyer, submitted for publication).

To better define the boundary of pp32 binding on LTR DNA from about R315 to L40 (which includes the joint region containing both mismatched 15-bp short inverted repeats, R315 to R330 and L1 to L15), we cleaved the SRA subclone with *Pvu*I to generate an internal fragment of 330 bp in length (Fig. 1). The DNA was end labeled and cleaved with *Eco*RI to generate a 275-bp molecule labeled at the L120 position on the minus strand. The pp32 protein did not induce any specific changes in the DNase I digestion pattern from L83 to approximately L34; diminished DNase I susceptibility started at approximately L34 and proceeded onward into the R terminal repeat (data not shown). The same general areas protected by pp32 on the plus strand (Fig. 5) also appear to be protected when the DNA is labeled on the minus strand.

Since the terminal repeats contain two complete copies of the U_3 and U_5 region joined together, pp32 could possibly interact with region L190 to L330 (Fig. 1; 901-bp fragment), which has the same sequence as region R190 to R330 (the 275-bp fragment). As observed in Fig. 6, pp32 apparently protected the same regions (for examples, see L222 to L230, L252 to L260, etc.) and caused the enhanced DNase I cutting pattern on the 901-bp fragment like that found on the 275-bp fragment (Fig. 4 and 5). The precise boundary of pp32 protection on the 901-bp fragment at the L330 position, adjacent to the primer binding site, is as yet undefined. However, pp32 can at least protect sequences from L325 to two nucleotides in on the adjacent primer site (Fig. 6).

In a series of control experiments, we wanted to determine the specificity of pp32 binding to LTR DNA. First, in similar studies with the AMV αβ DNA polymerase, the presumed precursor to pp32, we did not observe any periodicity of protection and enhancement under conditions which permitted pp32 to form a unique complex with the SRA 275- or 901-bp LTR DNA. One such analysis is shown in Fig. 7. A

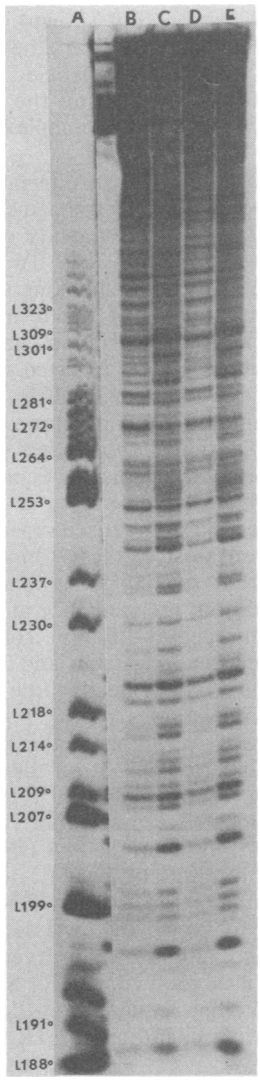

FIG. 6. DNase I footprint of pp32 binding to an SRA 901-bp restriction fragment. Conditions were similar to those described in Fig. 4 except that the pp32 concentrations were different and the samples were analyzed on 10% polyacrylamide gels. Lane A, chemical G (20); lane B, pp32 (7.0 μg/ml); lane C, pp32 (7.0 μg/ml) challenged with heparin; lane D, pp32 (12 μg/ml); lane E, pp32 (12 μg/ml) challenged with heparin.

comparison of Fig. 6 with Fig. 7 clearly reveals that there is no protection or enhanced DNase I cleavage observed when αβ DNA polymerase is complexed to SRA 901-bp LTR DNA. At protein concentrations comparable to those used with pp32, the αβ DNA polymerase did not promote the formation of specific complexes as observed with pp32. Rather, increasing concentrations of αβ DNA polymerase promoted a general reduction (Fig. 7; lanes D, E, and F) in DNase I-susceptible sites, suggesting nonspecific protection of DNA sequences by the polymerase.

Second, we determined whether other viral DNA restriction fragments permitted the formation of these pp32-mediated specific DNA complexes. As indicated in the DNA sequence diagram (Fig. 5), pp32 does not protect certain areas of the U_3 terminal repeat region. A 435-bp DNA fragment (Fig. 1) representing region R178

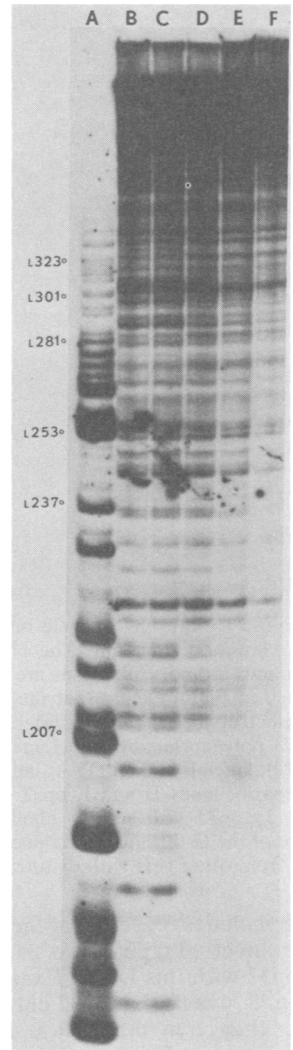

FIG. 7. DNase I footprints of AMV αβ DNA polymerase binding to the SRA 901-bp restriction fragment. Conditions were similar to those described in Fig. 4 except that pp32 was replaced with purified αβ DNA polymerase. Lane A, chemical G (20); lanes B and C, DNA only; lanes D, E, and F, αβ polymerase at 4.7, 7.3, and 9.5 μg/ml, respectively.

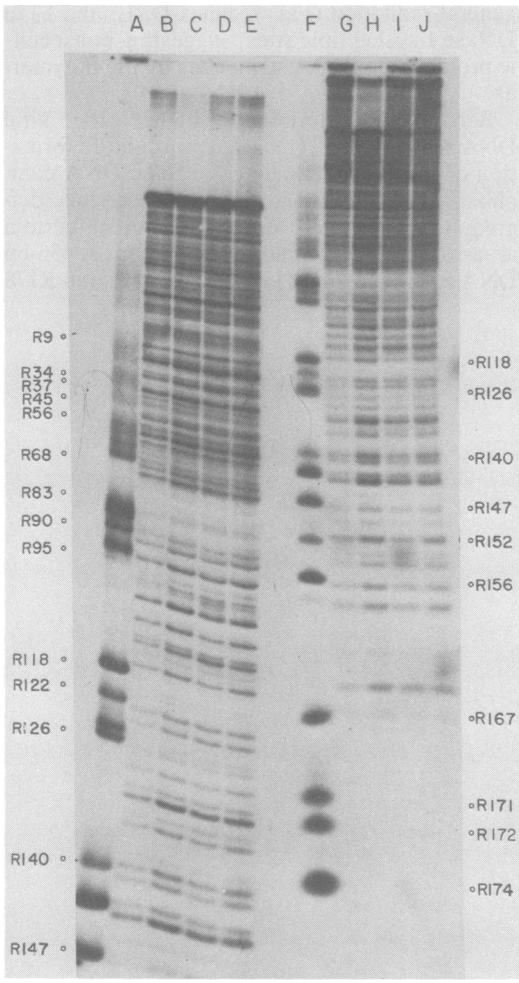

FIG. 8. DNase I footprints of pp32 binding to the SRA 435-bp restriction fragment. The DNA was labeled on the minus strand. Conditions are the same as those described in Fig. 4 except that the sample was subjected to electrophoresis for two different times on the same 15% polyacrylamide gel. Lanes A and F, chemical G (20); lanes B and G, DNA; lanes C and H, DNA plus heparin; lanes D and I, pp32 (7.0 μg/ml); lanes E and J, pp32 (7.0 μg/ml) challenged with heparin. Some of the G-terminated fragments (lane A) were derived from other gels with distinct bands.

to R1, which includes one copy of the terminally located short inverted repeat, was used to study binding of pp32 with this DNA. Examination of the gel in Fig. 8 reveals that pp32 did not induce any specific change in the DNase I digestion pattern of any unique U_3 sequences on the 435-bp fragment. Increasing the concentration of pp32 to 14 μg/ml (twofold increase) also resulted in no specific change in the DNase I digestion pattern. In addition, with pp32 there was no protection of DNA or enhanced DNase I cutting of the 55-bp fragment (L178 to L118) also located in the U_3 region (not shown). Finally, one DNA fragment derived from the *pol* gene of cloned RSV PrA DNA and labeled at the unique *Bgl*II site (10) did not permit the formation of these specific pp32-DNA complexes (data not shown).

pp32 binding sites in PrA terminal repeats. Does AMV pp32 bind to the same or different regions on the terminal repeats of PrA RSV DNA relative to SRA DNA? To answer this question, we analyzed a subclone of PrA DNA containing two copies of the terminal repeat. Figure 9 illustrates a restriction enzyme map of the PrA tandem repeats inserted into pBR322 DNA. This subclone (pXBm102) was derived from a permutated genomic clone of PrA RSV DNA which contained two tandem copies of the terminal repeat (10). This particular clone offered the advantage of a unique *Hin*dIII site located at the joint of the fused repeats, enabling examination of most of the U_5 binding regions from a different starting point than with SRA DNA. In addition, the sequence at the junction of the tandem repeat is different from that found in SRA (Gilmartin, Pugatsch, and Parsons, unpublished data). Specifically, the PrA DNA lacks seven nucleotides of the 14-bp inverted repeat on the U_5 terminus at the joint and the entire short inverted repeat on the adjacent U_3 terminus (Fig. 10). The adjacent U_3 short inverted repeat is located 22 nucleotides downstream at position L22. In total, the PrA DNA sequence is considerably different from L1 to about L60 (Fig. 10) as compared to SRA DNA (R322 to R330 and L1 to approximately L44; see Fig. 5). This sequence alteration observed with PrA DNA serves as a good control for SRA DNA to determine whether sequences at the fused joint are involved in recognition of LTR DNA by the pp32 protein.

For a positive control, the pXBm102 cloned DNA was cleaved with *Eco*RI and end labeled with T4 kinase. A fragment of approximately 300 bp labeled at position L196 on the plus viral DNA strand was isolated after *Sac*I digestion (Fig. 9). Binding of pp32 protein produced a similar ladder pattern and caused diminished DNase I susceptibility in the same DNA regions on the PrA 300-bp restriction fragment (data not shown) as those observed on the SRA 901-bp restriction fragment (Fig. 5).

We next investigated the interaction of the pp32 protein with a 285-bp PrA restriction fragment (R165 to L144, labeled on the 5′ end of plus strand DNA) which was similar in size and shared partial sequence homology with the SRA 275-bp fragment. As stated previously, the sequence of the PrA fragment deviates considerably from SRA DNA (Fig. 5) at positions L1 to approximately L60 (Fig. 10). The pp32 protein

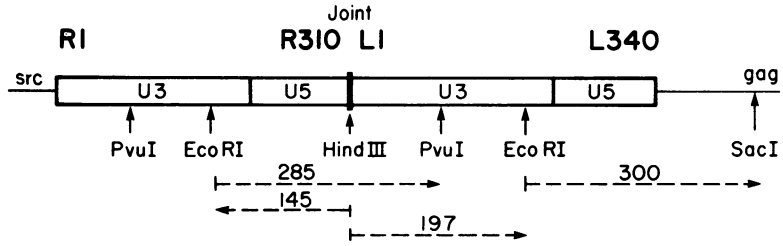

FIG. 9. PrA DNA restriction fragments used in DNase I footprinting experiments. The PrA DNA (pXBm 102) inserted into pBR322 DNA was digested with the indicated restriction enzymes, and the fragments were end labeled as described in Fig. 1. The ~300-, 285-, and 197-bp restriction fragments were labeled on the plus strand, whereas the 145-bp fragment was labeled on the minus strand.

again protected from partial DNase I digestion the same regions of the PrA DNA (approximately R180 to R260) (Fig. 11 and 12; some data not shown) as those previously observed with the corresponding SRA DNA (Fig. 5). However, the extent of pp32 protection of PrA DNA varied considerably from about R260 through the U_5-U_3 joint and into the U_3 region (to approximately L50). As demonstrated in Fig. 11 (lanes B, D, and F), pp32 was able to partially protect sequences to approximately R260, but thereafter it appeared that every DNase I cleavage point accessible in the control assay (lane B) and those protected with pp32 but challenged with heparin (lanes E or G) were also accessible in the presence of pp32 (lanes D and F). Interestingly, the association of pp32 with this PrA 285-bp restriction fragment stimulated DNase I nicking at numerous points from R260 onward (Fig. 11; compare lanes B, D, and E), but it protected DNA sequences from R180 to approximately R260. The pp32 protein did not protect any DNA regions from approximately R260 to L50 in this 285-bp fragment, which is in direct contrast to the results obtained with SRA DNA (Fig. 5). Apparently, the divergency of sequences from L1 to L60 at the PrA DNA joint region prevented the formation of a stable pp32-DNA complex, resulting in only partial protection of this restriction fragment (~80 bp). The association of pp32 from R180 to R260 might result in partial unwinding of the remaining DNA, thus promoting the enhanced DNase I nicking observed between R260 and L60 as compared to control DNA digests.

We were able to subdivide the PrA terminal repeats at the U_5-U_3 joint by *Hin*dIII digestion, separating the PrA sequences (L1 to L150, U_3 region), which are divergent from SRA DNA sequences, from the PrA DNA sequences (U_5 region), which are similar to SRA DNA sequences but lack most of the terminal 15-bp inverted repeat. The PrA subclone was digested with *Hin*dIII, labeled with T4 kinase, and subsequently cut with *Eco*RI. The PrA restriction fragment (145 bp) labeled on the minus DNA strand and containing sequence information similar to SRA sequences was examined first. pp32

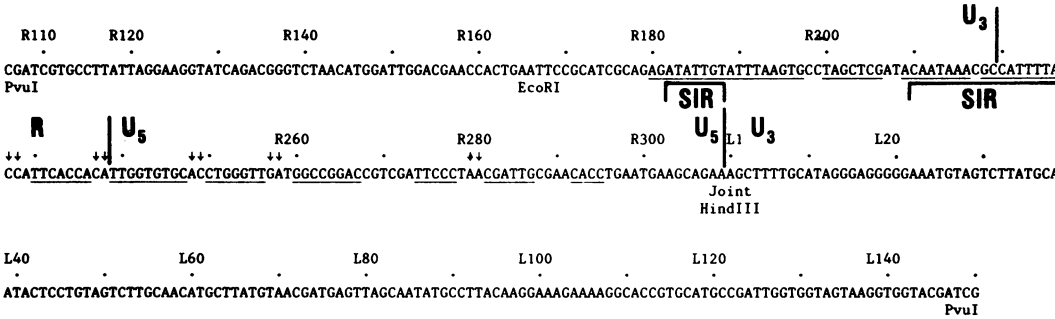

FIG. 10. Partial nucleotide sequence of PrA terminal repeats. The sequence information shown describes the sequences from the *PvuI* site (R110) on the right end of the viral linear map, through the tandem repeat joint, to the U_3 *PvuI* site on the left end of the linear DNA map. The regions protected by pp32 from partial DNase I digestion on the PrA 145-bp fragment (Fig. 12) are indicated by discontinuous underlining. The arrows indicate areas of enhanced DNase I cutting. The binding of pp32 to the PrA 145-bp fragment, labeled on the minus strand, is illustrated on the plus strand sequence. SIR, Short inverted repeats; R, terminal repeat present at both ends of the genomic DNA.

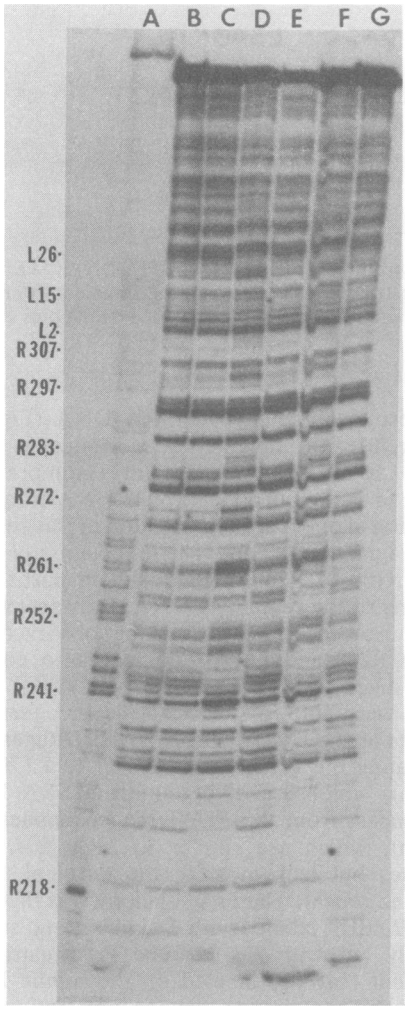

FIG. 11. DNase I footprint of pp32 binding to PrA 285-bp restriction fragment. Conditions were similar to those described in Fig. 4. Lane A, chemical G (20); lane B, DNA; lane C, DNA plus heparin; lane D, pp32 (6.5 μg/ml); lane E, pp32 (6.5 μg/ml) challenged with heparin; lane F, pp32 (9.8 μg/ml); lane G, pp32 (9.8 μg/ml) challenged with heparin. The numbers of the G-terminated fragments (lane A) were derived in part from the same gel exposed for a longer period of time.

was able to essentially protect (Fig. 12) the same DNA regions (R180 to R270) on the PrA DNA restriction fragment as those protected on SRA DNA (Fig. 5). In contrast to the approximate 10-bp protection patterns observed from R180 to R270, there was only partial protection from DNase I digestion of DNA from R270 to R300 by pp32 (Fig. 10 and 12). There was little or no pp32 characteristic protection from partial DNase I digestion of the sequence-divergent PrA (197-bp restriction fragment from L1 to L140) (data not shown).

DISCUSSION

Circular avian retrovirus DNA containing two copies of the LTR DNA possesses the structure *src*-U_3 U_5:U_3 U_5-*gag*. The AMV pp32 protein forms a complex with the most conserved part of the repeat structure. DNase I footprinting analysis of pp32-restriction enzyme fragment complexes revealed that the protein protects the DNA from partial DNase I digestion, resulting in regions of diminished DNase I susceptibility interspaced with sites of enhanced DNase I susceptibility. Starting in the U_3 region adjacent to *src*, the protected sequences extend proximal to the proposed promoter in U_3, through the U_5 region, and terminate approximately 34 bp past the joint, for a total of 170 ± 10 bp.

The avian retrovirus pp32 protein has two distinct capabilities: DNA binding and DNA

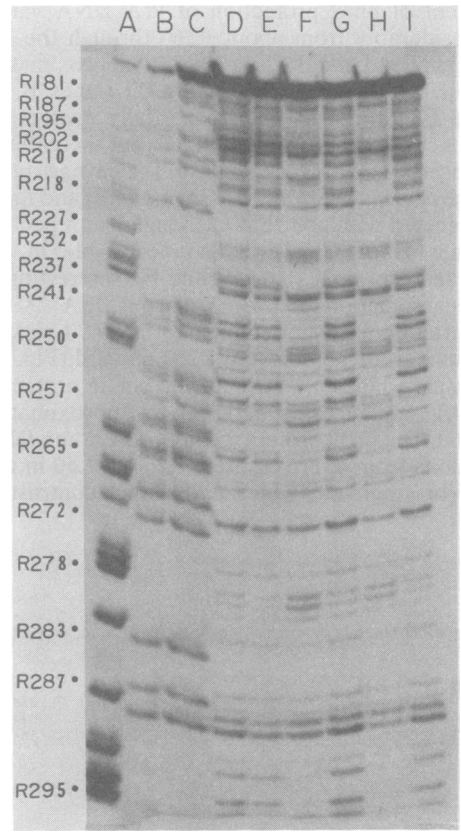

FIG. 12. DNase I footprint of pp32 binding to PrA 145-bp restriction fragment. Conditions were similar to those described in Fig. 4. Lane A, chemical G (20), lane B, chemical C + T (20) (reaction for T was incomplete); lane C, chemical C (20); lane D, DNA; lane E, DNA plus heparin; lane F, pp32 (6.5 μg/ml), lane G, pp32 (6.5 μg/ml) challenged with heparin; lane H, pp32 (9.8 μg/ml); lane I, pp32 (9.8 μg/ml) challenged with heparin.

endonuclease activity (8). In the presence of Mg^{2+}, the enzyme nicks supercoiled DNA, generating only unit-length DNA. Supercoiled viral DNA present in the nucleus of virus-infected cells, containing either one or two copies of the terminal repeat, could possibly be the immediate precursor to the integration event. The pp32-associated DNA-nicking activity could conceivably generate a staggered cut in either form of viral DNA, similar to mechanisms proposed for integration of transposable elements (31) and retrovirus DNA (33). Efforts are currently underway to determine whether pp32 or αβ DNA polymerase (5) is able to nick supercoiled viral DNA containing tandem copies of the terminal repeats at a specific location within the LTRs. It is also possible that linear DNA is the immediate precursor to the integrated provirus, eliminating the need for a site-specific nuclease.

The DNase I footprinting data suggest that the complex formed between the viral terminal repeat DNA restriction fragments (Fig. 4 and 6) and pp32 resembles eucaryotic nucleosome structures (19, 23) or procaryotic DNA gyrase-DNA complexes (15, 18, 21). The biological significance of these pp32-DNA complexes is presently unknown. Protein cross-linking studies should reveal a subunit structure for the protein in solution and bound to DNA in the above complexes. Evidence already exists indicating that the pp32 protein dimerizes in solution (8). By analogy with the other systems mentioned previously, it is plausible that 170 ± 10 bp of viral DNA could be wrapped around oligomers of pp32 containing one or two tetramers of pp32. Other physical techniques must be used to provide direct supporting evidence that pp32 forms nucleosome-like structures with LTR DNA.

What mechanisms are involved which permit pp32 to recognize selective regions (Fig. 5 and 10) of the LTR DNA? Apparently, nucleotide sequences may play a functional role, because pp32 does not form these complexes with certain regions of LTR DNA. A readily identifiable sequence common to all of the viral DNA fragments promoting pp32-DNA complexes is the terminally located 15-bp short inverted repeat structures or promoter sequences. One short inverted repeat structure (R1 to R15) located on the SRA 435-bp fragment (Fig. 8) and containing only U_3 sequences was insufficient to permit formation of a complex. However, pp32 can form a complex with the 901-bp fragment (Fig. 6), which contains one short inverted repeat (L316 to L330) and the entire U_5 region, including the proposed promoter region of U_3. Lastly, two adjacent short inverted repeats are present at the joint of the LTRs of the SRA 275-bp fragment (Fig. 4 and 5). The pp32 protein apparently is able to form a ~170-bp pp32-DNA complex with this fragment. Interestingly, the deletion of one short U_3 inverted repeat and partial deletion of the other U_5 inverted repeat along with a 21-bp insertion in this joint region of the PrA fragment prevented the formation of a complete stable complex (Fig. 10 and 11). This result suggests that the short inverted repeats may be necessary but not sufficient for the formation of a stable pp32-DNA complex. This preliminary observation that the short inverted repeats are necessary for the formation of these pp32-DNA complexes must be confirmed with other deletions in these repeats. Resection of the entire LTR may be necessary for identifying key sequences involved in formation of these pp32-DNA complexes. At present, we cannot exclude the possibility that pp32 may bind to other selective regions of avian retrovirus genomic DNA not yet tested (see below).

The preferential retention by pp32 on nitrocellulose filters of viral DNA restriction fragments containing LTR DNA sequences (Fig. 2 and 3) complements our DNase I footprinting analysis. What common parameters exist for pp32 recognition of viral LTR DNA and the four separate plasmid regions on supercoiled pPvuII-DG? The most outstanding common features among these six fragments (Fig. 1) are that they possess either potential promoters, palindromic sequences, or A-T-rich regions. We have not yet thoroughly analyzed which sequences or structural features permit pp32 recognition of these six DNA regions on pPvuII-DG. We have confirmed the mapping of the preferred binding sites for pp32 on supercoiled pPvuII-DG by the use of restriction enzymes *Hin*fI and *Hpa*II (unpublished data). The pp32 protein does not preferentially retain viral DNA fragments containing *src* or *gag* sequences in supercoiled pPvuII-DG (Fig. 1, 2, and 3; unpublished data). We are currently determining whether pp32 can bind preferentially to other viral DNA fragments derived from the rest of the RSV DNA genome and to LTR DNA derievd from AMV (25). Although most of the U_3 region of AMV LTR DNA is different from that of RSV, there is considerable homology between both viral LTR DNAs, starting in the conserved part of the U_3 region (promoter) and extending through the end of the U_5 region (25).

Supercoiled DNAs are required or preferred in a number of biological processes such as repair, recombination, and transcription. Therefore, a key to understanding the biological role of pp32 may be directly related to defining the parameters of pp32 binding to supercoiled unintegrated viral DNA. We can only speculate on the biological role, if any, pp32 has in the life cycle of avian retroviruses. The observation of a

similar DNA endonuclease in mammalian retroviruses (16, 22) lends support to the possibility that this particular protein is involved in the retrovirus life cycle. Our DNA binding data is consistent with the interpretation that pp32 might be involved in the transport of linear viral DNA from the cytoplasm to the nucleus or might promote the formation of a noncovalent circle by bringing the ends of the linear DNA together, or both. The binding of pp32 with selective regions of the terminal repeats of retrovirus DNA, which are actually involved in the in vivo integration event, is particularly suggestive of a functional integrative role for pp32. We cannot exclude the possibility that pp32 functions in viral DNA synthesis, transcription, or other undefined steps in the replication cycle. Functional virus mutants encoding a temperature-sensitive lesion in the pp32 moiety of the β subunit will surely be needed to establish a biological role for pp32 as well as for the polymerase-associated DNA endonuclease. Generation of these temperature-sensitive virus mutants by site-directed in vitro mutagenesis of cloned retrovirus DNA is currently underway.

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