PRIMASE ACTIVITIES CONSTANTLY PRESENT IN AVIAN MYELOBLASTOSIS VIRUS CORE ISOLATES: DETECTION AND BASIC CHARACTERISTICS

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Summary. – RNA-synthesizing activities (RNA-SAs) by its nature identical with primase activities (Pr-As) were found to be constantly present in avian myeloblastosis virus (AMV) core isolates. Their endogenous templates are molecules of the virus core-bound host cell DNA (AMV DNA) (Říman and Beaudreau, 1970) that have been recently recognized as a collection of still active early replicative structures (Říman *et al.*, 1993b). Like the Pr-As, the RNA-SAs are not inhibited by α-amanitin nor by aphidicolin and they show a mutually competitive affinity for ATP and GTP. Their reaction products treated with DNase I are short RNAs similar in length to initiator RNAs (iRNAs), their precursors and degradation products. In AMV core proteins separated in isopycnic CsCl gradients, they are chiefly located in the density region of reverse transcriptase activities (RT-As) but with a distinct peak fraction. Like Pr-As, they are able to use poly(dT) as template and to form, in the presence of [α-^{32}P]ATP, products that after DNase I treatment consist of poly(rA) molecules similar in length to iRNA monomers and multimers. Like the Pr-As, they are able to complement *E. coli* DNA polymerase (pol) I reactions. They occur in the analyzed AMV core proteins as six distinct sedimentation species (PrA-SS). This, together with other relevant properties, indicates the presence of Pr-As associated with molecules of a primase-α DNA polymerase enzyme complex, its degradation products and 'free' primase monomers.

Key words: AMV core-bound DNA; primases; initiator RNAs

Introduction

It has only recently been recognized that the host cell 7 S DNA constantly present in the AMV complex (AMV DNA) (Říman and Beaudreau, 1970) in association with the virus core (Deeney *et al.*, 1976; Dvořák and Říman, 1980) represents a collection of still active minute early replicative structures (Říman *et al.*, 1993a,b; Korb *et al.*, 1993). These unique features implicate that molecules of such a DNA might enter the virus core in association with special proteins (enzymes and factors) relevant to the early stages of eukaryotic cell DNA replication (for review see Heintz *et al.*, 1992). To test this possibility evidently important for elucidation of the role of AMV DNA and for a better understanding of the actual enzyme composition of the AMV core nucleoprotein complex, we searched the AMV primarily for the enzymatic activities crucially important for the initiation of DNA synthesis. In prokaryotes as well as in eukaryotes these activities prime the bidirectional synthesis of DNA in both its strands (Wang, 1991). In contrast to prokaryotes, in eukaryotes the relevant enzymes termed

Abbreviations: Aldo = aldolase; AMV = avian myeloblastosis virus; BPB = bromophenol blue; BSA = bovine serum albumin; Cat = horse liver catalase; Cyt c = cytochrome c; DNA pol-As = DNA polymerase activities; DNase = deoxyribonuclease; dNTPs = deoxyribonucleoside triphosphates; DTT = dithiothreitol; iRNAs = initiator RNAs; NA = nucleic acids; NP-40 = Nonidet-P40; NTPs = ribonucleoside triphosphates; PMSF = phenylmethyl sulfonyl fluoride; pol = polymerase; poly(dT) = polydeoxythymidylate; Pr = primase; Pr-As = primase activities; PrA-SS = primase activity sedimentation species; Pr-α DNA pol = primase-α DNA polymerase; RNase = ribonuclease; RNA-SAs = RNA-synthesizing activities; RT-As = reverse transcriptase activities; SB = solubilizing buffer; Topo I-As = topoisomerase I activities; XC = xylene cyanol

primases (Pr) are most tightly associated with other components making up a Pr-α DNA polymerase (Pr-α DNA pol) enzyme complex (Gronostajski *et al.*, 1984). For detection of Pr-As in AMV, we used as starting material virus core isolates only in order to rule out an accidental non-specific external contamination by host cell components. Using different methods for detection of Pr-As including analysis of the reaction products as recommended by Roth (1987), we present here the first evidence that the AMV core isolates and a certain defined small portion of AMV core proteins are associated with RNA-SAs whose properties fully correspond to those of Pr-As. We show further that the endogenous templates of these Pr-As in virus core isolates are virus core-bound AMV DNA molecules. The constant occurrence of six distinct PrA-SS detected in virus core proteins indicates that Pr-As present in AMV core isolates represent the Pr-As associated with molecules of a Pr-α DNA pol complex, its degradation products and with molecules which may be 'free' Pr hetero-monomers. Implications of these findings are discussed.

Materials and Methods

Chemicals. Phenylmethylsulfonyl fluoride (PMSF), urea, bromophenol blue (BPB), xylene cyanol (XC), and α-amanitin were from Serva. Cesium chloride AnalaR was from BDH Chemicals. Nonidet-P40 (NP-40) was from LKB. Bovine serum albumin (BSA), aphidicolin, and glutathione (reduced form) were from Sigma. Unlabelled ribonucleoside triphosphates (NTPs), deoxyribonucleoside triphosphates (dNTPs) and dithiothreitol (DTT) were from Calbiochem. All other chemicals used were of A grade.

Radioisotopes. [α-^{32}P]adenosine-5'-triphosphate ([α-^{32}P]ATP) and [α-^{32}P]deoxyadenosine-5'-triphosphate ([α-^{32}P]dATP), 110 TBq/mmol each, were from Amersham.

Enzymes. Deoxyribonuclease (DNase) I (ribonuclease-free) and ribonuclease (RNase) A were from Worthington. *E. coli* DNA pol I was from Sigma. Horse liver catalase (Cat) was from Calbiochem, aldolase (Aldo) from Reanal and cytochrome c (Cyt c) from Light. Rabbit polyclonal antibodies against AMV αß-RT molecules were kindly provided by Prof. D.P. Grandgenett.

Templates. DNA template was calf thymus DNA (Worthington) activated (Maniatis *et al.*, 1982) or activated and thermally denatured. RNA template was AMV 65 S RNA as described earlier (Říman, 1971). Synthetic template was polydeoxythymidylate (poly(dT), Sigma) with chain length of about 100 residues.

Virus and virus core isolation. The AMV complex, virus BAI strain A (Říman and Beaudreau, 1970) was isolated from leukemic chicken blood plasma successively by discontinuous and isopycnic sucrose gradient centrifugation (Říman *et al.*, 1993a). Fractions of a density 1.160 g/cm^3 were selected for virus core isolation accomplished according to Stromberg and Litwack (1973) with modifications (Říman *et al.*, 1993a). AMV core isolates with a density 1.25 g/cm^3 consisted electron microscopically of homogenous populations of virus core structures (Korb *et al.*, 1993). One batch of AMV core isolates (6 equal sediments) derived from AMV present in 150-180 ml of leukemic chicken blood plasma (about 10^{14} virus particles) contained 3.5 – 4.5 mg protein. Enzymatic activities of AMV core isolates kept as sediments in cellulose nitrate tubes at -70 °C did not change over 6 months.

Virus core preparation. For detection of RNA-SAs (Pr-As) in core isolates or for separation of virus core proteins, 2 to 3 virus core sediments were suspended at 0 °C in aliquots (up to 0.9 ml) of the solubilizing buffer (SB) of Weber *et al.* (1973) containing NP-40, reduced glutathione, DTT and glycerol but enriched in PMSF (0.1 mmol/l). After 30 mins of gentle mixing at 0 °C, the detergent-solubilized virus core samples were ready for experiments. Separation of virus core proteins was accomplished according to Weber *et al.* (1973). Aliquots of detergent-solubilized core isolates (1 to 2 mg of protein) were run in isopycnic CsCl gradients (starting CsCl density 1.40 g/cm^3). Centrifugation conditions: 44 hrs 30 mins at 37,000 rpm, Spinco rotor SW 50.1 at 2 °C. By piercing the tubes the fractions were collected at 0 °C into Eppendorf tubes calibrated for 210 µl. At 0 °C, the fractions showed no substantial loss of RNA-SAs (Pr-As) for about 7 days.

Sedimentation characteristics of Pr-As. The relevant CsCl gradient fractions (see Results and Discussion) were dialyzed at 0 °C in SB without NP-40 and then subjected to velocity centrifugation (Spinco rotor 50.1) at 37,000 rpm for 20 hrs at 2 °C in 35 to 15% glycerol gradients supplemented with SB without NP-40. In parallel, a mixture of Cat (3,600 units), Aldo (0.8 mg) and Cyt c (0.4 mg) was run as sedimentation markers. Fractions collected and kept as described above were without any substantial loss of enzymatic activities for 6 days. The sedimentation position of Cat was determined enzymatically (Martin and Ames, 1961), and that of Aldo and Cyt c by determining the protein concentration (Weber *et al.*, 1973). Approximate S values (sedimentation coefficients) at peak positions of the enzymatic activities were determined according to Martin and Ames (1961).

Assay of RNA-SAs (Pr-As) in solubilized virus core isolates (Assay 1). The reaction mixture (50 µl) contained 0.05 mol/l Tris-HCl pH 7.2, 0.01 mol/l $MgSO_4$, 0.10 mmol/l DTT, 0.05% BSA, 0.1% NP-40, 40 µmol/l unlabelled CTP, UTP and GTP each, 1.5 µCi [α-^{32}P]ATP and an aliquot (about 100 µg of protein) of solubilized virus core isolate.

Assay of RNA-SAs (Pr-As) in CsCl gradient fractions of virus core proteins (Assay 2). The reaction conditions were the same as in Assay 1, except that the reaction mixture was without NP-40 and that activated and denatured template DNA (5 µg) and aliquots (5 µl) of CsCl gradient fractions were added.

Assay of RNA-SAs (Pr-As) in CsCl gradient fractions of virus core proteins with poly(dT) as template (Assay 3). The reaction mixture was the same as in Assay 2, except that unlabelled NTPs were omitted and poly(dT) (0.22 µg) was added. [α-^{32}P]ATP (2.0 µCi) and an aliquot (5 µl) of CsCl gradient fraction were added.

Assay of RT-DNA pol-As with activated DNA template (Assay 4). The reaction mixture (50 µl) contained 0.05 mol/l Tris-HCl pH 8.2, 0.005 mol/l $MgCl_2$, 0.040 mol/l KCl, 0.002 mol/l DTT, 40 µmol/l dCTP, dTTP, dGTP each, 4 µmol/l unlabelled dATP,

1.5 μCi [α-^{32}P]dATP, and 5 μg activated DNA (Weber *et al.*, 1973). Two and five μl of CsCl- and glycerol-gradient fractions, respectively, were used for the reaction.

Assay of RT-DNA pol-As with RNA template (Assay 5). The reaction mixture was the same as in Assay 4, except that the template was 65 S AMV RNA (10 μg) (Grandgenett *et al.*, 1976).

Assay of DNA pol-As under conditions stimulating α DNA pol-As according to Banks et al. (1979) (Assay 6). The reaction mixture was that used in Assay 4, except that 0.04 mol/l $(NH_4)_2SO_4$ instead of KCl was added.

E. coli DNA pol I complementary assay (Conaway et al., 1982) (Assay 7). The reaction mixture (50 μl) contained 0.05 mol/l Tris-HCl pH 7.2, 0.010 mol/l $MgSO_4$, 0.10 mmol/l DTT, 0.05% BSA, 0.22 μg poly(dT), 12.5 μmol/l unlabelled dATP, 3.03 mmol/l unlabelled ATP (Gronostajski *et al.*, 1984), 2.5 μCi [α-^{32}P]dATP, 0.5 units *E. coli* DNA pol I, and 5 and 10 μl of CsCl- and glycerol-gradient fraction, respectively.

Unless otherwise stated all enzymatic assays were carried out at 37 °C for 30 mins, stopped with EDTA (2 μl 0.5 mol/l per 50 μl), supplemented with yeast tRNA (100 μg) and precipitated at 0 °C with Spiegelman's mixture modified by Weber *et al.* (1971). The material insoluble in 5% TCA was collected and thoroughly washed on Synpor No. 6 filters. The radioactivity of dried filter discs was counted on the basis of the Cerenkov effect or in toluene-based scintillation fluid in a Beckman spectrophotometer LS 6000 SE.

Preparation of reaction products. Solubilized virus core sediments (100 μg protein) were incubated for 30 mins at 37 °C in 30 μl reaction mixture used in Assay 1 but with 50 μCi of [α-^{32}P]ATP. Then EDTA was added and the reaction samples were supplemented with SDS (to 0.2%) and proteinase K (10 μg) and once more incubated for 15 mins at 37 °C. The samples were then extracted by phenol-chloroform and divided into two equal portions that were ethanol-precipitated according to Chang *et al.* (1984) with yeast tRNA (50 μg) at -20 °C for 24 hrs, sedimented by centrifugation, and dried in vacuum. One of these sediments was further treated with DNase I from 6 to 10 hrs at 37 °C. The treatment with DNase I (10 μg) was performed in 5 μl of digestion buffer (Maniatis *et al.*, 1982). The reactions were stopped by EDTA and heating for 2 mins at 100 °C. Then both the DNase I-treated and the untreated (native) portions of the reaction products were prepared in final volumes of 12 μl in formamide mixture (Maniatis *et al.*, 1982) for electrophoretic analysis.

Preparation of products of the reactions performed with the peak CsCl gradient fraction of the RNA-SAs (Pr-As) was closely similar, except that the reaction mixture (100 μl) of Assay 3 contained 100 μCi [α-^{32}P]ATP, 2 μg poly(dT) and 15 μl of the relevant CsCl gradient fraction.

Polyacrylamide gel electrophoresis (PAGE) of the reaction products in 12% slabs (17 x 12 x 0.04 cm) with 7 mol/l urea in TBE buffer of Maniatis *et al.* (1982) was accomplished at 300 V for 210 mins at +8 °C. Native and DNase-treated samples were run in parallel. Radioactivity distribution was estimated by counting the radioactivity on the basis of the Cerenkov effect in 4 mm thick gel slices dried on filter discs. Positions of the radioactive label were estimated using BPB, XC and synthetic markers as described previously (Říman *et al.*, 1993b). PAGE of the reaction products in 20% gel with 7 mol/l urea (Singh and Dumas, 1984; Singh *et al.*, 1986) was performed in slabs (15 x 35 x 0.03 cm) at 1000 V and +15 °C for 5 hrs. Then the gels were subjected to autoradiography with Foma X-ray film at -70 °C in the presence of an intensifying screen. Positions of the radioactive label were estimated using XC, BPB and *Eco*RI linker-ladder size standards of Singh and Dumas (1984).

Protein in AMV core isolates was estimated by the Lowry method (Říman *et al.*, 1993b). In CsCl- and glycerol-gradient fractions, it was estimated spectrophotometrically at 400 nm measuring the turbidity of aliquots of gradient fractions and BSA standards precipitated with 3% TCA (Weber *et al.*, 1973). One A_{400} unit was equal to 400 μg of protein.

Results and Discussion

Basic properties of RNA-SAs associated with AMV core isolates

Data relevant to these characteristics are depicted in Fig. 1. Incubation of aliquots of detergent-solubilized AMV core isolates in the reaction mixture with [α-^{32}P]ATP and other three common unlabelled NTPs led to a time-dependent incorporation of [α-^{32}P]AMP into acid-insoluble material. Treatment of the reacted samples with RNase A prior to acid precipitation rendered the reaction products soluble. Thus we had to deal with RNA-SAs associated with AMV

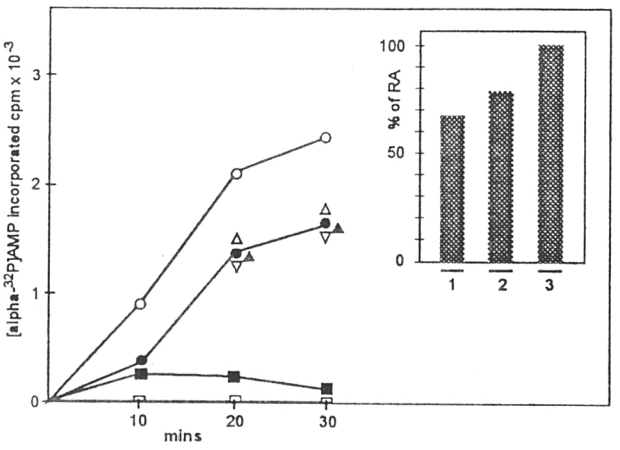

Fig. 1

RNA-SAs of the detergent-solubilized AMV core isolates

Incorporation of [α-^{32}P]AMP into acid-insoluble material under the reaction conditions of Assay 1. Reactions without added template (●) in the presence of α-amanitin (1 μg/ml (△) and 100 μg/ml (▲)), aphidicolin 2.5 μg/50 μl) (▽) or DNAse I (20 μg/50 μl) (■). Reaction without added template followed by RNAse A (2 μg/50 ml) treatment at 37 °C for 15 mins (□). Reaction with added activated and denatured calf thymus DNA (4 μg/50 μl) (○). Insert: Incorporation of [α-^{32}P]AMP expresses in % radioactivity (RA) in the presence of labelled ATP only (1), labelled ATP and unlabelled CTP and UTP (2), and the complete NTP-set (3) in reaction taken as 100% of incorporated RA.

core isolates. These activities were DNA-dependent. On one hand the inclusion of DNase I into the reaction samples led to a gradual inhibition of the endogenous reactions, on the other hand the addition of activated and denatured calf thymus DNA stimulated the radioisotope incorporation by 40%. As evident from the inhibitory effect of DNase I on the endogenous reactions, the RNA-SAs present in AMV core isolates used as their natural endogenous template the DNA molecules occurring in AMV core isolates, i.e. those of the AMV DNA, since this DNA is the only DNA species which was found to be constantly present in AMV core isolates (Deeney *et al.*, 1976; Dvořák and Říman, 1980). This conclusion was also tested directly by checking the physicochemical properties of DNA isolated from the same batch of AMV core isolates used in these experiments. This DNA exhibited the same properties as did the core-bound AMV DNA (Říman *et al.*, 1993a,b; Korb *et al.*, 1993). An about 40% stimulatory effect of added denatured DNA on the endogenous reactions may indicate that the enzyme molecules relevant to virus core-associated RNA-SAs are not saturated with the endogenous template (Chang *et al.*, 1984) using only its suitable single-stranded portion (Singh and Dumas, 1984). This might be the case of virus core-bound AMV DNA molecules, up to 30% of which were found to be single-stranded (Dvořák and Říman, 1980; Říman *et al.*, 1993a). To distinguish between AMV core-associated RNA-SAs and the other cellular RNA polymerases, we used the α-amanitin inhibition (Manley, 1987). This drug in concentrations inhibiting RNA pol II and III did not affect the virus core-associated RNA-SAs (Fig. 1). Since the sequence properties of AMV DNA molecules (Říman *et al.*, 1993b) make an expression of RNA pol I activities unlikely, the behaviour of the virus core-associated RNA-SAs toward α-amanitin is similar to that of Pr-As (Gronostajski *et al.*, 1984). As in the case of Pr-As, these RNA-SAs were not inhibited by aphidicolin in concentrations inhibiting the DNA pol-As, but not the Pr-As of a Pr-α DNA pol complex (Gronostajski *et al.*, 1984). Resemblance of RNA-SAs was found also by comparing the influence of various combinations of unlabelled common NTPs on the incorporation of [α-^{32}P]AMP from labelled ATP. As shown in the insert in Fig. 1, a mere presence of labelled ATP is sufficient to achieve about 70% of the maximum activity obtained with the complete set of NTPs. Such a phenomenon observed in Pr-As of tissue extracts reflects extra demands of Pr-As for ATP and GTP in a ratio of 4:1, respectively, for the initiation of the iRNA synthesis (Roth, 1987; Reichard *et al.*, 1974). Consequently, in the case of the virus core-associated RNA-SAs, this characteristic may reflect multiple initiation events taking place on virus core-associated AMV DNA molecules which were found, in addition, to be relatively AT-rich (Říman *et al.*, 1993a). Preferential initiation sites for Pr reactions seem to be, generally, the pyrimidine-rich regions and more specifically the ARS-like sequence motifs (Roth, 1987; Heintz *et al.*, 1992). Actually both sequence properties were found to be present in cloned AMV DNA molecules (Říman *et al.*, 1993b).

Characteristics of the products of RNA-SAs in virus core

The reaction products, the labelled nucleic acids (NAs) isolated from virus core samples assayed for RNA-SAs (see *Materials and Methods*), were analyzed untreated (native) and DNase I-treated by PAGE in denaturing conditions (Fig. 2) and, in addition, by velocity glycerol gradient centrifugation. The RNA label (the newly synthesized RNAs labelled with [α-^{32}P]AMP) of the native products resided mostly at electrophoretic mobility positions of molecules about 40 – 100 b in length besides a smaller portion of RNA label situated in the migration zone of very short RNAs up to 6 b long. In contrast, DNase I treatment of the reaction products led to the disappearance of RNA label in the zone of 40 – 100 b and to its accumulation in the zone of short RNAs about 8 to 3 b in length that are compatible in size with the iRNAs, their precursors and degradation products (Roth, 1987). This indicates that RNA label of the native products located at positions of the molecules about 40 and 100 b long monitors the presence of the newly synthesized very short RNAs linked with the newly synthesized short

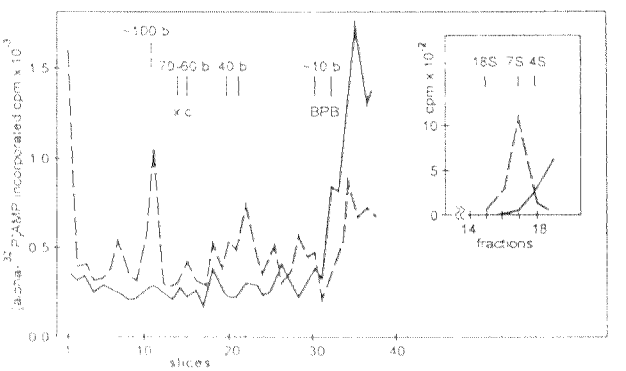

Fig. 2
Electrophoretic mobility pattern of [α-^{32}P]AMP-labelled products of RNA-SAs of detergent-solubilized AMV core isolates in denaturing conditions

Reaction conditions of Assay 1. Native (broken line) and DNase I-treated (continuous line) samples of isolated reaction products run in parallel in 12% PAGE in 7 mol/l urea. XC and BPB used as size markers. Positions of 100 and 40 b long molecules estimated according to Říman *et al.* (1993b). Insert: sedimentation characteristics of the untreated (broken line) and DNase I-treated (continuous line) samples of reaction products analyzed above. A linear 40 – 10% glycerol gradient supplemented with TNE buffer of Maniatis *et al.* (1982) run at 35,000 rpm for 3 hrs at +4 °C. Chicken myeloblastic 18 S rRNA and 4 S tRNA, and 7 S AMV DNA used as size markers (Říman *et al.*, 1993a).

strands of DNAs (made on the same endogenous template molecules), reflecting in this way the presence of RNA-DNA molecules that are reminiscent by size of the Okazaki fragment precursors (Nethanel *et al.*, 1989) and Okazaki fragments, respectively (Nethanel and Kaufmann, 1990; Bullock *et al.*, 1991). Actually the covalently linked RNA-DNA molecules similar in length to those present in the RNA-SAs reaction products of AMV core isolates were found to be constantly present in the single-stranded portion of the virus core-bound AMV DNA (Říman *et al.*, 1993a). The occurrence of newly synthesized RNA-DNA molecules in RNA-SAs reaction products suggests that these activities may act in virus core isolates similarly as Pr-As associated with the Pr-α DNA pol complex (Chang *et al.*, 1984). Complementary to these data are the sedimentation characteristics of the native and DNase I-treated reaction products (insert in Fig. 2). RNA label of the native products sedimented with a distinct peak at about 7 S whereas DNase I treatment led to a dramatic shift of the RNA label profile into the region of the lowest S values. Thus the presence of RNA label at position 7 S simulating the presence of a 7 S RNA species virtually represents newly synthesized very short RNAs linked with newly synthesized DNAs into RNA-DNA molecules about 100 b in length. Interestingly, a 7 S RNA was detected in retroviruses using labelling for RNA (for review see Coffin, 1982).

To achieve a better resolution of the short RNAs synthesized by virus core-associated RNA-SAs, we analyzed the native and DNase I-treated reaction products by PAGE in denaturing conditions (7 mol/l urea) followed by gel autoradiography. In this way, we characterized the reaction products of two tested virus core samples originating from two different batches of AMV core isolates designated further as A and B (Fig. 3A and 3B). Their radioactive label visible by autoradiography, being RNase A-sensitive, was of RNA nature (Fig. 3A, lane 0). This RNA label present in native reaction products was mostly bound with molecules 40 to 100 b long (see Fig. 3A and 3B, lanes 1). A small portion of this label situated at positions of short molecules 3 to 6 b in length was visible in the native reaction products of sample A (Fig. 3A, lane 1). DNase I treatment of the reaction products of both virus core samples originating from different isolation batches led to closely similar characteristics (compare lanes 2 in Fig. 3A and 3B): an appearance of a ladder-like assortment of RNA label in the zone of molecules 3 – 12 b in length with its distinct accumulation in a band corresponding to positions of molecules 7 – 10 b long with the iRNAs of a full-size basic unit (Singh *et al.*, 1986). The less evident presence of the label at positions of 14 to 26 b and a distinct one at position of about 50 – 60 b and below the start most likely reflect the presence of iRNAs still linked with short DNA fragments resisting DNase I digestion (Singh and Dumas, 1984). A complete DNase I digestion of the reaction products originating from the activities of a Pr-α DNA pol complex is always a problem. Attempts were made to solve it by using very long digestion times (Gronostajski *et al.*, 1984). This seems to be also the case of the products of AMV core-associated RNA-SAs in our study, and it may reflect the special properties of their endogenous templates represented by AMV DNA molecules. Interestingly, AMV DNAs are also unsatisfactorily labelled by the „nick" translation procedure using DNase I treatment (J. Říman, unpublished data). In

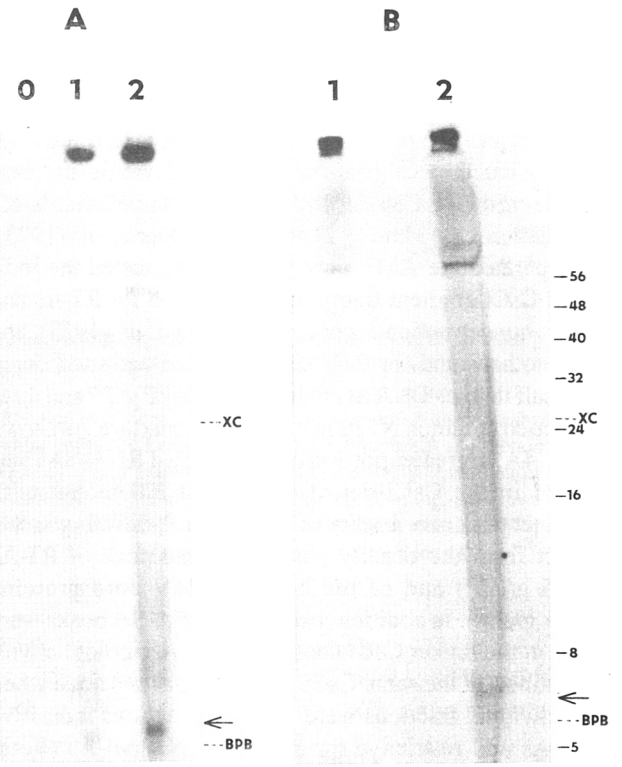

Fig. 3

Autoradiograms of the electrophoretic pattern in denaturing conditions of [α-^{32}P]AMP-labelled reaction products of RNA-SAs (Pr-As) of detergent-solubilized AMV core isolates

Reaction conditions of Assay 1, 20% PAGE in 7 mol/l urea. Two samples (A and B) originated from different batches of AMV core isolates. Lanes 1: the label distribution characteristic of the native (untreated) reaction products. Lanes 2: reaction products treated with DNAse I at 37 °C for 7 hrs. Lane 0: RNA nature of reaction products. In this case the products treated with DNase I were additionally treated with RNase A (5 μg/50 μl) at 37 °C for 30 mins. Size distribution of the reaction products evaluated graphically using XC and BPB indicating positions of polynucleotides 26 and 6 b long (Singh *et al.*, 1987) and positions of *Eco*RI linker-ladder size standards given for the same experimental conditions (Singh and Dumas, 1984). Arrows indicate accumulation of ribopolynucleotides 7 b long in both DNase-treated samples (lanes 2, A and B).

conclusion, the characteristics of the reaction products of RNA-SAs associated with AMV core isolates, together with other data obtained in this Section, suggest that these RNA-SAs resemble the Pr-As associated with a Pr-α DNA pol complex.

Detection of RNA-SAs of the Pr nature in AMV core proteins

Results obtained by reactions accomplished with virus core isolates prompted us to search for these RNA-SAs in AMV core proteins. The problem of separating scarce amounts of AMV core proteins (3 – 4 mg of protein per batch of AMV core isolates) by column chromatography (Grandgenett *et al.*, 1978) was resolved by centrifugation of the detergent-solubilized AMV core isolates in isopycnic CsCl gradients according to Weber *et al.* (1973). Using this method it is possible to separate from the bulk of AMV core proteins a natural template-primer-free reverse transcriptase (RT) with well preserved enzymatic activities and physicochemical properties of an enzyme purity comparable with the CM-Sephadex step of the Kacian's RT purification procedure as checked by Weber *et al.* (1973). We separated the AMV core proteins and tested the individual CsCl gradient fractions on one hand for RT-As and protein concentration according to Weber *et al.* (1973), and on the other hand for RNA-SAs using an activated denatured calf thymus DNA as template and [α-^{32}P]ATP and three unlabelled common NTPs in the reaction mixture. As shown in Fig. 4A, a greater portion of the detected RNA-SAs was located in the CsCl-density region of RT-As but with a distinct peak at a density of 1.265 g/cm^3, differing in this respect from the density position of the peak of RT-As (1.275 g/cm^3) and of the bulk of AMV core proteins (1.230 g/cm^3). In addition, two minor RNA-SA peaks were found in the higher CsCl density region. A similar activity distribution in the same CsCl gradients was obtained when the individual fractions were tested in reactions indicative of Pr-As and restrictive for all other RNA pol-As (Roth, 1989), i.e., in the presence of poly(dT) as template and [α-^{32}P]ATP (Fig. 4B). The activities expressed under these conditions were more pronounced over the whole density region of the RT-As. To complete this characteristic with another test indicative of Pr-As, we tested the individual gradient fractions of the density region of RT-As for their ability to stimulate the *E. coli* DNA pol I reaction in the complementary assay with poly(dT) as template and [α-^{32}P]dATP at high concentrations of ATP (Conaway *et al.*, 1982). As evident in Fig. 5, the activity profile of the stimulated *E. coli* DNA pol I, reflecting the distribution of Pr-As in the individual fractions tested, resembled, including the position of the activity peak, the activity profile characteristics obtained by previous tests (compare Fig. 4A and 4B).

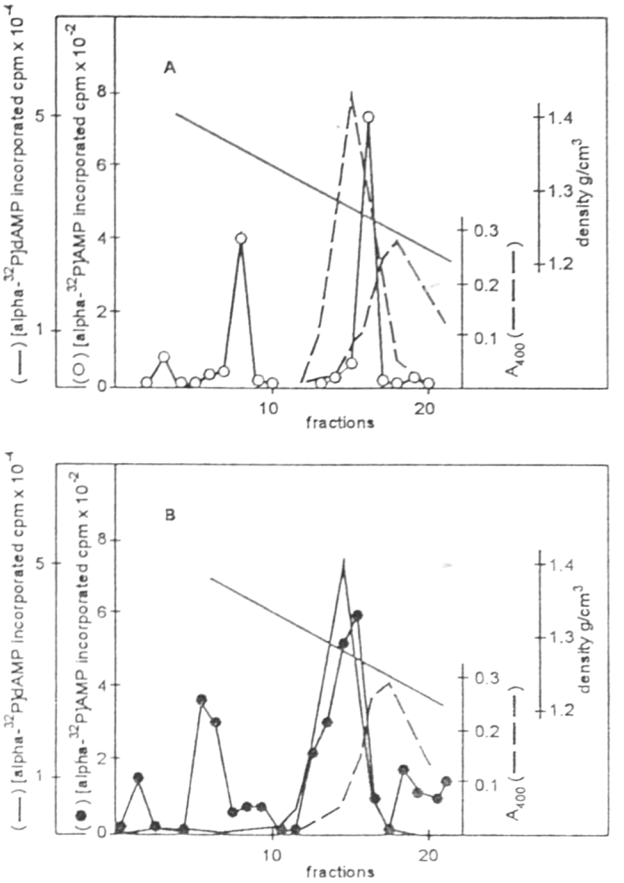

Fig. 4
Equilibrium CsCl density gradient distribution of RNA-SAs (Pr-As) and RT-DNA pol-As in AMV core proteins
Detergent-solubilized AMV core isolates subjected to sedimentation analysis. A: RNA-SAs (O) determined by Assay 2, RT-DNA pol-As (continuous line) determined by Assay 4, and concentration of virus core proteins (A_{400} units, broken line). One A_{400} unit is equal to 400 μg of protein. B: RNA-SAs (●) determined by Assay 3, RT-DNA pol-As (continuous line) determined by Assay 4, and concentration of virus core proteins (broken line).

Fig. 5 shows further that, in accordance with the properties of Pr-As (Conaway *et al.*, 1982), the absence of ATP completely abolished the stimulatory effect of aliquots of the individual fractions tested in this complementary assay. It also shows a strong inhibitory effect of GTP which competes efficiently for ATP in reactions of the Pr type (Gronostajski *et al.*, 1989). Using this test we also compared directly its sensitivity with that of the assay using poly(dT) as template and [α-^{32}P]ATP. From closely similar activity profiles at identical specific radioactivities of the relevant radioisotopes used per reaction it is evident that *E. coli* DNA pol I complementary assay was at least three times more sensitive than the poly(dT) test (Fig. 5).

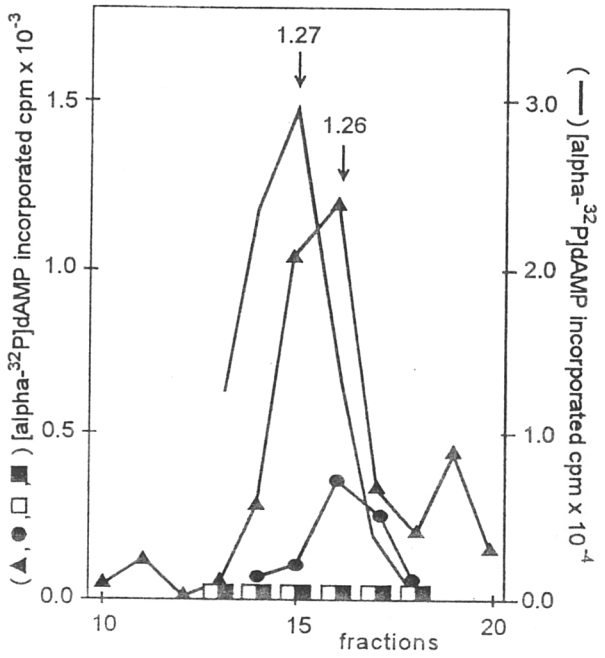

Fig. 5
Equilibrium CsCl density gradient distribution of Pr-As and RT-DNA pol-As in AMV core proteins
Another batch of detergent-solubilized AMV core isolates used. Fraction aliquots tested in *E. coli* DNA pol I complementary assay (Assay 7) (▲), in poly(dT) assay (Assay 3) (●) and in RT-DNA pol assay (Assay 4) (continuous line). Addition of GTP (0.8 mmol/l) (□) or omission of ATP (■) in reactions of Assay 7. To compare the sensitivities of Assays 7 and 3, the concentrations of the relevant radioisotopes were adjusted to the same specific radioactivity per reaction.

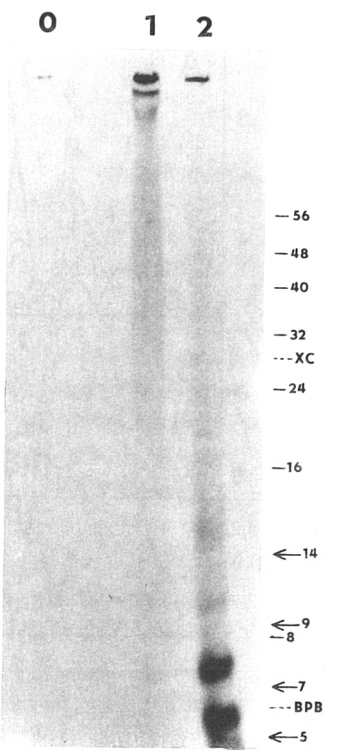

Fig. 6
Electrophoretic mobility pattern of [α-^{32}P]AMP-labelled products of RNA-SAs (Pr-As) of the CsCl density peak fraction (density 1.265 g/cm^3) of AMV core proteins
Reaction conditions of Assay 3. Lane 0: reaction accomplished without added poly(dT). Lanes 1 and 2: native (untreated) and DNase I-treated (37 °C, 6 hrs) reaction products, respectively. Positions of labelled products evaluated as described in the legend to Fig. 3. Arrows to lane 2 indicate a ladder-like assortment of newly synthesized ^{32}P-poly(rA) molecules 5, 7, 9, and 14 b long.

Characteristics of the reaction products of RNA-SAs (Pr-As) associated with AMV core proteins

To prove decisively the Pr nature of RNA-SAs associated with AMV core proteins, we analyzed by PAGE in denaturing conditions (7 mol/l urea) followed by autoradiography the native and DNase I-treated products of the reactions accomplished with the RNA-SAs (Pr-As) peak fraction (CsCl density 1.265 g/cm^3) with poly(dT) as template and [α-^{32}P]ATP. As shown by autoradiography, the synthesis of the reaction products was completely dependent on poly(dT) (Fig. 6, lane 0). The RNA (poly(rA)) label of the native products showed a diffuse gel distribution in the zone of molecules 20 – 100 b in length with a minor portion of the label in the zone of molecules 6 – 3 b long (Fig. 6, lane 1). The label distribution of the DNase I-treated products showed (Fig. 6, lane 2) a ladder-like assortment of poly(rA) molecules 5, 7, 9, and 14 b in length, suggesting formation of iRNA multimers typical of Pr-As expressed under such reaction conditions. This characteristic is considered significant for Pr-As expressed in association with a Pr-α DNA pol complex (Singh and Dumas, 1984; Singh *et al.*, 1986).

Sedimentation properties of Pr-As associated with AMV core proteins

Sedimentation properties of proteins associated with enzymatic activities represent an important basic characteristic. According to their origin the Pr-As associated with virus core proteins should reveal sedimentation properties common to Pr-As in animal cells. Accordingly, the expected S values of virus core Pr-As, if associated with a Pr-α DNA pol complex, should be from about 12 S to 9 S (Banks *et al.*, 1979). Pr-As associated with molecules of primase hetero-monomers of a M_r range of 46 – 50 K and 50 – 60 K (Roth, 1987) should reveal the S values of about 3 – 3.5 S and 4 – 4.5 S, respectively. Finally, the Pr-As of

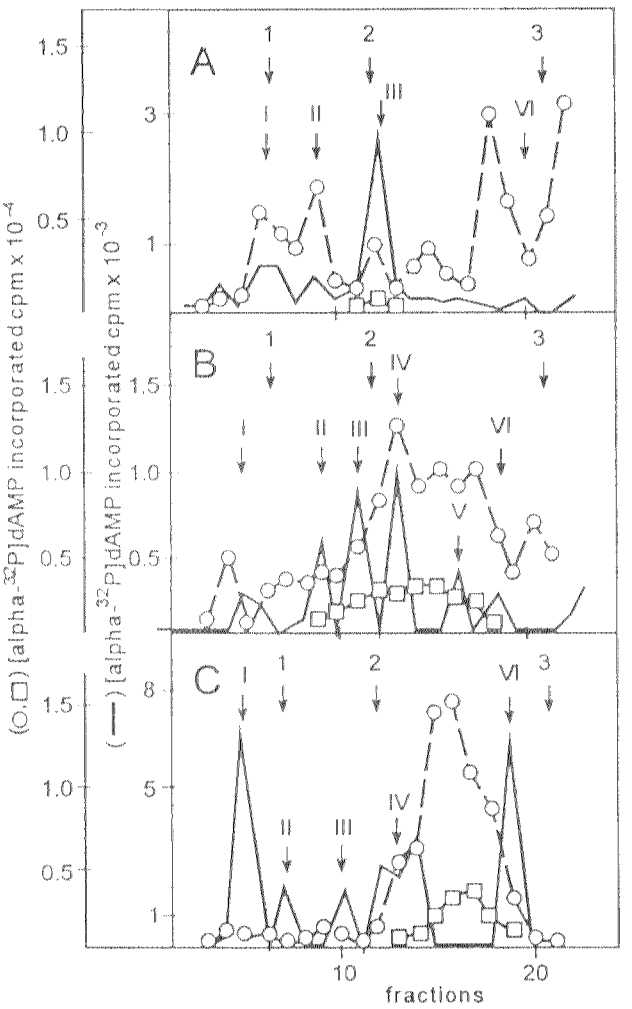

Fig. 7
Sedimentation properties of Pr-As present in CsCl density region of RT-DNA pol-As

Three selected representative fractions A, B, C with densities 1.290 g/cm^3, 1.275 g/cm^3 (the peak fraction of RT-DNA pol-As), and 1.265 g/cm^3 (the peak fraction of RNA-SAs (Pr-As)), respectively, were dialyzed and subjected to velocity centrifugation in linear 35 – 15% glycerol gradients (A, B, C). In parallel, size marker mixtures of Cat (1), Aldo (2) and Cyt c (3) were run. Their positions are indicated by vertical bars (1, 2, 3). Fraction aliquots of gradients A, B, and C were tested for Pr-As (continuous line) in Assay 7, and for RT-DNA pol-As in Assay 4 (○) and Assay 5 (□). Roman numbers (I – VI) designate the six PrA-SS. For their S values see Table 1.

primase hetero-dimer molecules were found to sediment at about 5.5 – 5.7 S (Roth, 1987). To test this assumption by sedimentation analysis, the AMV core proteins associated with Pr-As residing in the density region of RT-As appeared to be most representative, as evident from data obtained in this study. For this reason, we selected three CsCl gradient fractions designated further as A, B, and C with densities of 1.290, 1.275 (RT-As peak fraction), and 1.265 g/cm^3 (Pr-As peak fraction), respectively. These fractions were dialyzed and subjected to velocity sedimentation in glycerol gradients. The individual gradient fractions were then tested for Pr-As using *E. coli* DNA pol I complementary assay and for RT-As using an activated DNA as template and also the 65 S AMV RNA (Grandgenett, 1976) to delineate the actual region of RT-As. Fig. 7A-7C show that the analyzed representative CsCl region contained altogether (samples A, B, C) 6 distinct sedimentation species associated with Pr-As (PrA-SS I to PrA-SS VI). Their mean S and M_r values with maxima and minima of variations are given in Table 1. The PrA-SS I and II most prominent in sample C (Fig. 7C) with mean S values of 11.3 S and 9.2 S, respectively, indicate an association of Pr-As with a protein complex reminiscent of sedimentation properties of Pr-α DNA pol complex. In contrast, the PrA-SS V and VI (both are present in sample B) with mean S values of 4.3 S and 3.1 S, respectively, are reminiscent of both Pr hetero-monomers dissociated from a Pr-α DNA pol complex. The PrA-SS III and IV with mean S values of 7.4 S and 5.6 S, respectively, located always in the region of RT-As (Fig. 7A-7C), may represent the Pr-As associated, surprisingly, with two distinct degradation products of a Pr-α DNA pol complex. The PrA-SS IV may indicate also that Pr dimer molecules do occur. This explanation of the occurrence of six distinct PrA-SS in the analyzed AMV core proteins is based on the postulation that molecules of a Pr-α DNA pol complex are present, as indicated not only by the sedimentation properties of the PrA-SS I and II, but also by the properties of the reaction products of Pr-As associated with virus core isolates and their proteins, as described earlier in this study. Consequently, a demonstration was needed that PrA-SS I and II, and then also PrA-SS III and IV, are accompanied by activities of α DNA pol type. Since the glycerol-gradient fractions of the analyzed

Table 1. PrA-SS present in AMV core proteins

PrA-SS	S value (in S)			M_r		
	Max.	Min.	Mean ± SD(n)	Max.	Min.	Mean
I	12.7	10.5	11.4 ± 0.9 (5)	278 K	205 K	240 K
II	9.7	8.7	9.2 ± 0.6 (5)	197 K	168 K	180 K
III	7.4	6.6	7.2 ± 0.6 (4)	137 K	119 K	127 K
IV	6.0	5.2	5.6 ± 0.6 (5)	96 K	77 K	88 K
V	4.6	4.0	4.4 ± 0.4 (4)	64 K	50 K	56 K
VI	3.4	3.0	3.1 ± 0.5 (3)	38 K	30 K	30 K

S and M_r values were estimated according to Martin and Ames (1961) using positions of the peak fractions of Pr-As detected by Assay 7 in five velocity glycerol gradients of selected CsCl density fractions of the RT-DNA pol As region. Cat (12.3 S) (Martin and Ames, 1961), Aldo (7.4 S) (Chang *et al.*, 1984), and Cyt c (2.1 S) (Weber *et al.*, 1973) were used as standards for calculation of S and M_r values. SD = standard deviation. n = the number of PrA-SS of one size category.

virus core proteins always revealed the presence of DNA pol-As tested under RT reaction conditions in the region of PrA-SS I and II, and III and IV (Fig. 7A-7C), we decided to examine, first of all, the relevant fractions in the zone of PrA-SS I and II and comparatively those in the RT-As region for activities of α DNA pol type. For this reason, we separated the proteins from a new batch of core isolates and characterized their dialyzed fraction of CsCl density of 1.290 g/cm^3 in velocity glycerol gradients for distribution of Pr-As and RT-As, as it was done previously (Fig. 8A). Banks *et al.* (1979) have shown that ammonium sulfate profoundly stimulates the DNA pol-As of Pr-α DNA pol complex. We therefore tested comparatively the gradient fractions from the region of PrA-SS I and II and those from the region of RT-As (Fig. 8B). Under these conditions specific changes in the distribution of DNA pol-As occurred in the zone of PrA-SS I and II (Fig. 8A and 8B). A single component profile characteristic of DNA pol-As tested under RT conditions was rendered to a bi-component one that paralleled the profiles of PrA-SS I and II, thus suggesting an association of Pr-As with DNA pol-As of α type. An association of PrA-SS III and IV with similar DNA pol-As indicated a bilateral broadening of the profile of DNA pol-As in the RT region exceeding the profile of the actual RT-As (Fig. 8B) tested either with a DNA template or a viral RNA template (Fig. 8A). For further distinction of DNA pol-As associated with the relevant Pr-As, we used aphidicolin, a specific inhibitor of DNA pol-As of the α family (Wang, 1991), which at a concentration of 5 μg/ml inhibits the DNA pol-As associated in a Pr-α DNA pol complex by 85% (Banks *et al.*, 1979). This antibiotic at the same concentration inhibited to the same extent the DNA pol-As associated with PrA-SS I and II whereas it had little effect on these activities in the region of the actual RT-As (Fig. 8B). Similarly, little effect of aphidicolin has been described in the case of the adenovirus DNA pol-As in reactions using also an activated DNA as template (Encomoto *et al.*, 1981). A more distinct inhibitory effect of aphidicolin in the RT zone flanked by PrA-SS III and IV may indicate that these supposed degradation products of a Pr-α DNA pol complex are still associated with activities of α DNA pol type. In addition, we tested tentatively the activities of both compared types with polyclonal rabbit antibodies against AMV αß-RT developed by Grandgenett *et al.* (1985). While these antibodies in a titer of 1:20 inhibited the DNA pol-As in the RT zone up to 45%, they had no effect on DNA pol-As associated with PrA-SS I and II (data not shown), thus indicating their origin difference. Any association of the PrA-SS V and VI with activities of α DNA pol type was not detected, even in separate experiments. Thus, the data obtained in this Section support our explanation of the occurrence of 6 distinct PrA-SS which we found to be constantly present in AMV core proteins. Consequently, these data,

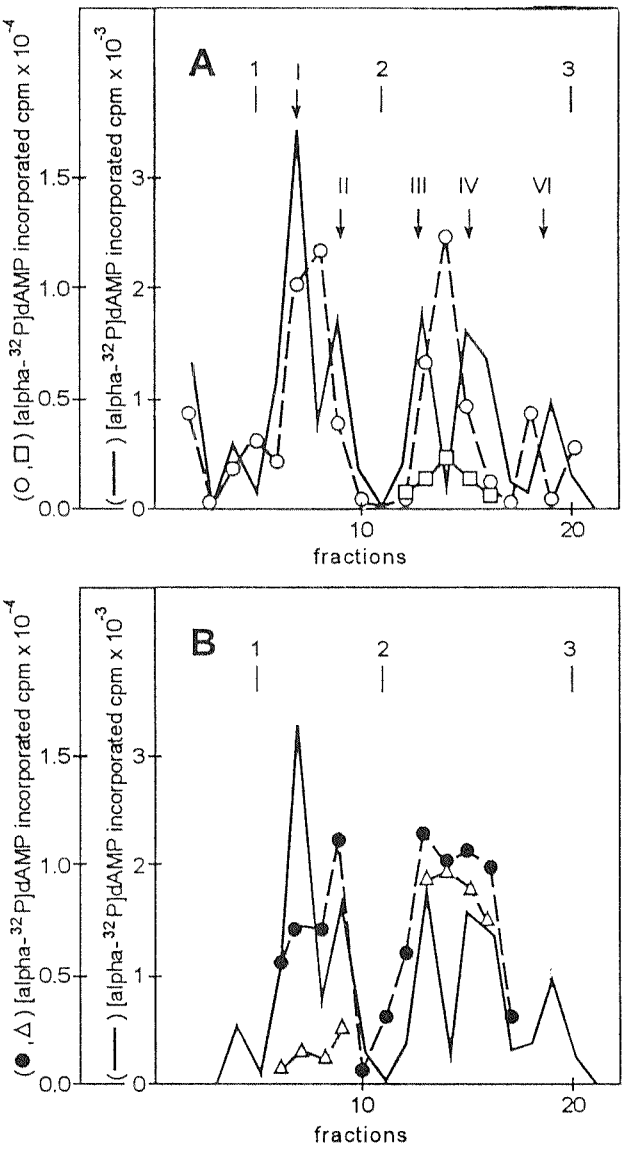

Fig. 8
Search for activities of α DNA pol type
Selected CsCl gradient fraction (density 1.290 g/cm^3) of AMV core proteins separated from a freshly prepared batch of AMV core isolates was dialyzed and sedimented in a glycerol gradient. A: Fraction aliquots were tested for Pr-As in Assay 7 (continuous line), and for RT-DNA pol-As in Assay 4 (O) and Assay 5 (□). B: Fraction aliquots of the selected regions of the same gradient were tested for α DNA pol-As in Assay 6 (●). In parallel, the same assay was done in the presence of aphidicolin (5 μg/ml) (Δ). Roman numbers designate PrA-SS and vertical bars indicate the positions of Cat (1), Aldo (2), and Cyt c (3).

together with other findings presented in this paper, show that the reaction machinery of the AMV nucleoprotein core complex is constantly enriched in special proteins of early cell DNA replication, a concrete example of which represent the activities of Pr and Pr-α DNA pol types. As shown in this study, their template dependence on virus core-bound

AMV DNA indicates that this DNA serves as a carrier transporting the relevant special cell proteins from the cell into the virus core complex. Moreover, it permits us to expect that an association of proteins relevant to early cell DNA synthesis with virus core may be significant for retroviruses, because a DNA similar to AMV DNA was found in all retroviruses so far studied (Říman *et al.*, 1993a). This expectation is supported by the occurrence of topoisomerase I activities (Topo I-As) in Rous sarcoma virus (Weiss and Faras, 1981) and, as shown more recently, also in virus core isolates of the equine infectious anemia virus and human immunodeficiency virus (Priel *et al.*, 1990). In analogy to the role of Topo I-As in the synthesis of a full-size copy of adenovirus DNA polymerase (Nagata *et al.*, 1983), one can imagine their role in the synthesis of the retroviral DNA, the formation of a double-stranded full-size copy of which still remains a privilege of the virus core nucleoprotein complex only (Grandgenett and Mumm, 1990). In the case of the Pr-As „free" or associated with a Pr-α DNA pol complex, it is possible to expect that they may play a role in the formation of RNA or „DNA" primers (Linn, 1991), suitable for certain steps of the reverse transcription, the mechanism of which is not yet fully understood. Attention deserves also the synthesis of the new AMV DNA molecules taking place in reactions accomplished with virus core isolates in the context with other reactions of the virus core machinery responsible for the replication and integration of retroviral information (Grandgenett and Mumm, 1990). These as well as the other questions, which remained unresolved in this study performed with virus core isolates that ensured virus specificity of the data obtained but was limited by scarce amounts of this material, can now be studied in more detail, starting from unfractionated AMV isolates.

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