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Characterization of Endonuclease Activities in Moloney Murine Leukemia Virus and Its Replication-Defective Mutants

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To study Moloney murine leukemia virus (M-MuLV) proteins associated with the integration of proviral DNA into the host chromosome, we isolated endonuclease activities from purified virion preparations of the wild type and two of its replication mutants. A major endonuclease activity was identified in virions of M-MuLV; the enzyme catalyzed nicks in double-stranded DNA in the presence of either Mn^{2+} or Mg^{2+} and was stimulated by ATP. The endonuclease nicked DNA adjacent to all four nucleotides with some preference for G and C. The same enzyme, and in comparable amounts, was isolated from two virus replication mutants: *dl*2905, deficient in the processing of $Pr65^{gag}$ and $Pr200^{gag\text{-}pol}$, and *dl*5401, deficient for the virus integration function. In the process of these experiments, the residual reverse transcriptase in mutant *dl*2905 was shown to be the mature size, implying that the uncleaved precursor lacks enzymatic activity. It appears that the major endonuclease activity found in virions of M-MuLV is not encoded by either the *gag* or *pol* genes.

Genetic studies have implicated at least two regions of the retrovirus genome as necessary for the integration of proviral DNA into the host chromosome: (i) the long terminal repeat (LTR) circle junction U_5-U_3 (*att*), which is needed in *cis* (1, 14), and (ii) a region at the 3′ end of the *pol* gene which appears to code for a protein termed integrase which acts in *trans* (3, 13, 15). Biochemical studies of avian sarcoma leukemia viruses have indicated the presence of a protein (pp32) which is a natural product during processing of the reverse transcriptase β subunit into the α subunit (7, 8). The pp32 and the β subunit of the avian sarcoma leukemia virus reverse transcriptase each possess an endonuclease activity which, under defined conditions, preferentially cleaves in vitro to form a staggered cut three or two nucleotides from the proviral LTR DNA circle junction U_5-U_3, the *att* site (9, 18). Characterization of the murine retrovirus proteins involved in the integration step has been less advanced. The reverse transcriptase, which is a single subunit of 80,000 molecular weight, does not appear to possess an endonuclease activity. However, endonuclease activities have been reported in Friend leukemia virus (12) and Rauscher leukemia virus (11). The specificity of these enzymes and their origin was, however, not clear. In this work, we characterized endonucleases in virions of Moloney murine leukemia virus (M-MuLV). To identify the origin of these proteins and their possible involvement in the integration reaction, we used two defined replication mutants of M-MuLV.

To identify endonuclease activities in M-MuLV virions, extracts were chromatographed through a phosphocellulose column. As a sensitive assay for endonuclease DNA activity, conversion of supercoiled form I DNA to form II was monitored. The endonuclease DNA substrate pMLTR contained sequences from the junction of two tandem LTRs (ΔR-U_5-ΔU_3) cloned in pUC13. This region of the unintegrated proviral DNA participates in the integration process (17).

A major nicking activity was eluted from phosphocellulose at a salt concentration of 0.35 M (Fig. 1A, fractions 33 and 36). Under the reaction conditions (10 mM $MnCl_2$), most of the substrate was converted to form II DNA. Some endonuclease and reverse transcriptase activities were also detected in the flowthrough fractions (fractions 5 and 8). However, in repeated chromatography, the ratio of the endonuclease and reverse transcriptase activities in the flow fraction was constant, indicating some leakage through the phosphocellulose. In addition, the enzymatic properties of the flowthrough endonuclease were compared with the major activity eluted at 0.35 M NaCl and were found to be similar, suggesting that it represents the same enzyme (data not shown).

To further characterize the endonuclease activity isolated from the phosphocellulose column, its divalent ion requirement was analyzed. The endonuclease purified from M-MuLV nicks form I DNA in the presence of Mg^{2+} (Fig. 2A, lanes B and C), and ATP significantly stimulates this activity (lanes D and E). This endonuclease is clearly more active in the presence of Mn^{2+}, converting the substrate into both form II and form III DNA (lanes F and G). In the presence of Mn^{2+} and ATP, the enzyme attains maximal activity to degrade the plasmid into small and heterogeneous DNA fragments (lanes H and I). Therefore, the amounts of product forms II and III in lanes H and I appear to be reduced.

Is this endonuclease a virus-coded protein or a cellular enzyme encapsidated during assembly of the virion? The enzyme appears to reside in the core of the virus, because proteinase K-treated virions or virus core preparations still retain this enzyme. To further explore the origin of this endonuclease, we analyzed its activity in two virus mutants. (i) Mutant *dl*2905 was mutated in the 5′ region of the *pol* gene by deleting 126 nucleotides. Cells transfected with this deleted virus DNA produce virions composed of unprocessed viral polyproteins, $Pr65^{gag}$ and $Pr200^{gag\text{-}pol}$ (2). If the endonuclease is normally encoded as part of these viral precursor polyproteins, its specific activity and chromatographic properties should differ in mutant *dl*2905. (ii) The *dl*5401 virus DNA was mutated at the 3′ region of the *pol* gene by deleting 91 base pairs (15). After transfection with

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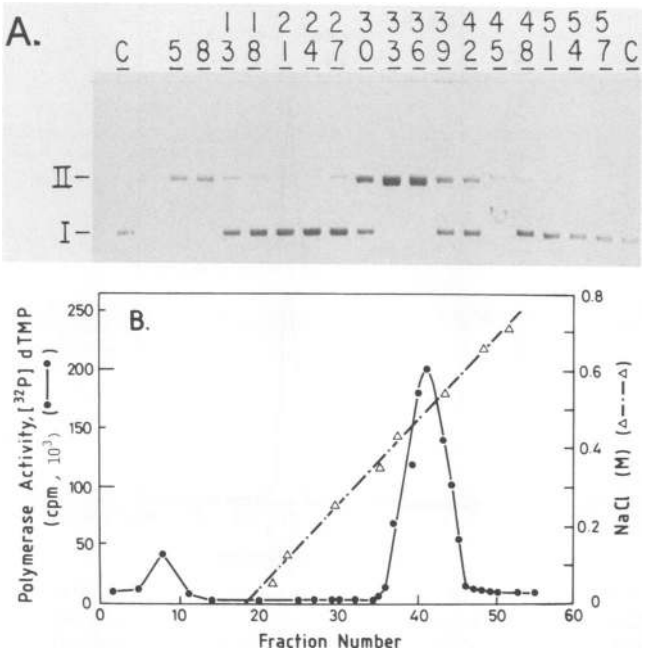

FIG. 1. Fractionation of endonuclease and reverse transcriptase activities by phosphocellulose column chromatography. Purified M-MuLV (4 mg) was isolated from 24-h culture fluids of NIH 3T3 cells infected with M-MuLV and purified by sucrose gradient centrifugation as described previously (5). Virions were disrupted by incubation for 30 min at 4°C in 450 μl of 0.5% Triton X-100–10 mM Tris hydrochloride (pH 7.5)–0.3 M NaCl–10 mM dithiothreitol. The lysate was diluted in 2 ml of buffer A (10 mM Tris hydrochloride (pH 7.5), 10 mM β-mercaptoethanol, 5% glycerol, 0.1% Triton X-100) and centrifuged at 12,000 × *g* for 10 min. The supernatant was loaded onto a phosphocellulose (P11) column (2-ml bed volume) equilibrated with buffer A. After being washed with buffer A (5 ml), the column was developed with a linear gradient (40 ml) of NaCl (0 to 0.8 M in buffer A). Fractions (0.8 ml) were collected and assayed for reverse transcriptase and endonuclease activity. Reverse transcriptase activity was determined (6) by using poly(A) · oligo(dT) as template primer. A unit of activity was defined as the amount of enzyme which catalyzed the incorporation of 1 pmol of [^{32}P]dTMP per min. Endonuclease reactions (25 μl) contained 20 mM Tris hydrochloride (pH 7.5), 5 mM dithiothreitol, 0.1% (vol/vol) Triton X-100, 0.1 mM EDTA, 10 mM $MnCl_2$ or 10 mM $MgCl_2$, pMLTR (450 ng), and viral proteins. After incubation for 1 h at 37°C, the reactions were stopped by 5 μl of 0.1 M EDTA and 50% (vol/vol) glycerol. The mixtures were applied to a 0.7% agarose gel in 90 mM Tris-borate–1 mM EDTA, containing 0.5 μg of ethidium bromide per ml, and electrophoresis was done at 100 V for 4 h. To construct the DNA substrate pMLTR, plasmid pMLV containing the entire M-MuLV sequences with two LTRs in tandem (10) was digested with *Xba*I. The permutated LTR DNA (592 base pairs) (ΔU_3-R-U_5-ΔU_3) was isolated and digested with *Xma*I. The fragment of 414 base pairs (ΔR-U_5-ΔU_3), *Xma*I nucleotide 28 to *Xba*I nucleotide 8113 was cloned into a pUC13 vector digested at the polylinker with *Xma*I and *Xba*I. Numbering of M-MuLV nucleotide sequences was according to Shinnick et al. (16). (A) Fractions of 6 μl assayed for endonuclease with supercoiled pMLTR (form I) DNA as substrate. C, Control (without enzyme). Numbers at top indicate column fractions. (B) Fractions of 5 μl assayed for reverse transcriptase activity.

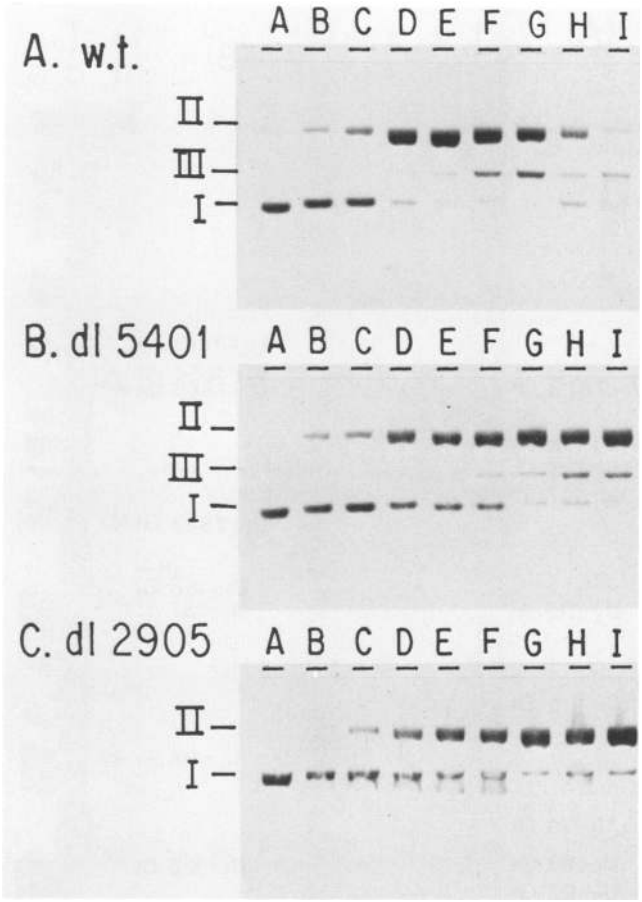

FIG. 2. Characterization of endonucleases from M-MuLV and its replication-defective mutants *dl*2905 and *dl*5401. Purified M-MuLV and the mutant virions (4 mg) were disrupted and fractionated on identical phosphocellulose columns as described in the legend to Fig. 1. The endonuclease activities, eluted at 0.35 M NaCl from the three phosphocellulose columns, were pooled (3.2 ml) and dialyzed against column buffer A for 3 h. The partially purified endonucleases of the three viruses (A, M-MuLV wild type (w.t.); B, *dl*5401; and C, *dl*2905) were compared in a standard endonuclease reaction mixture (see the legend to Fig. 1). Lanes: A to C, with 10 mM $MgCl_2$; D and E, with 10 mM $MgCl_2$ and 2.5 mM ATP; F and G, with 10 mM $MnCl_2$; H and I, with 10 mM $MnCl_2$ and 2.5 mM ATP. Lane A, without enzyme; lanes B, D, F, and H, with 5 μl of endonuclease; lanes C, E, G, and I, with 15 μl of endonuclease.

the deleted DNA, assembly and release of progeny virus appeared to be normal. However, infection with the mutant virus was aborted after synthesis of the proviral DNA, probably because of a deficiency in the integrase function. If this region of the *pol* gene encodes the endonuclease described in this work, then *dl*5401 virions would be expected to be deficient in the nicking activity.

Purified virions of *dl*2905 and *dl*5401 displayed comparable levels of endonuclease activity after disruption with Triton X-100. To compare the enzyme to that of M-MuLV wild type, the mutants were extracted and chromatographed on identical phosphocellulose columns as described in the legend to Fig. 1. A major endonuclease activity was eluted from these two columns at 0.35 M NaCl, and the levels of enzymes were similar to that of wild type M-MuLV. To compare enzymatic properties of the endonuclease isolated from the three viruses (*dl*2905, *dl*5401, and wild type), the divalent ion requirements and ATP stimulation were analyzed (Fig. 2). The endonucleases isolated from the three viruses preferred Mn^{2+} over Mg^{2+} (compare lanes B and C to F and G) and were similarly stimulated by ATP (lanes D, E, H, and I). Because similar amounts of the same nicking

FIG. 3. Specificity of DNA cleavage catalyzed by the purified endonuclease. Reaction mixtures with the phosphocellulose column-purified endonuclease of wild-type virus and 5′ [^{32}P]DNA (30,000 cpm) were incubated for 45 min at 37°C. For preparation of a linear DNA containing the *att* site (U_5-U_3 circle junction), pMLTR was digested with *Xma*I or *Eco*RI at the polylinker, treated with calf intestine phosphatase, and radiolabeled at the 5′ end of the plus strand (*Xma*I site, nucleotide 28) with [α-^{32}P]ATP (3,500 to 7,000 Ci/mmol) and T4 polynucleotide kinase. The plasmid was then digested with *Eco*RV. The resulting fragment (261 base pairs; *Xma*I nucleotide 28 to *Eco*RV nucleotide 7959) contained the circle junction, ΔR-U_5-ΔU_3. The endonuclease reactions were stopped by 200 μl of 0.2 M sodium acetate–50 mM EDTA–5μg of tRNA per ml and extracted once with phenol-chloroform. Nucleic acids were recovered by ethanol precipitation, denatured, and fractionated on 6% sequencing polyacrylamide gels, alongside of markers cleaved chemically at G and C+T. Nucleotide numbers were according to Shinnick et al. (16). *att* designates the U_5-U_3 junction. (A) Reactions with 10 mM $MnCl_2$. Lanes: 1, without enzyme; 2, 3 μl of endonuclease; 3, 10 μl of endonuclease; 4, 3 μl of endonuclease and 2.5 mM ATP; G, chemical cleavage ladder. (B) Lanes: 1, reaction with 10 mM $MgCl_2$–2.5 mM ATP and 10 μl of endonuclease; T+C, chemical cleavage; G, chemical cleavage.

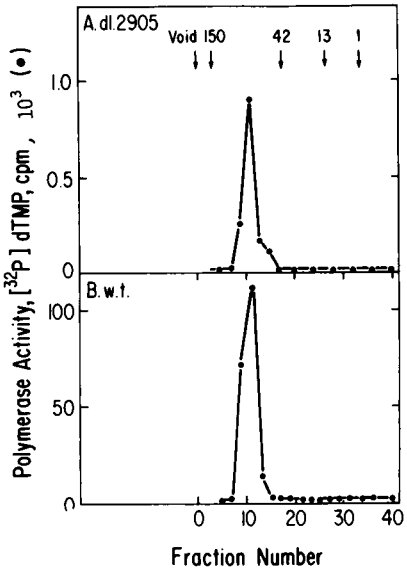

FIG. 4. HPLC for sizing the reverse transcriptase activity of *dl*2905 virus. Wild-type (w.t.) M-MuLV (450 μg) or the mutant *dl*2905 (430 μg) was disrupted with Triton X-100 (0.3%, vol/vol) in a volume of 100 μl. After 30 min of incubation at 4°C, the lysates were centrifuged at 12,000 × *g* for 10 min. The clear supernatant was injected into an HPLC column (GF 250) preequilibrated with buffer B (10 mM Tris hydrochloride [pH 7.5]–0.1 mM dithiothreitol–5% glycerol–0.005% Triton X-100) at room temperature. Buffer B was pumped through the column at a rate of 1 ml/min, and 0.25-ml fractions were collected. Reverse transcriptase activity was determined on 10-μl portions. The column was calibrated with globular protein markers (Bio-Rad Laboratories) of 150, 42, 13, and 1 kilodaltons, indicated by arrows at the top of the figure.

activity were present in both the mutant and wild-type viruses, this endonuclease appears not to be encoded in the *gag* or the *pol* genes.

We next analyzed the cleavage specificity of the purified endonuclease. Because the endonuclease activities associated with the avian sarcoma leukemia virus reverse transcriptase and pp32 were shown to cleave preferentially near the U_5-U_3 junction (4, 9), it was of interest to determine the sequence specificity of the major M-MuLV endonuclease. As a substrate, we used a DNA fragment containing the U_5-U_3 junction of M-MuLV. The position of the cleavage relative to the ^{32}P-labeled 5′ end was determined by fractionation of the degradation products on sequencing gels, alongside DNA markers cut chemically at G or T+C sites.

In the presence of Mn^{2+}, the enzyme cleaved at multiple preferred sequences indicated by the dots in Fig. 3A, lane 2. However, none of the preferred cleavage sites was near the U_5-U_3 junction (*att*). Greater amounts of enzyme (lane 3) increased the level of cleavage but at the same preferred sites. The addition of ATP (lane 4) further increased enzyme activity without changing the specificity. Substituting Mn^{2+} for Mg^{2+} in the presence of ATP gave similar cleavage specificity (Fig. 3B). The enzyme appeared to cut mostly

near G and C. At a lower frequency, some of the cuts were found near A and T.

Because equal levels of endonuclease activity were detected in the mutant and wild-type virions, it was of interest to compare the protein composition and reverse transcriptase enzyme of the three virus preparations. As reported by Goff and co-workers, *dl*2905 virus consisted mostly of $Pr65^{gag}$ and $Pr200^{gag\text{-}pol}$ (2) and *dl*5401 contained mostly processed *gag* proteins but, in addition, more $Pr65^{gag}$ compared with the wild-type virus (15). The reverse transcriptase specific activities varied among the three purified virus preparations. Whereas the wild-type virus had an enzyme activity of 103 U/μg of protein, the two virus mutants *dl*2905 and *dl*5401 expressed a consistently much lower reverse transcriptase specific activity of 1.75 and 1.3 U/μg of protein, respectively. For *dl*2905 virions, this could be caused by the lower enzyme specific activity of $Pr200^{gag\text{-}pol}$ or by residual proteolysis to produce some mature enzyme of 80,000 daltons. To resolve this question the size of the reverse transcriptase in *dl*2905 virus was compared with that of the wild-type enzyme by using high-performance liquid chromatography (HPLC).

The resin GF 250 (Du Pont Co.) can resolve a wide range of proteins according to their molecular weights. When wild-type virus extract was chromatographed through such a sizing column, a high yield of reverse transcriptase was measured in fractions corresponding to a protein of 80,000 molecular weight (Fig. 4). When *dl*2905 viral extract was chromatographed through the HPLC column, only a single peak of reverse transcriptase activity was evident, with a molecular weight, like that of the wild type, of 80,000. Although equal amounts of viral protein were loaded on the two HPLC columns, the enzyme activity in fractions of the wild-type virus was much higher than that of the mutant, reflecting the low specific activity of the later virus (Fig. 4). We confirmed these observations by using another method, glycerol gradient centrifugation, to estimate the size of the reverse transcriptase activity in both the wild-type and *dl*2905 mutant virus (data not shown).

Biochemical characterization of avian sarcoma leukemia virus *pol* gene products has revealed a specific endonuclease encoded at the 3′ end of the *pol* gene. This enzyme activity was found in virions as part of the β subunit of the reverse transcriptase or as a degradation product of 32,000 molecular weight, pp32 (7, 18). In the present study, we analyzed the possible existence of an analogous protein in virions of M-MuLV and two of its replication mutants. A major endonuclease was readily identified in extracts of M-MuLV. As a substrate, we used supercoiled or linear DNA containing the *att* sequence (U_5-U_3 junction); however, the enzyme appeared to catalyze nicks nonspecifically, with some preference for G and C phosphodiesters. The endonuclease described in this work is similar in several respects to the enzyme isolated from Friend MuLV (12). (i) It has a similar molecular weight (30,000 to 40,000), determined by velocity sedimentation in glycerol gradients (data not shown). (ii) It is eluted from a phosphocellulose column at 0.35 M NaCl. (iii) It is stimulated by ATP. (iv) It prefers Mn^{2+} to Mg^{2+} as a divalent ion for activity. It is not clear whether this enzyme is also similar to the endonuclease isolated from Rauscher MuLV (11). The latter enzyme had a similar molecular weight, but in contrast to the endonuclease described here, it was fully active on double-stranded linear DNA in the presence of Mg^{2+}.

Because retroviruses are known to incorporate a variety of cellular macromolecules during assembly, we used two defined virus mutants to investigate the origin of this endonuclease. The integration-defective mutant *dl*5401 (15) appeared to contain wild-type levels of the same endonuclease, suggesting that this enzyme may not be correlated with the integrase function and is perhaps of cellular origin. If the major endonuclease was a product of cleavage of either the *gag* or *pol* genes, its precursor polyprotein would be expected to have altered chromatographic and enzymatic activities. Our results indicated normal levels of the same endonuclease in the mutant *dl*2905, which was defective in the processing of *gag* and *pol* gene products (2).

Virions of *dl*2905 mutants contained relatively large amounts of unprocessed $Pr200^{gag\text{-}pol}$, and on this basis, it has been suggested that this polyprotein expressed some reverse transcriptase activity (2). Size analysis of the residual reverse transcriptase activity in *dl*2905 virions, however, revealed an enzyme of 80,000 daltons. In three preparations of purified *dl*2905, the enzyme specific activity was about 50-fold lower than that of wild-type virions, which probably represented some residual proteolysis in *dl*2905. This observation indicates that processing of $Pr200^{gag\text{-}pol}$ is crucial for the activation of reverse transcriptase enzyme, as suggested previously by Witte and Baltimore (19).

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