Proteins Antigenically Related to Peptides Encoded by the Mouse Mammary Tumour Virus Long Terminal Repeat Sequence Are Associated with Intracytoplasmic A Particles

By G. H. SMITH, L. J. T. YOUNG, E. BENJAMINI, D. MEDINA1 and R. D. CARDIFF2

Laboratory of Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892, 1Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030 and 2Department of Pathology, University of California, Davis, California 95616, U.S.A.

(Accepted 30 October 1986)

SUMMARY

Intracytoplasmic A particles (CAP), previously identified as cytoplasmic nucleocapsid precursors to mouse mammary tumour virus (MMTV), reacted strongly in immunodiffusion tests with polyclonal antibodies raised against synthetic oligopeptides derived from the open reading frame (ORF) in the long terminal repeat (LTR) of MMTV. In Western blots, several CAP proteins (p80, p72-68, p36, p32, p18-12) were reactive with polyclonal antibodies raised against three separate LTR ORF synthetic peptides. Disrupted MMTV virions did not react with the anti-LTR ORF peptides suggesting that ORF proteins were excluded from mature virions during maturation. Serial dilution of anti-LTR ORF antibody demonstrated that the most reactive CAP proteins in Western blots migrated as a doublet band with estimated molecular weights of 68,000 and 72,000. Reactivity of anti-LTR ORF serum with these and other CAP proteins was removed upon preincubation with free synthetic peptide. Absorption with LTR synthetic peptides did not affect the reactivity of antibodies directed against MMTV gag proteins with similarly sized CAP polyproteins. LTR ORF-related proteins with molecular weights similar to those associated with CAP were also detectable in Western blots of total cytoplasmic extracts of MMTV-infected mammary tumour cells.

INTRODUCTION

Intracytoplasmic A particles (CAP) are extensively studied morphological entities found within the cytoplasm of cells infected with mouse mammary tumour virus (MMTV) (Kerchaert et al., 1970; Smith & Wivel, 1972, 1973; Tanaka et al., 1972; Smith & Lee, 1975; Tanaka, 1977; Smith, 1978). CAP are MMTV pronucleocapsid structures and possess all the antigenic determinants identified as MMTV gag gene products, i.e. MMTV p27, pp20, p14, p10 and p8 (Smith & Lee, 1975; Tanaka, 1977; Arthur et al., 1978; Cardiff et al., 1978; Smith, 1978). These gag protein determinants mainly reside on precursor polyproteins of 75,000 mol. wt. (75K) to 82K. Partially processed gag precursor polyproteins are also found in purified A particle preparations (Smith, 1978). Proteolytic digestion of CAP proteins results in the production of gag polyproteins similar in molecular size to those found in mature virion nucleoprotein cores (Smith & Lee, 1975; Tanaka, 1977; Smith, 1978). Antibodies raised against the high mol. wt. CAP polyproteins contain precipitating activity for MMTV virion structural proteins p27, pp20, p14 and p10 but not for the major virion envelope (env) proteins, gp52 and gp36 (Arthur et al., 1978; Smith, 1978). Purified preparations of unprocessed A particles also contain relatively large amounts of low mol. wt. (12K to 18K) proteins with little or no antigenic relationship to known MMTV structural polyproteins (Arthur et al., 1978; Smith, 1978).

MMTV is unique among the retroviruses because, in addition to genes encoding the major
structural virion proteins (*gag*, *env*) and the virus-specific RNA-dependent DNA polymerase (*pol*), its genome contains a long terminal repeat (LTR) with a 960 base pair open reading frame (ORF) in the U₃ region (Dickson *et al.*, 1981; Donehower *et al.*, 1981). This ORF appears to have coding capacity sufficient to encode a 36K protein. Sequence analysis of the entire ORF coding sequence demonstrates the existence of several internal AUG codons which could initiate the translation of LTR peptides smaller than 36K, e.g. 32.7K, 23.3K, 21K, 18.6K, 6.5K, 3.8K and 0.7K. Several bands corresponding to these have been observed following *in vitro* translation of MMTV genomic RNA (Dickson *et al.*, 1981; Racevskis & Prakash, 1984). Identification of LTR ORF-encoded proteins in MMTV-infected or transformed tissue has not as yet been accomplished.

In this paper we report that polyclonal antibodies raised against several different synthetic peptides predicted from the published LTR ORF nucleic acid sequence cross-react with proteins associated with purified MMTV preprocapsid structures (CAP). CAP proteins of 43K, 36K, 32K and 18K to 12K reacted with polyclonal antibodies raised in five separate hosts against three different LTR ORF peptides. Several higher mol. wt. CAP proteins also reacted with anti-LTR peptide sera. The most abundant and strongly reactive LTR ORF-related CAP proteins possessed molecular weights in the range of 68K to 72K.

**METHODS**

Isolation of MMTV and CAP. MMTV C3H virus isolated from the cell line MmSmt/Cl (Owens & Hackett, 1972) was used as the source of purified MMTV and was kindly provided by Dr Larry O. Arthur, Frederick Cancer Research Center, Frederick, Md., U.S.A.

CAP were isolated from Leydig cell tumors produced in BALB/c 4-NIV male mice by diethylstilbestrol treatment and carried by subcutaneous transplant in BALB/c 4-NIV female mice or from primary tumors arising in C3H/Sm MMTV breeding female mice (Graham *et al.*, 1984). CAP were separated from cellular components on discontinuous and linear sucrose gradients (Smith & Wivel, 1972, 1973).

**Antisera.** Antisera to purified MMTV, MMTV p27, MMTV p14 and MMTV p10 were prepared in rabbits as described by Arthur *et al.* (1978). Antisera against purified CAP were prepared similarly and possessed precipitating activities for p27, pp20, p14 and p10 (Author *et al.*, 1978; Cardiff *et al.*, 1978). Antibody raised in rabbits against CAP low mol. wt. proteins p18-12 (previously designated as CAP p20-18; Smith, 1978) was found to have no precipitating activity to MMTV virion proteins, but formed immunoprecipitin lines with disrupted purified CAP in immunodiffusion plates and had demonstrable activity against CAP DNA unwinding activity (Smith *et al.*, 1980).

Antisera against the MMTV LTR ORF synthetic peptides were raised in rabbits given subcutaneous and footpad injections of 0.2 mg LTR peptide–keyhole limpet haemocyanin (KLH) conjugates emulsified in complete Freund's adjuvant followed at 3 week intervals by booster injections of 0.1 mg conjugate in incomplete Freund's adjuvant. Serum samples were obtained 10 to 14 days after the last immunization. An additional LTR ORF antigen was raised against a 23 amino acid synthetic peptide was kindly supplied by Dr H. Diggelmann (Fasel *et al.*, 1982).

**LTR peptide synthesis and preparation of immunogens.** Peptides representing reading frames (I) 772 to 798, (II) 829 to 852 and (III) 799 to 819 of the LTR (Fasel *et al.*, 1981; Racevskis & Prakash, 1984). CAP were separated from cellular components on discontinuous and linear sucrose gradients (Smith & Wivel, 1972, 1973).

The peptides were synthesized by the solid-phase method of Merrifield (1964). The following amino acids (Peninsula Laboratories, Belmont, Ca., U.S.A.) were used for synthesis: t-Boc nitroarginine; t-Boc aspartic acid, β-benzyl ester; t-Boc leucine; t-Boc ε-carboxbenzoxyl lysine; t-Boc proline; t-Boc phenylalanine; t-Boc o-benzyl threonine; t-Boc o-benzyl tyrosine.

Following cleavage from the resin with HBr–trifluoroacetic acid, peptides 1 and III were subjected to catalytic hydrogenation using Pd in BaSO₄ to reduce the nitroarginine to arginine. The peptides were then purified by ion-exchange chromatography as described by Stewart & Young (1969).

Each peptide was conjugated to KLH as described by Fearney *et al.* (1971) using water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide–HCl. After coupling, the conjugates were dialyzed extensively against cold H₂O and then lyophilized. This method of conjugation results in a mixture of KLH–peptide molecules in which the peptide could be conjugated to KLH through its N terminus, C terminus, ε-aminolysine and carboxylic groups of aspartic or glutamic acid residues. All of these molecular species would be expected to be present in the final preparation.

**Cell-free translation and immunoprecipitation.** Translation of cellular and viral polyadenylated RNA was carried out using a rabbit reticulocyte lysate kit (NEK-001, [³⁵S]methionine) purchased from New England Nuclear.
Briefly, 0·1 to 1 µg of cellular or viral RNA was added to the (25 µl) translation mixture and the reaction was allowed to proceed for 60 min at 37°C. Thereafter, 10% (2·5 µl) of the reaction mixture was immediately removed and placed in 30 µl of electrophoresis sample buffer (0·0625 M-Tris-HCl, 5% 2-mercaptoethanol, 2·5% SDS, 10% glycerol, pH 6·8) and heated to 100°C for 3 min in preparation for analysis by gel electrophoresis. The remainder of the reaction mixture (22·5 µl) was diluted with 777·5 µl of lysis buffer (0·154 M-NaCl, 0·05 M-Tris-HCl, 0·5% Nonidet P40, 0·05% SDS, pH 7·4) and after brief mixing, the lysates were clarified at 12000 g for 5 min. The supernatant was then removed and incubated with 2 to 4 µl of anti-peptide or control antiserum. Immunoprecipitation was performed using Staphylococcus aureus as described by Gottesman & Cabral (1981). Electrophoresis was performed in 10 or 15% polyacrylamide gels as described by Laemmli (1970) except that the separating gel contained 10% glycerol and 0·5% linear acrylamide.

**Electrophoretic blotting procedures.** Proteins and viral particles were placed in 2% SDS, 60 mM-Tris-HCl, 5% 2-mercaptoethanol and 10% glycerol, pH 6·8, incubated at 80°C for 3 min, and then subjected to electrophoresis in one-dimensional gels of 10 or 15% acrylamide in the presence of 0·1% SDS. The gel was then soaked for 15 min in 25 mM-Tris·192 mM-glycine, 20% methanol at pH 8·3. The proteins in the gel were transferred electrophoretically to nitrocellulose as described by Towbin et al. (1979). Electrophoresis was carried out overnight at 250 mA in buffer (25 mM-glycine, 20% methanol, pH 8·3).

**Immunological detection of proteins on nitrocellulose blots.** The electrophoretic blots (unstained) were soaked in 3% bovine serum albumin in 100 mM-NaCl, 10 mM-Tris·HCl, 3 mM-2-mercaptoethanol at pH 7·4 (soak buffer) for several changes at room temperature. The protein blots were stored at −25°C after being wrapped while still moist in clear plastic film (Saran Wrap). The sheets were immersed in soak buffer plus 10% foetal calf serum for 30 min, rinsed in saline, and then exposed to primary antiserum diluted in soak buffer overnight at room temperature with constant agitation. The blots were subsequently washed in 100 ml of Tris-buffered saline pH 7·2, for five changes and then exposed to affinity-purified biotin-conjugated secondary antibody (biotin-labelled goat anti-rabbit gammaglobulin; Vector Laboratories, Burlingame, Ca., U.S.A.) diluted 1:500 in Tris-buffered saline with shaking. Antibody-antigen immunocomplexes were detected by incubating the washed blots with avidin-biotin-horseradish peroxidase conjugates (1:80) (Vector Laboratories) and immunochemical localization of peroxidase enzyme activity with diaminobenzidine (60 µg/ml) as described by Hogan & Smith (1982). The protein blots were then washed in buffer and dried.

**RESULTS**

The LTR region of the MMTV genome is known to contain an ORF (Dickson et al., 1981; Donehower et al., 1981). From the predicted amino acid sequence, we selected three small regions in the 3' portion of the LTR ORF for synthesis of representative peptides. The position of these three regions relative to internal initiation codons within the LTR ORF is shown in Fig. 1.

Two rabbits were immunized with each ORF peptide-carrier conjugate and sera were tested by solid-phase radioimmune assays for reactivity against the peptide conjugates. Antisera were also tested for their ability to immunoprecipitate [35S]methionine-labelled polypeptides synthesised by MMTV virion RNA in a rabbit reticulocyte cell-free translation system. One rabbit serum raised against LTR peptide II (see Fig. 1) showed significant activity in both tests and was selected for further study. Antibodies raised to LTR ORF peptides I, II and III were tested for immunoprecipitating activity against radioactively labelled polypeptides translated in vitro from MMTV virion RNA and from polyadenylated RNA from C37-1, a spontaneous C3H/Sm mouse mammary tumour which expresses a 2·2 kb LTR ORF RNA transcript from an endogenous MMTV provirus. In C37-1 RNA, this transcript represents the only polyadenylated MMTV-specific RNA (Graham et al., 1984; G. H. Smith, unpublished data). Antiserum against LTR peptide II precipitated approx. 36K and 24K polypeptides synthesized in vitro from MMTV RNA and occasionally smaller proteins (18K and less) not readily seen in Fig. 2 (lanes g and h). When tested for reactivity to radioactive proteins translated from C37-1 RNA, anti-LTR II recognized several polypeptide species whose apparent mol. wt. (36K, 32K, 30K, 21K) closely approximated the sizes of the polypeptides synthesized from LTR ORF cRNA (Dickson et al., 1981) and several of considerably smaller size (less than 14K). Anti-LTR III showed weak activity against similar sized proteins translated from C37-1 RNA, but anti-LTR I was essentially unreactive at similar dilutions (Fig. 2). Antibodies against MMTV or CAP which were highly reactive with polypeptides synthesized from MMTV RNA in Fig. 2 (i, j) were unreactive with proteins translated from C37-1 RNA with the exception of a polypeptide of approx. 8K (not shown). Antiserum against MEP, a transformation-dependent glycoprotein
secreted abundantly from mouse tumour cells (Gottesman & Yuspa, 1981; Doherty et al., 1985), precipitated C37-l RNA-encoded polypeptides of sizes similar to those reported earlier in translation of unselected polyadenylated RNAs from transformed mouse fibroblasts. Preincubation of anti-LTR serum with free peptide II abolished its reactivity with polypeptides synthesized in vitro from MMTV RNA (not shown).

In immunodiffusion experiments, anti-LTR II was tested against disrupted, gradient-purified CAP, disrupted, gradient-purified MMTV and undiluted normal mouse serum (Fig. 3). Anti-LTR II reacted strongly with CAP in all of these tests and formed immunoprecipitin lines with antibodies raised against purified CAP, CAP p18-12, MMTV p27 and a 23 amino acid LTR ORF (LTR 23) synthetic peptide. However, essentially no reaction was observed between anti-LTR II and gradient-purified MMTV. Preincubation of anti-LTR II with free peptide abolished its immunoprecipitin reaction with CAP (Fig. 3). These tests demonstrated that anti-LTR II, anti-LTR 23, anti-CAP 18-12 and anti-MMTV p27 all recognize epitopes in disrupted CAP. Significantly, the synthetic peptide used by Fasel et al. (1982) to raise anti-LTR 23 contains the entire amino acid sequence of our peptide II in its N-terminal domain.

The SDS–polyacrylamide gel patterns of purified CAP are complex (Smith & Lee, 1975; Tanaka, 1977; Smith, 1978). The most abundant polyproteins present as determined by Coomassie Brilliant Blue staining are between 95K and 65K and between 20K and 10K. Numerous partially processed CAP polyproteins were present with apparent mol. wt. ranging from 50K to 30K. Other experiments (Smith & Lee, 1975; Tanaka, 1977; Smith, 1978) have shown that the high mol. wt. polyproteins can be processed by endogenous or exogenous proteolytic activity to proteins of mol. wt. similar to the major MMTV nucleocapsid proteins encoded by the MMTV gag gene (p27, p20, p14, p10, p8).

Following gel electrophoresis, CAP proteins were transferred to nitrocellulose sheets by electroblotting and incubated with antisera from five rabbits which received injections of LTR ORF peptides I, II or III (Fig. 4). For comparison similar dilutions of anti-MMTV p27 and anti-CAP p18-12 were included. Hyperimmune serum raised against MEP was also reacted with CAP proteins. All the sera reacted with CAP low mol. wt. proteins (p18-12) except anti-MEP. In addition, all LTR ORF antisera bound to CAP proteins of approximate mol. wt. 36K and 32K and somewhat more weakly with a CAP protein at 43K. Antiserum to MMTV p27 reacted strongly with the major CAP polyprotein (mol. wt. approx. 80K), CAP p68-72 and p18-12. CAP proteins at p68-72 were also reactive with anti-LTR II, anti-LTR I (2853) and anti-CAP p18-12. Subsequent titration of these antisera against Western blots of other purified CAP preparations demonstrated that the most abundant LTR ORF-reactive CAP proteins migrated as a doublet with apparent mol. wt. of 68K and 72K. Reactions with CAP p18-12 polypeptides were not demonstrable with any of the antisera shown in Fig. 4 at dilutions greater than 1:500. Likewise, activity against CAP p36 and p32 was only occasionally observed at dilutions of 1:500 and greater. In contrast, activity against CAP p72-68 was present in anti-LTR II serum up to
MMTV LTR-ORF proteins in A particles

Fig. 2. Immunoprecipitation of [35S]methionine-labelled proteins translated from polyadenylated C37-1 mammary tumour RNA and from MMTV virion RNA. Lanes (a) to (f) represent proteins immunoprecipitated from translation of 1 µg poly(A)+ C37-1 RNA by (a) 4 µl non-immune rabbit serum, (b) 2.5 µl anti-MEP (see text for description), (c) 4 µl anti-LTR peptide II, (d) 2 µl anti-LTR peptide II, (e) 4 µl anti-LTR peptide III or (f) 4 µl anti-LTR peptide I. Lanes (g) to (k) contain radioactive proteins translated from approx. 0.2 µg total MMTV virion RNA and immunoprecipitated with (g) 3 µl anti-LTR peptide II, (h) 1.5 µl anti-LTR peptide II, (i) 3 µl anti-MMTV or (j) 3 µl in vivo absorbed anti-CAP. Lane (k) contains radioactive mol. wt. marker proteins.

dilutions of $1.2 \times 10^5$ (Fig. 5). All reactivity of anti-LTR II antibody to CAP proteins on Western blots was abolished by preincubation of the serum with free synthetic peptide II, but was unaffected upon incubation with peptide I (Fig. 5).

Comparison of the relative sensitivities of anti-MMTV p27 and anti-LTR II for CAP polyproteins indicated that anti-p27 reactivity with high mol. wt. CAP proteins was detectable at dilutions as great as $1.2 \times 10^5$ (Fig. 6). Anti-p27 reactivity with high mol. wt. CAP
Fig. 3. Ouchterlony analyses of immunoprecipitin activity in antisera raised to LTR ORF synthetic peptides II, LTR 23, MMTV, CAP and normal mouse serum (NMS). All sera were used undiluted; the protein concentrations in the antigen wells were: MMTV, 1.8 mg/ml; CAP, 2.0 mg/ml; NMS 60 mg/ml. Antigen wells are labelled 'ag'; antibody wells are labelled 'ab'. The reactions were carried out at room temperature in a humidified chamber. The plates were washed for 3 h in several changes of buffered physiological saline. Absorption of anti-CAP serum ('abs. in vivo') was accomplished by inoculation into normal mice (1 ml intraperitoneally) and incubation overnight in vivo. The mice were subsequently bled out. The serum samples were pooled and used as primary CAP antiserum in all subsequent tests. Anti-LTR II (+ pep II) serum was absorbed by incubation overnight at room temperature with free synthetic peptide II which had been blotted onto nitrocellulose paper.

polypeptides was unaffected by preincubation of anti-p27 with free LTR ORF peptide II. To assess specificity, anti-p27 and anti-LTR II sera were absorbed with 'cores' prepared from gradient-purified MMTV. p27 represents approximately 40% of the protein mass of MMTV 'cores' (Dickson & Peters, 1983). The reactivity of anti-p27 against CAP in Western blots was completely abolished by preincubation with MMTV 'cores' whereas the activity of anti-LTR II was not detectably affected. Preincubation of either antiserum with dissociated CAP completely removed their respective reactivities with CAP Western blots (data not shown).

In an effort to identify the CAP proteins co-reacting with anti-LTR II, anti-LTR 23 and anti-p27 and responsible therefore for the formation of the immunoprecipitin line (Fig. 3c, d), these sera were titrated against the same CAP Western blot (Fig. 7). CAP proteins co-reactive with each of these antisera were detected. These co-reactive proteins apparently included the major
Western blot of total gradient-purified CAP reacted with antisera (diluted 1:500) raised against MEP (lane 1), MMTV gag p27 (lane 2), low mol. wt. CAP proteins (p18-12) (lane 3), and LTR synthetic peptides (II, lane 4; pep III 3157, lane 5; pep III 3158, lane 6; pep I 2852, lane 7; pep I 2853, lane 8). The relative positions of prestained mol. wt. markers run in the same gel are indicated in the left margin. The dissociated CAP proteins were separated on a 15% SDS-polyacrylamide gel before blotting on nitrocellulose.

Fig. 4. Western blot of total gradient-purified CAP reacted with antisera (diluted 1:500) raised against MEP (lane 1), MMTV gag p27 (lane 2), low mol. wt. CAP proteins (p18-12) (lane 3), and LTR synthetic peptides (II, lane 4; pep III 3157, lane 5; pep III 3158, lane 6; pep I 2852, lane 7; pep I 2853, lane 8). The relative positions of prestained mol. wt. markers run in the same gel are indicated in the left margin. The dissociated CAP proteins were separated on a 15% SDS-polyacrylamide gel before blotting on nitrocellulose.

high mol. wt. CAP polyprotein (approx. 80K), CAP p68 and variously sized partially processed CAP polypeptides.

Western transfer blots of MMTV proteins were incubated with all the antisera raised against the LTR ORF peptides. Of these, only anti-LTR peptide II was responsive, reacting weakly with single protein bands in different MMTV virion preparations at 32K, 21K and 16K (data not shown). However, we were unable to demonstrate this activity reproducibly. This observation is consistent with our failure to detect immunoprecipitin activity against disrupted
MMTV virions. Therefore, it seems unlikely that significant amounts of LTR ORF protein are present in mature MMTV virions.

To determine whether LTR ORF peptide II-reactive proteins were synthesized and stable in cells where virus assembly was absent, we compared Western blots of cytoplasmic extracts from MMTV-induced C3H/Sm mouse mammary tumour cells with those from chemically induced or spontaneous C3H/Sm mammary tumours which are negative for MMTV virion and CAP production but positive for MMTV LTR ORF RNA transcription (Graham et al., 1984). Anti-LTR peptide II-reactive proteins of 68K to 72K were detectable to dilutions of greater than 1:4000 in virus-positive C3H/Sm tumour cell immunoblots (Fig. 2) whereas the other tumours gave Western immunoblots which reacted weakly with anti-MMTV p27 (1:500). This result was expected as these tumours are immunologically negative for MMTV structural gene products (Smith et al., 1978, 1981). Nevertheless, high mol. wt. protein(s) (p68-72 in DMBA-induced tumours and p80 in a spontaneous C3H/Sm tumour) reacted with anti-LTR ORF peptide II at dilutions of 1:2000 (Fig. 8). Anti-LTR II at low dilution (1:500) also detected numerous low mol. wt. proteins (≤ 21K) in C37-1 tumour blots, similar in size to those immunoprecipitated by this serum from in vitro translation of C37-1 mRNA. Western blots containing extracts from normal uninfected mammary tissue and from transformed mouse NIH 3T3 tissue culture cell
MMTV LTR-ORF proteins in A particles

MMTV LTR-ORF proteins in A particles

Fig. 6. Titration of anti-MMTV gag p27 with strips from the same CAP immunoblot. (a) anti-CAP absorbed in vivo diluted 1 : 5000; (b) to (e) anti-MMTV gag p27 diluted 1 : 5000, 1 : 25000, 1 : 125000 and 1 : 250000 respectively; (f) anti-LTR II diluted 1 : 2000. The last two dilutions of anti-p27 shown (d and e) were preincubated overnight with nitrocellulose strips containing free LTR peptide II. No difference between preabsorbed and untreated anti-MMTV p27 dilutions was observed. Mol. wt. standards are indicated.

lines which produce high levels of MEP (Gottesman & Cabral, 1981) showed no reactive proteins (data not shown).

DISCUSSION

Until now, candidates for the protein(s) encoded by the ORF of the LTR of MMTV have been scarce. We have detected several polypeptides in CAP preparations which are antigenically related to LTR ORF peptides.
Fig. 7. Comparison of the activities of antisera raised to MMTV gag, p27 LTR peptide II and LTR peptide 23 on the same Western immunoblot of CAP proteins. The antisera and their dilutions were (a to f): anti-p27 (1:1000), anti-LTR II (1:2000), anti-LTR 23 (1:1000), anti-LTR II (1:5000), LTR 23 (1:2000) and (1:4000). Mol. wt. standards are indicated.

Immunodiffusion shows that purified CAP preparations possess antigenic determinants readily detectable by anti-LTR peptide II serum. In addition, a polyclonal rabbit antibody raised in H. Diggelmann's laboratory against a 23 amino acid peptide synthesized from the LTR ORF (Fasel et al., 1982) gives identical results in immunodiffusion tests.

Immunoblots containing CAP proteins revealed that the LTR peptide II cross-reacting antigenic determinants were primarily limited to CAP proteins of mol. wt. 68K to 72K, although peptides at 43K, 36K, 32K and 18K to 12K also cross-reacted at low dilutions of antibody (1:500). Further analysis of CAP immunoblots showed that antisera raised against each of the
LTR ORF synthetic peptides (I, II and III) also reacted with CAP p18-12 and with CAP proteins whose approximate sizes are 43K, 36K and 32K. However, only one of these, anti-LTR peptide I (2853), reacted with CAP p68.

These data taken together strongly suggest that LTR ORF-encoded polypeptides are closely associated with intracellular MMTV preprocapsid inclusion bodies (CAP). The three CAP polypeptides detected at 43K, 36K and 32K by all anti-LTR peptide antisera are very close to the size predicted from the complete LTR ORF (Fig. 1) and correspond in size to those detected by Dickson et al (1981) in cell-free translation studies of LTR ORF RNA.

The fact that antisera raised to different but closely clustered LTR ORF peptides react with the same CAP proteins is compelling evidence that portions of all these proteins are encoded by
the MMTV LTR ORF. The cross-reactivity of CAP proteins (68K to 72K and 18K to 12K) with anti-MMTV gag sera is more difficult to explain. The cross-reactions of CAP p18-12 with antibodies raised against MMTV gag proteins and the synthetic LTR ORF peptides do not represent the major fraction of LTR ORF reactive proteins and are not detectable at high dilutions of antibody. Unfortunately, this result also obtains for the CAP LTR ORF reactive proteins at p43, p36 and p32. The most abundant CAP proteins cross-reactive with anti-LTR peptide antisera migrate with apparent mol. wt. of 68K and 72K. Higher mol. wt. CAP proteins (p80) are detected by anti-LTR II and anti-LTR 23 sera even at high dilutions of antibody (Fig. 6). Although reactivity of anti-LTR II with CAP proteins is abolished by preincubation with free peptide II, it is difficult to account for the large size of the LTR ORF-related CAP proteins on the basis of what is known from LTR sequence data and in vitro translation studies of RNAs containing the LTR open reading frame. Several possible explanations may be proposed: first, post-translational modification of the LTR ORF protein results in its large apparent increase in mol. wt.; second, the LTR ORF gene product exists as a dimer highly resistant to denaturation; third, there is more than one MMTV RNA transcript that encodes proteins containing LTR ORF peptide sequences and this transcript is similar in size to one of the three known intracellular MMTV mRNAs (8.0 kb, 3.8 kb and 1.8 kb). The translation product of this hypothetical transcript might exist as a gag–LTR fusion protein subject to post-translational processing. A precedent for this latter point has been raised by Broome & Gilbert (1985), who described a transcriptional activator (enhancer) encoded within the gag gene of Rous sarcoma virus (RSV). The protein is encoded by a genome-sized mRNA distinguishable from the known RSV genomic transcript only by a different splice site and by translation from an alternative reading frame. The translation product of this RNA is predicted to be a 124 residue polypeptide which includes the first six amino acids from gag. The target of this transcriptional modulator (presumably mediated through binding to the DNA) lies within the RSV LTR, 111 to 620 nucleotides upstream from the cap site. If a similar mechanism applies to MMTV, it is conceivable that the 68K to 72K protein, detected by our anti-LTR ORF sera and perhaps also by anti-MMTV gag antisera, represents the product of a similar alternative transcript in MMTV-infected cells. A MMTV gag–LTR ORF polypeptide could give rise through proteolytic processing to one or more of the low mol. wt. CAP proteins (18K to 12K) detected by both antisera.

What is the significance of finding LTR ORF peptide determinants in association with intracytoplasmic A particles? These morphological entities are invariably present in the cytoplasm of cells replicating MMTV, and considerable evidence has accumulated demonstrating that these structures represent preprocapsids of MMTV. They possess a Mg$^{2+}$-dependent reverse transcriptase with all the functional attributes of that found in the mature virion (Kohno & Tanaka, 1977) and have been shown to contain newly synthesized DNA (Smith et al., 1975). They also contain MMTV-specific RNA with a complexity sufficient to protect full-length radiolabelled MMTV cDNA from S1 nuclease digestion and they fractionate with DNA enriched for MMTV genomic sequences (Michalides et al., 1977; Henry & Smith, 1979). In addition, CAP preparations have significant DNA binding and destabilizing (unwinding) activities (Smith et al., 1980).

Recent studies of transposable elements in Drosophila and yeast (Ty element) have clearly established the importance of subviral entities similar to MMTV CAP in the production of efficiently transposed DNA copies of these genes and reverse transcription of their RNA transcripts (Boeke et al., 1985; Clare & Farabaugh, 1985). Since CAP share many of the features of these virus-like structures which Garfinkel et al. (1985) have shown to be indispensable to reverse transcription and transposition of Ty, sequestration of one or more of the LTR ORF gene products in CAP may suggest a role for LTR ORF in one of the following viral functions: encapsidation, integration, reverse transcription, or the regulation of post-translational proteolytic processing of gag and gag-pol gene products. Transfection of LTR ORF sequences under the control of a heterologous promoter into cells containing MMTV proviral genes defective for one or more of these viral functions may serve to provide clues to the resolution of this hypothesis.
MMTV LTR-ORF proteins in A particles

The authors gratefully acknowledge the skilful technical assistance of Clifford Parkison in the preparation and analysis of the Western blots. Paul V. Rossitto contributed importantly to the early characterization of the LTR peptide antiseras.

REFERENCES


*(Received 19 June 1986)*