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DNA-JOINING ACTIVITY ASSOCIATED WITH A MURINE RETROVIRUS

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SUMMARY

A DNA-joining activity associated with a murine retrovirus, the Friend murine leukemia virus (F-MuLV), has been identified. DNA-joining by the virus associated enzyme, as measured by the circularization of $d(A-T)_n$, did not require the addition of ATP. The enzyme was partially purified by glycerol gradient centrifugation and its molecular weight was determined to be about 40 000.

Covalently closed circular double-stranded viral DNA may be synthesized in an endogenous reaction using purified avian retroviruses (1). It thus appears that all the enzymatic activities which are necessary for the conversion of the viral RNA genome into a covalently closed circular DNA duplex are present in the virus particle (1). Consequently, in addition to the well characterized RNA/DNA-directed polymerase and RNase H activities of the virus coded reverse-transcriptase, the virus must also contain a DNA-joining activity for the circularization of the viral DNA. Such an activity has earlier been reported to be present in avian sarcoma virus (ASV) extracts (2). Though the DNA-joining activity detected in the ASV extracts may be responsible for the circulari-

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zation of the viral DNA when synthesized in vitro, the significance of this activity for the in vivo replication of the virus DNA is uncertain. The fact that an endonuclease which preferentially nicks supercoiled DNA duplexes appears to be coded for by avian retroviruses (3,4) suggests that the in vivo replication of the viral DNA may in addition to the viral reverse-transcriptase also involve other virus associated enzymes. In order to gain an understanding of the relevance of the avian retrovirus associated DNA-joining activity for in vivo viral DNA replication, a further characterization of this activity is required. Furthermore, the identification and characterization of similar activities in other retroviruses is also necessary. With this in mind we have attempted to identify a DNA-joining activity in murine retroviruses. We report here that such an activity is associated with the Friend murine leukemia virus (F-MuLV). As is the case for the DNA-joining activity associated with the avian retroviruses (1,2), the F-MuLV associated activity functioned without the addition of ATP. However, this may be due to the presence of a sufficient amount of ATP in the virus or substrate preparations, rather than being a characteristic of the virus associated enzyme. The molecular weight of the enzyme was estimated by sedimentation studies to be about 40 000.

MATERIAL AND METHODS

Materials: Oligo and polynucleotides were from P.L. Biochemicals (dT_{10} and dA_n) and Sigma ($d(A\text{-}T)_n$). Radioactive [γ-^{32}P] ATP and [^{3}H]-dTTP were from the Radiochemical Centre, Amersham, UK, and the specific activities were 3000 Ci/mmole and 50 mCi/mmole respectively.

Enzymes: T_4 polynucleotide ligase was prepared by the method of Tait et al. (5) from λ NM 989 infected E. coli K 1150 cells. One unit of the enzyme is defined as the amount which in one minute at 20°C catalyzes the transformation of one picomole 5'-[^{32}P]-termini of 5'-[^{32}P]dT_{10} to a form insusceptible to bacterial alkaline phosphatase (6). T_4 polynucleotide kinase was

from Boehringer Mannheim and bacterial alkaline phosphatase (BAP) from Worthington Biochemical Company.

Virus purification and preparation of virus extract: F-MuLV was propagated in Eveline cells and purified from the cell culture as described earlier (7). Purified viruses were treated with proteinase K for the digestion of extraneous nonviral proteins in addition to viral membrane associated proteins (8), and finally disrupted by the addition of Triton X-100, all performed according to earlier published procedures (8,9). The protein concentration in the virus preparation was determined using the Bio-Rad protein Assay Kit and lyophilized bovine gamma globulin as standard protein.

Preparation of substrate for the detection of DNA-joining activity: $[^3H]$-d(A-T)$_n$ (0,13 cpm/mmole) ($n \simeq 1000$) was prepared essentially according to the procedure of Modrich and Lehman (10). Phosphorylation of $[^3H]$-d(A-T)$_n$ was as previously described (6). The fraction of $[^{32}P]$-$[^3H]$-d(A-T)$_n$ molecules which could be circularized by ligase was determined by measuring the percentage of molecules rendered BAP resistant by adding an excess of purified T_4-ligase. In agreement with earlier observations (10) this fraction varied between 20 and 50% in the various $[^{32}P]$-$[^3H]$-d(A-T)$_n$ preparations, and it reflects the amount of active substrate in the preparations.

Assay for DNA-joining and exonuclease activities: The standard reaction mixture (50 µl) contained 66 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP unless otherwise indicated, 5 µM $[^{32}P]$-$[^3H]$-d(A-T)$_n$ (phosphate) and the amount of virus extract indicated in the legend to figures. The reaction mixture was incubated for 10 min at 30°C. BAP (2µg/ml) was then added and the incubation was continued for 30 min at 65°C. The reaction mixture was subsequently spotted directly onto strips of DE 81 paper and developed for 1 h in 0.35 M ammonium formate. In this system dimeric and larger oligonucleotides will stay at the origin whereas mononucleotides and phosphate will move with the front. The amount of DNA-joining was then determined by measuring the amount of radioactive phosphate at the origin. The exonuclease activity was determined by measuring the decrease in $[^3H]$-labelled polynucleotides at the origin. The radioactivity was measured in a Packard Tri-Carb 460 CD liquid scintillation counter using a toluene based scintillation liquid. The virus associated DNA-joining activity is in all figures in this study presented as the percentage of active substrate in the reaction mixture which was rendered BAP resistant due to DNA-joining.

RESULTS AND DISCUSSION

Initial attempts at detecting a virus associated DNA-joining activity using $[^{32}P]$-$dT_{10} \cdot dA_n$ as substrate failed because the substrate was extensively degraded by a virus associated exonuclease. Since similar problems were apparently also encountered when studying the ASV associated DNA-joining enzyme (2), an assay pro-

cedure somewhat similar to that which was needed for the detection of the ASV associated activity was devised. In this assay [5'-^{32}P]-[^{3}H]-d(A-T)$_n$ was used as substrate. This substrate is largely double-stranded and contains a single nick with ^{32}P in the 5'-position. A single joining event renders it insusceptible to the action of exonucleases and BAP, since the product is circularized. Moreover, the double labelling of the substrate enables one to detect simultaneously both DNA-joining activity and any degradation of the substrate by exonucleases (see Materials and Methods).

Prior to assaying for a F-MuLV associated DNA-joining activity the virus particles were treated with proteinase K such that the virus membrane protein (gp 71) and all nonviral proteins were completely digested (8). Subsequent disruption of the proteinase K treated virus particles by Triton X-100 was necessary in order to detect significant DNA-joining activity (Fig. 1). This indicates the presence of a F-MuLV associated DNA-joining enzyme which is located internally in the virus particle.

The time course of the DNA-joining reaction in the presence and absence of ATP shown in Fig. 1 indicates that the DNA-joining activity in the virus extract does not require the addition of ATP. The same observation has been made with the avian retrovirus associated DNA-joining activity (1,2), and this most likely reflects the presence of a sufficient amount of cofactor in the virus and/or substrate preparations (2,11). Purified T_4-ligase ordinarily requires ATP as a cofactor (12). However, when [^{32}P]-[^{3}H]-d(A-T)$_n$ was used as substrate the addition of ATP was not required in order to obtain DNA-joining with purified T_4-ligase (data not shown), suggesting the presence of a sufficient amount of cofactor in the substrate preparation.

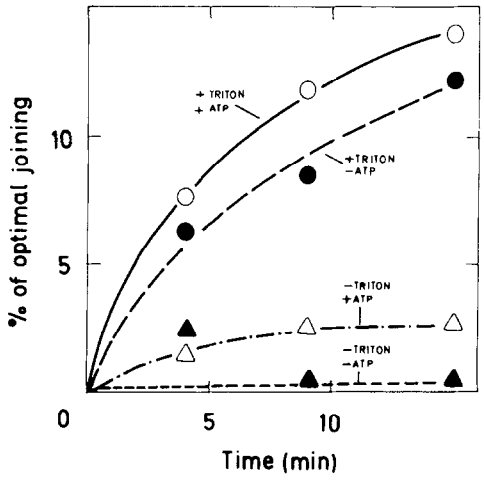
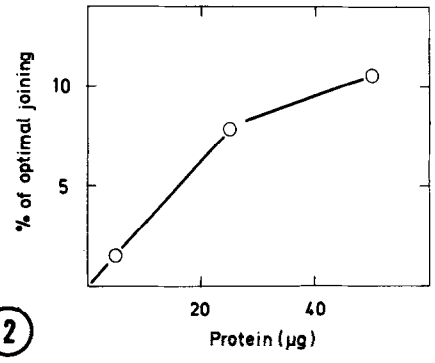


Fig. 1. Time course of the DNA-joining activity in a F-MuLV preparation in the presence and absence of Triton X-100, and with and without the addition of ATP. The reaction mixture (0.2 ml) was as described in Materials and Methods, and the reactions were initiated by adding 250 µg of F-MuLV. Aliquots of 50 µl were withdrawn at different times and the joining activity determined as described in Materials and Methods. (△---△) undisrupted virions in the presence of ATP (100 µM), (▲---▲) undisrupted virions in the absence of added ATP, (○—○) disrupted virions in the presence of ATP (100 µM), and (●—●) disrupted virions in the absence of added ATP.

From Fig. 2, which shows the DNA-joining activity as a function of the amount of viral protein in the reaction mixture, it can be seen that about 50 µg of protein resulted in the circularization of about 10% of the substrate. This is the amount circularized by approximately 0.2 units of T_4-ligase when incubated under the same conditions (data not shown). Cell extracts of T_4-infected E. coli have a specific activity of about 50 units of DNA-joining activity per mg protein, which implies that the specific DNA-joining activity in the proteinase treated F-MuLV is about one order of magnitude less than in T_4-infected E. coli extracts. The specific DNA-joining activity in the F-MuLV is nevertheless relatively high, considering that cell extracts of T_4-infected E. coli appear to have 100-1000 times more DNA-joining activity than mammalian cell extracts (13).



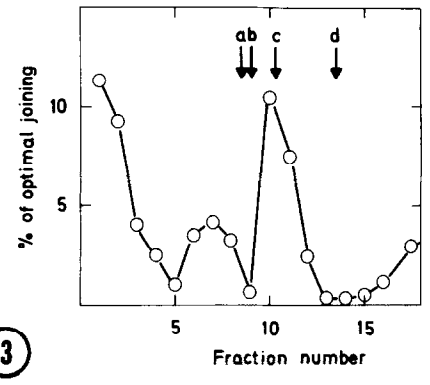


Fig. 2. DNA-joining activity as a function of the amount of F-MuLV protein. The assay for DNA-joining activity was as described in Materials and Methods.

Fig. 3. Glycerol gradient centrifugation of the F-MuLV DNA-joining enzyme. Virus extract (200 µl, 5 mg protein/ml) to which NaCl had been added to a final concentration of 1 M was loaded onto a 3.5 ml 10-30% glycerol gradient in 20 mM Tris-HCl (pH 7.5), 3 mM 2-MSH and 1 M NaCl. The gradient was centrifuged at 59 000 rev/min for 25 h at 2°C in an International SB-405 rotor. Fractions of 200 µl were collected from the bottom of the gradient and 5 µl aliquots from each fraction were tested for DNA-joining activity as described in Materials and Methods. Arrows indicate the positions of the following marker proteins: (a) F-MuLV reverse-transcriptase (molecular weight 84 000), (b) T_4-ligase (molecular weight 68 000), (c) F-MuLV associated endonuclease (molecular weight 40 000), (d) cytochrome C (molecular weight 12 500). F-MuLV associated reverse-transcriptase and endonuclease activities were assayed as earlier described (14, 8).

The virus associated DNA-joining enzyme was partially purified and a rough estimate of its molecular weight was obtained by velocity sedimentation of the enzyme through a 10-30% glycerol gradient (Fig. 3). More accurate methods for determining the molecular weight and further purification of the enzyme failed due to the high lability of the enzyme and the small amount of starting material at our disposal. The DNA-joining activity appeared to sediment together with the F-MuLV associated endonuclease whose molecular weight is about 40 000 (8), and slightly behind the F-MuLV reverse-transcriptase and T_4-ligase whose molecular weights are about 84 000 and 68 000 respectively (14,15). Based on the sedimentation study, the molecular weight

of the DNA-joining enzyme was estimated to be 40 000±15 000. The DNA-joining activity observed in the first three fractions of the glycerol gradient (Fig. 3) was presumably due to a pelleting of viral particles which had not been completely solubilized by Triton X-100.

The molecular weight of the viral DNA-joining enzyme is surprisingly small considering that mammalian cell ligases all appear to have molecular weights above 80 000 (13). In view of the fact that there is some uncertainty in molecular weight determinations obtained by velocity sedimentation studies, our data do not completely rule out the possibility that the virus associated enzyme is a cellular ligase. Moreover, partial degradation of the enzyme similar to that which occurs with the F-MuLV reverse-transcriptase upon freezing the virus or by the action of proteases (14) could account for a low estimate of the molecular weight of the DNA-joining enzyme. The apparent low molecular weight, however, could also suggest that the enzyme is infact a bonafide viral protein.

Though the virus associated DNA-joining enzyme probably circularizes viral DNA *in vitro* (1), its function in the *in vivo* replication of the viral DNA is not obvious. Further characterization of the retrovirus associated DNA-joining activity is consequently necessary in order to establish the biological significance of the activity.

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