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STUDIES OF MOUSE MAMMARY TUMOR VIRUS COMPOSITION AND REPLICATION

by

Frank Maldarelli

A dissertation presented to the Graduate Faculty in Biomedical Sciences in partial fufillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1985

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ABSTRACT

STUDIES OF MOUSE MAMMARY TUMOR VIRUS

COMPOSITION AND REPLICATION

by

Frank Maldarelli

Advisor: Dr. M. J. Yagi

Studies of mouse mammary tumor virus (MMTV) replication revealed virions purified from spent culture medium of MJY-mammary tumor cells consistently contained 7 polypeptides. Principal protein constituents were products of MMTV env gene (gp52 and gp37,7-33), and gag gene ($p24$, $p14$, p8), which were present at approximately 1850-2350 molecules each per MMTV particle. A fourth gag protein, p17, was detected, but was present in only 1079 molecules per virion. MMTV protein $p44$, was present in fewest numbers (350 molecules/virion), and was similar to the cytoskeletal protein actin in electrophoretic mobility and in DNase I-binding and polymerization activities. To determine whether the cytoskeleton was involved in MMTV replication, effects of microfilament-disrupting cytochalasins B and D, and of microtubule-disrupting colcemid were examined. Cytodisruptive agents altered MMTV production and polypeptide composition, but did not specifically inhibit MMTV polypeptide synthesis. Cytochalasin treatment reduced or eliminated budding MMTV particles, although levels of MMTV cell surface antigens were only slightly decreased. These data indicated proper MMTV production required an intact cytoskeleton. Exposure of mammary cells to 14μM

in 2-5 fold increases in synthesis of MMTV polyprotein precursors $Pr76^{env}$ and $Pr75^{gag}$, in levels of cell surface MMTV antigens, and in virion production. HC treatment also decreased env precursor $Pr76^{env}$ half-life, and altered incorporation of ^3H-monosaccharides into precursors $Pr76^{env}$ and $Pr79^{env}$. These data suggested HC effects were not restricted to MMTV transcriptional stimulation. Prolonged HC exposure decreased MMTV production to levels obtained from untreated cells. HC-induced increases in MMTV production were obtained with increased doses of HC, or by cell passage in HC-free medium, followed by restimulation using 14 μM HC. These data indicated prolonged HC exposure reversibly altered HC responsiveness. The necessity of monolayer orientation of mammary tumor cells for MMTV production was assessed by maintaining MJY-alpha cells in suspension culture. MMTV production in MJY-beta cultures was increased 10-200 fold over MJY-alpha cells. These data indicated mammary tumor cell growth and MMTV production do not require solid substratum, and that MMTV production can be stimulated by alternate growth conditions.

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Finally, I wish to thank my family, who cheerfully endured, and constantly encouraged this work; they are the silent co-authors of this thesis. To the members of my All-Star family, new members and old, those in heaven, and those on their way, I respectfully dedicate this work.

TABLE OF CONTENTS

LIST OF TABLES

List of Figures

Chapter I

INTRODUCTION

A. RETROVIRUSES.

Mouse mammary tumor virus (MMTV) is an oncogenic member of the Retroviridae family, defined as that group of enveloped RNA-containing animal pathogens which replicate via a DNA intermediate (replication class VI; Luria, et al., 1978; Mattnews, 1982). Retroviruses induce persistent infections in mammals, birds, and reptiles, which result in neoplastic, inflammatory, or spongiform disease states. Four Retrovirus groups have been identified based on appearance and location of the virion core, and the presence and shape of surface projections (Bernhard, 1958). However, as shown in Table 1, there is no apparent correlation between the morphology of the virion particle, and the disease induced.

B. BIOLOGY OF MMTV.

Table 1. Correlation of virion type with Retrovirus subgroup.

		Virion morphologic type			
Subgroup	associated disease	A	B	C	D
Oncoviridae	neoplastic	+	+	+	+
Spumaviridae	neuro-degenerative	-	-	+	-
Lentiviridae	inflammatory	-	-	+	-

Early investigations of mammary carcinoma in mice identified females of several strains with high and low incidences of mammary adenocarcinoma (Murray, 1911; Dmochowski, 1953; Heston, 1949). Foster nursing studies among the inbred mouse strains suggested participation of extrachromosomal factors in mammary tumor induction; mammary tumor-inciting activity was identified as a milk-borne agent horizontally transmitted from lactating females to suckling newborns (Staff of Roscoe B. Jackson Memorial Laboratory, 1933; Bittner, 1936). Mammary tumors were induced in agent-free, low tumor incidence (<1%) mouse strains, such as BALB/c, by foster nursing pups on high tumor incidence (>90%) strains, such as C3H; over 90% of BALB/cfC3H females developed mammary tumors at 3-8 months. Reciprocally, tumor incidence in C3HfBALB/c pups was decreased by 0-60% (Medina et al., 1970; Heston et al., 1950).

Although these experiments demonstrated that milk was a primary vector in mice, other natural modes of transmission, including vertical (through germinal cells), and horizontal (by sperm, aerosol, or arthropod-borne transmission), were also reported (Hilgers and Bentvelzen, 1978). Experimental transmission of mammary tumor inciter activity was accomplished by inoculating milk or cell-free tumor extracts into susceptible mice. Identification of inciter activity as a true virus followed purification of virus-like particles from milk of infected females, and demonstration that inocu-

lation of these purified preparations reproduced mammary adenocarcinomas with incidence, latency, and histopathology characteristic of the naturally induced disease (Lyons, and Moore, 1962, 1965).

Foster nursing and experimental transmission studies identified strains such as BALB/c which were easily infected with MMTV in contrast to other low incidence strains, such as C57Bl/6, which were refractory to MMTV infection and oncogenesis. Crosses of high tumor incidence females with low tumor incidence males resulted in intermediate tumor incidence in hybrids (Bittner, 1944), suggesting that genetic components were involved in susceptibility to mammary inciter-induced tumors.

Development of mammary tumors after MMTV exposure was also dependent on the hormonal state of the host. MMTV expression and mammary tumor development was restricted to females; removal of principal source of sex steroids by oophorectomy resulted in decreases in mammary tumor incidence (Lathrop and Loeb, 1916). Experimental administration of hormones related to pregnancy and lactation (estrogens, progesterone, prolactin) to MMTV-infected, castrated,

C3H female or male mice resulted in high incidence of mammary tumors (Dmochowski, 1953). Studies of steroid therapy after surgical removal or transplantation of endocrine and reproductive organs documented the role of pregnancy-related hormones in MMTV viremia and virus-induced tumors (Nandi and McGrath, 1973).

The mode of MMTV transmission, tumor latency, and histopathology of MMTV induction of mammary tumors in inbred mouse strains have been employed as taxonomic determinants to classify four MMTV virus strains: 1) MMTV-S (standard) is naturally transmitted via milk in mouse strains such as C3H or A. Alveolar and papillary adenocarcinomas arise from MMTV-S-induced sites of acinar proliferation (hyperplastic alveolar nodules, HANs; Dunn, 1959; Nandi and McGrath, 1973). 2) MMTV-P (plaque-forming), found in European strains GR, RIII, DD, is transmitted via milk or germinal cells. MMTV-P induces alveolar and papillary adenocarcinomas, as well as, preneoplastic, pregnancy-dependent plaques adjacent to mammary ductal epithelium (Bentvelzen and Daams, 1969). 3) MMTV-L (low, nodule-inducing virus, NIV) is transmitted by milk or germinal cells. MMTV-L induces HANs, but few of these lesions progress to mammary adenocarcinomas, and tumor incidence is low (Nandi and McGrath, 1973). 4) An endogenous, integrated MMTV provirus, MMTV-O, has been identified in the the genome of uninfected BALB/c mice. MMTV-O is beleived to be the cause of

rare adenocarcinomas in retired female breeders (Varmus, et al., 1972). Cohen and coworkers (1979) identified 3 provirus-like elements in uninfected BALB/c liver DNA by restriction mapping, but it is not known which site(s) are expressed in tumorigenesis. Characteristics determining individual virulence and histopathology of MMTV-O have not been identified.

C. DETECTION AND DISTRIBUTION OF MMTV.

Identification of a viral etiology in mouse mammary tumorigenesis led to studies determining the organ distribution of MMTV. The methods employed were visual electron microscopic detection of viral particles and bioassays which measured tumorigenicity of organ extracts from infected animals inoculated into susceptible mice (Nandi and McGrath, 1973). The 6-7 month long bioassay detected infectious MMTV in milk, mammary glands, mammary tumors, HANs, and blood of infected, lactating females. However, complex titration curves obtained from *in vivo* assays were not useful in determining MMTV titer. Limitations of tissue sampling for electron microscopy restricted sensitivity, and the accuracy of particle counting varied +/-100%, even with large sample sizes.

These early studies were later repeated using sensitive enzyme assays detecting virion-associated reverse transcriptase, and immunoassays for virion proteins. These methods confirmed the bioassay results and also detected MMTV in spontaneous renal adenocarcinomas (Felluga, et al., 1969), pulmonary adenoma (Calafat, 1969) , reticulum cell sarcomas (Seman and Dmochowski, 1973), leydig cell sarcomas (Pourreau-Schneider, et al., 1968), and carcinogen-induced lung or ependymal tumors (Rogney,et al., 1975; Moore, et al., 1969). Whether MMTV is present in these non-mammary tumors as an etiologic agent or is incidentally expressed is unknown.

In addition to neoplastic tissue, MMTV antigens and intracellular particles were identified in normal tissues of infected animals, including salivary gland, thymus, and epididymis. MMTV antigens were also detected in brain, heart, spleen, kidney, liver, and bone marrow, but this could be due to blood-borne MMTV contamination (Rogney, et al., 1975, Hendrick, et al., 1978; Zangerle, et al., 1977). It is not known why MMTV is expressed in epithelial cells of several endoderm-derived organs, but induces neoplasia only in mammary glands. All MMTV-infected organs undergo rapid cellular proliferation during the exposure period to MMTV (1-4 weeks post partum). However, the mammary gland is the only organ of this group which responds to cyclic steroid hormone stimulation by undergoing repeated cellular proliferation.

Presumably, these factors contribute to mammary carcinogenesis, although specific events which result in mammary adenocarcinomas have not been described.

D. VIRION MORPHOLOGY

Early electron microscopic observations of Porter and Thompson (1946), and Bang (1953) identified intracytoplasmic and extracellular virion-like particles in thin sections of C3H mammary tumor tissue. Subsequent studies of infected mammary glands and tumors identified three viral structures: intracellular type A particles, particles budding at the cell surface, and extracellular type B virions. Intracytoplasmic type A particles were described as doughnut-shaped, consisting of two concentric, electron-lucent shells, 70 nm in diameter, which were identified in perinuclear aggregates, and near intracellular vacuoles, or cell surfaces (Bernhard, 1958, 1960; Bang, et al., 1956a; Pitelka, 1964). Particles budding at the tips of microvilli were associated with cellular filaments and consisted of electron-dense, centrally located cores enveloped by a patch of plasma membrane containing spike-like projections (Bang, et al., 1956a,b). Thin-section preparations of purified B-type extracellular MMTV revealed spherical, 120-140 nm, particles

with a 70 nm, electron-dense, eccentrically located core surrounded by a thin shell (Sarkar and Moore, 1974; Sarkar et al., 1975). As revealed by negative stain and freeze-fracture/freeze-etch techniques, the outer membrane of the B-type virion contains a regular arrangement of spike-like projections, 95 A long and 15 A wide, consisting of a stalk and knob. (Clarke and Attridge, 1970; Sarkar and Moore, 1974; Calafat and Hageman, 1968).

E. VIRION COMPOSITION.

The chemical composition of MMTV particles is 27% lipid, 0.8-1% RNA, 2-3% carbohydrate, and 65-70% protein by weight (Lyons and Moore, 1965). Three principal virion-associated RNA classes were identified by sucrose gradient fractionation: 1) MMTV 70S RNA, composed of 2 non-covalently linked copies of capped, polyadenylated (poly A+), positive-stranded, noninfectious, MMTV genomic RNA (Duesberg and Blair, 1966). 2) The 5S cellular tRNA, demonstrated to participate in reverse transcription (Haseltine, et al., 1976). 3) MMTV virions also contain a diffuse, 13-30S RNA of unknown origin and function. This RNA species consists of poly A+ and poly A- RNA, some of which may be partially degraded virion genomes and/or ribosomal in origin (Sen et

al., 1981; Dickson and Peters, 1981; Dickson, et al., 1981). Intracytoplasmic type A virion particles contain 70S MMTV-related RNA and DNA which is not a cDNA copy of MMTV RNA, but is apparently cellular in origin (Smith and Wivel, 1973; Tanaka, 1977).

The polypeptide composition of MMTV B-type particles, as analyzed by SDS-PAGE, consists of 7-8 proteins and glycoproteins (Dickson and Skehel, 1974; Yagi and Compans, 1977; Teramoto, et al., 1973; Sarkar et al., 1977). Relative levels of principal MMTV polypeptides, including gp60, gp52, p44, gp37.7-33, p24, p17, p14, and p8, were comparable in MMTV derived from milk and tissue culture sources. Viral origin of gp52, gp37.7-33, p24, p17, p14, and p8 was elucidated by demonstrating: 1) immune responses to these proteins following infection or inoculation (Schlom, et al., 1978a; Blair, 1981). 2) synthesis of these proteins in vivo only in MMTV-infected cells (Nandi and McGrath, 1973) and by 3) synthesis of these species in vitro using translation systems primed with 70S genomic MMTV RNA (Dickson and Peters, 1981). Virions purified from milk contained several additional high molecular weight proteins not observed in MMTV from tissue culture sources, which were identified as contaminants adsorbing to the virion surface.

Investigations of the structure, location, and function of MMTV proteins identified gp60, gp52, and gp37.7-33 as membrane-associated glycoproteins: 1) Labeling of intact

virions by galactose oxidase tritiation resulted in 3H-labeled carbohydrate side chains in all three glycoproteins (Sheffield and Daly, 1976), although lactoperoxidase-catalyzed iodination of intact MMTV labeled only gp52 (Witte, et al., 1973). 2) Detergent treatment disrupted the virion membrane and removed gp60, gp52, gp37.7-33 from intact virions (Teramoto, et al., 1973). 3) Virion-associated gp60 and gp52, but not gp37.7-33, were cleaved after exposure of intact MMTV to trypsin (Yagi and Compans, 1977). These studies suggested that gp52 and gp60 were both exposed on the virion surface, and gp37.7-33 was probably an integral membrane protein, with only a small portion of the molecule, principally carbohydrate, exposed to the exterior. However, gp52 and gp37.7-33 apparently comprise the virion surface projection. Removal of spikes from MMTV by treatment with Streptomyces griseus protease type VI resulted in loss of gp52 and gp37.7-33 from the remaining "bald" particles (Cardiff, et al., 1974). MMTV spikes, extracted as aggregates (rosettes) from virions by Tween 80/ether treatment, were composed of both gp52 and gp37.7-33.

The function of MMTV spikes is unknown; analogous projections on influenza, VSV, and MuLV viruses interact with specific cell surface receptors, suggesting the spike is involved in host recognition and virion attachment. Although the MMTV spike may participate in recognition and virion attachment, the route of infection of MMTV (by inges-

tion) may result in spike degradation before particles reach the target organ. In vitro exposure of purified MMTV to trypsin or chymotrypsin resulted in cleavage of gp52, as demonstrated by SDS-PAGE, but electron microscopic observations revealed protease-treated virions retained characteristic spike morphology, and immunologic assays demonstrated gp52 antigenicity in treated virions (Yagi and Compans, 1977). Bioassay revealed MMTV infectivity was neither enhanced nor reduced by protease treatment. These data indicated exposure to proteolytic agents similar to those found in the mouse digestive tract did not destroy functional characteristics of the MMTV virion (Sheffield, et al., 1977).

MMTV surface proteins, especially gp52, have been implicated in the host immunologic response to MMTV. Pretreatment of MMTV with anti-gp52 antisera neutralized infectivity, and neonatal immunization with gp52 decreased the incidence and prolonged the latency of MMTV-induced tumors (Charney and Moore, 1978, Sarkar and Moore, 1978). However, naturally circulating IgG antibodies to gp52, identified in lactating C3H mice, did not protect the infected mother or the suckling pups from subsequent tumor development. The presence of circulating gp52 IgM blocking antibody, and an altered cellular immune response as measured by in vitro cellular cytotoxicity assays may contribute to an immunologically tolerant state permitting tumor proliferation (Blair, 1981).

The interior of the MMTV particle contains p28, p17, p13, p8, and the MMTV reverse transcriptase. P14 was reported to be a basic protein with nucleic acid-binding activity which was suggested to participate in packaging of nucleic acid into virions (Arthur, et al., 1978b). Reverse transcriptase is present in virions at one copy per particle, and is apparently associated with virion RNA.

The virion protein p44 has been suggested to be cellular actin, since it has an electrophoretic mobility identical to actin in SDS-PA gels. The close association and alignment of budding B-type particles with actin-containing microfilaments has further supported the concept that actin is incorporated into virions (Damsky, et al., 1977; Tyrrell, and Ehrnst, 1979). Functional identification of this protein as actin has not been obtained, and its location within the virion particle has not been assigned. Why this putative host-encoded cytoskeletal protein is incorporated into virion particles with the exclusion of other cellular proteins is not known.

Virion proteins from MMTV strains have been compared to determine whether the observed biologic differences correlate with detectable changes in polypeptide structures. Comparative tryptic peptide mapping of gp52 and p24 from MMTV-P and MMTV-S suggested strain variations in both the major internal and external virion proteins (Gautsch, et al., 1977), but no differences in the tryptic peptide maps

of gp36, p14, or p10 of MMTV-P or MMTV-S were detected. Comparison of the amino acid compositions of the polypeptides from MMTV-P and -S revealed that gp52 or p24 of MMTV-S could not be distinguished from corresponding polypeptides of MMTV-P (Dion, et al., 1979a; Yagi, et al., 1978c). Greatest variation in amino acid composition was observed in gp34, which appeared identical in tryptic peptide maps.

F. ANTIGENICITY OF MMTV POLYPEPTIDES.

Antigenicity of MMTV proteins was identified by Lezhneva (1961) and Blair (1965), who detected specific lines of immunoreactivity between purified MMTV and antisera from infected mice or rabbits immunized with purified virions, using agar immunodiffusion. Subsequent investigations revealed 2-6 additional precipitin bands in immunodiffusion assays (Blair, 1969), and electron microscopic examination of the precipitin front identified MMTV envelope material reacting with the antisera (Blair, 1965). Radioimmunoassay (RIA) techniques revealed that principal MMTV polypeptides (gp52, gp37.7-33, p24, p14, p8) were antigenically distinct. Common group-specific determinants were detected for each protein from MMTV-S, -L, and -P variants. Direct and competition RIA also identified unique type-specific determinants

in gp52 and p24 from MMTV-S, -L, and -P, confirming and expanding data obtained from the tryptic peptide mapping studies (Arthur, et al., 1978a,b; Arthur, et al., 1981). However, type specificities were also detected in gp36 and p10 of MMTV-S, -L, and -P, previously found to have identical tryptic peptide maps. Future studies using restriction enzyme and sequencing analysis of cDNA copies of MMTV genomes should identify additional differences among MMTV strains.

G. INTRACYTOPLASMIC TYPE A PARTICLES.

The polypeptide composition of intracytoplasmic type A particles from infected mammary tumors or tumor cell lines has been analyzed by SDS-PAGE (Tanaka, 1977; Smith, and Wivel, 1972, 1973). The degree of purity of these preparations was not determined, and a consistent polypeptide profile has not been reported; 3-7 polypeptides were detected in all reports although only two proteins were consistently present. The molecular weights of A particle proteins do not correspond to polypeptides of B-type particles. However, a 70,000 mw species present in A particles was immune precipitated by antisera to the major MMTV core protein p24, and may represent a precursor to this gag product. (Smith and Wivel,

1973). Common structural features of intracellular type A particles and extracellular B virions suggested either a precursor/product relationship, or that type A particles represented products of abortive assembly.

H. CULTURE OF MMTV PRODUCING CELLS.

In vitro methods for culturing MMTV-producing cells were developed to obtain a reproducible source of MMTV virions and to enable a detailed dissection of MMTV replication. Maintenance of organ cultures of mammary glands from MMTV-infected animals identified strain-dependent steroid and polypeptide hormone requirements for mammary gland maintenance, (Elias, 1959; Lasfarques and Murray, 1959; Lasfargues and Feldman, 1963; Singh, et al., 1970). Low levels of MMTV production were detected from organ cultures compared to that from milk; highest MMTV yields were detected when glands were developed and secreting casein, a state requiring insulin, estradiol, progesterone, prolactin, and cortisol (Lasfarques and Feldman, 1963). This hormone cocktail was developed empirically, and the relative contribution of each component to gland differentiation or MMTV production was not determined. Establishment of primary cultures of mammary tumor cells provided a useful tissue culture system

to produce and analyze MMTV (Lasfargues and Lasfargues, 1975; McGrath, 1971; Nandi, et al., 1972; Kimball, et al., 1976). McGrath (1971) demonstrated proliferation and organization of dissociated mammary tumor epithelial cells into characteristic pulsating, three-dimensional, acinar-like "dome" cultures after insulin and glucocorticoid exposure. Primary cultures produced relatively large amounts of MMTV for up to 50 days (Kimball, et al., 1976), following which domes collapsed, MMTV production declined, and the remaining epithelioid cells were overgrown by contaminating fibroblasts. McGrath (1971) suggested MMTV maturation and release required dome formation.

Several epithelial-like mammary tumor cell lines producing MMTV-S, -L, and -P were derived using techniques to selectively remove fibroblast contamination; however, most of these lines (except MJY-alpha) also produce detectable levels of mouse leukemia virus (MuLV; Yagi, 1973; Owens and Hackett, 1972; Arthur, et al., 1979; Fine, et al., 1974). Cloning of several lines has been unsuccessful in removing C particle contamination, and in some cases resulted in complete loss of detectable MMTV expression (Sykes, et al., 1968). Tumorgenicity, latency, and pathology induced by MMTV derived from tissue culture sources were identical to that obtained from infected milk (Arthur, et al., 1979). Yields of MMTV from tissue culture sources are unexplainably variable and cell line-dependent. Culture supplements for

in vitro MMTV production are unclear; serum requirements for proliferation of Mm5mt/c1 cells have been replaced by a chemically defined supplement including thyroxine, mannitol, oleic acid, lecithin, and cholesterol (Bauer, et al., 1976; Nagle and Fine, 1978). It is not known whether elements of chemically defined medium support MMTV replication directly, or indirectly, by promoting cell replication. All currently available producer lines are stimulated by glucocorticoid treatment (Dickson, et al., 1974). Dexamethasone and hydrocortisone are the most potent stimulants of MMTV, and are active at 1×10^{-8} M; corticosterone, aldosterone, and deoxycorticosterone are active at 1×10^{-4} - 1×10^{-6} M. High concentrations (1×10^{-5} M) of testosterone, estradiol, and deoxycorticosterone inhibit glucocorticoid induction of MMTV (Dickson, et al.,1974).

Heterologous cell lines in which MMTV genomes were established by molecular cloning and transfection did not produce extracellular virions, but MMTV RNA and polypeptides were synthesized in a hormone-dependent manner (Firestone, et al., 1982, Buetti and Diggelmann, 1981).

I. MOLECULAR BIOLOGY

1. GENOME STRUCTURE

Retrovirus infection is initiated by particle uptake and virion uncoating, and proceeds by transcription of viral RNA to DNA using a virion-encapsulated reverse transcriptase; newly generated DNA is inserted in heterogeneous sites within the host genome. The MMTV genome consists of three genes coding for virion components: 1) *env*, for envelope proteins, 2) *gag*, for internal viral proteins, and 3) *pol*, for reverse transcriptase. The genome structure of MMTV is similar to other retroviruses in size (8.5-9 kbp) and gene order (5'*gag*-*pol*-*env*3'). *Src* sequences, or their corresponding polypeptides, have not been identified in MMTV-infected cells, and the MMTV genome bears a closer resemblance in genetic structure to mouse leukemia virus than to the sarcoma viruses. As in all naturally occurring RNA tumor viruses, long terminal repeats (LTRs), generated during reverse transcription, have been found in integrated MMTV genomes. MMTV LTR of endogenous or exogenous MMTV proviruses is 3-4 times longer (1300 bp) than LTRs of other retroviruses, and contains an additional open reading frame (denoted the MMTV orf gene) coding for 198 amino acids. The corresponding RNA or protein products to this putative gene have not been conclusvely identified (Dickson *et al.*, 1981; Donehower, *et al.*, 1981; Kennedy *et al.*, 1982).

2. TRANSCRIPTION

Transcription of the MMTV genome is similar, although more complex, than that observed in other RNA tumor viruses.

Intection with most retroviruses results in production of two classes of virion-specific cell-associated RNA, 24S and 35S (Hayward, 1977; Fan and Baltimore, 1973), whereas, MMTV-specific, poly A-containing RNAs have at least three sizes: 35S, 24S, and 13S (Robertson and Varmus, 1979). Intermediate-sized RNA species have been reported as well (Groner, et al., 1980), and size heterogeneity exists in the largest class of RNA (28-35S; Dickson and Peters, 1980). Mapping of RNA transcripts to the MMTV genome has not been reported; analogous mapping studies with C-type viruses demonstrated that the 35S mRNA is a full length copy of the retrovirus genome. The 24S RNA encodes the 5' terminus of the genome and the env gene, with the intervening gag-pol genes reported to be spliced out. The viral or cellular origin of the 13S RNA has not been determined. Identification of sequences for proper initiation, capping, and polyadenylation of MMTV mMRNA in long terminal repeats (LTRs) suggested these regions may participate in MMTV transcription regulation (Donehower, et al., 1981; Varmus, 1982).

Rate of MMTV transcription is regulated by glucocorticoid hormones (Parks, et al., 1975). Exposure of MMTV-producing cells to 1 x 10^{-5} to 1 x 10^{-8} M steroid results in 10-260 fold increases in production of extracellular reverse transcriptase activity, and in synthesis of MMTV RNA, as determined by hybridization of ^{3}H-labeled, 35S MMTV RNA (Parks, et al., 1977; Parks, et al., 1975, Ringold, et al., 1979).

Steroid-modulated increases in MMTV RNA does not represent traditionally defined induction of RNA, since exposure increases synthesis only in cells already expressing MMTV. However, demonstration of steroid binding to intracellular receptors, transport of hormone-receptor complexes to nuclei, and a correlation between the binding affinity of steroids and their potency in MMTV stimulation, suggest that the mechanism of MMTV mRNA amplification is similar to that observed in classical mRNA induction by hydrocortisone (Ringold, 1978). Recent studies by Payvar, (1982), Hager (1983), Hynes (1983), Pfahl (1983), and Scheidereit (1983) have attempted to locate specific sites on the MMTV genome which are required for steroid stimulation or which bind activated steroid-receptor complexes, but consistent data have not been obtained.

Following mRNA amplification, increases in intracellular MMTV protein synthesis and extracellular virion production were detected, although the magnitude of stimulation (2-10 fold) is typically less than stimulation of MMTV RNA (Parks, et al., 1975; Dickson, et al., 1974). The basis for this difference is unknown, and other effects of glucocorticoids on MMTV expression and mammary tumor cells have not been extensively investigated.

3. TRANSLATION AND PROCESSING.

Translation of MMTV mRNA *in vivo* or *in vitro* yields 4 high molecular weight precursors: $Pr160^{gag/pol}$, $Pr110^{gag}$,

$Pr76^{gag}$, and $Pr76^{env}$. $Pr160^{gag/pol}$ contains gag proteins p28, p8, p14, and reverse transcriptase (Dickson and Atterwill, 1979, 1980; Racevskis and Sarkar, 1978). $Pr76^{gag}$ contains p24, p14, and p8; $Pr110^{gag}$ contains these species and an additional 30,000 m.w. protein. $Pr76^{env}$ consists of env proteins gp52 and gp37.7-33. Pactomycin mapping studies revealed that gag proteins are arranged NH2-p24-p8-p14-COOH and env proteins are arranged NH2-gp52-gp37.7-33-COOH (low levels of $Pr110^{gag}$ prevented mapping of p30; Massey and Schochetman, 1979; Dickson and Atterwill, 1979). However, principal precursors $Pr160^{gag/pol}$, $Pr110^{gag}$, $Pr76^{gag}$, $Pr76^{env}$ may not be the only products of translation of MMTV mRNA. MMTV polypeptides with short (5-10 min) half lives, or with high molecular weights (>200,000) may be synthesized, but not detected, due to technical limitations of labeling or identification. MMTV polypeptide intermediates and products may be primary products of mRNA translation and not the result of precursor processing, since significant levels of intermediates and products were detected using in vitro translation of genome-length 35S MMTV mRNA, and by immune precipitation of pulse-labeled, MMTV-infected mammary tumor cells (Sen et al., 1981; Dickson and Peters, 1981). MMTV-encoded proteins which do not react with existing antisera to virion proteins will not be identified in immune precipitation studies. An illustration of this point may be the recently described virion 13S RNA which was reported to

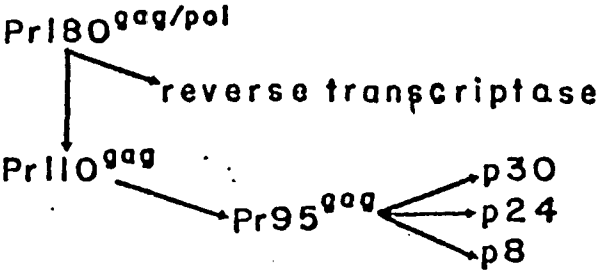
encode proteins of mw 18,000-31,000 not antigenically related to known MMTV virion proteins (Sen et al., 1981; Dickson et al., 1981). Comparison of existing information with sequencing data and analysis of precursor processing kinetics would be useful in determining the actual number and size of MMTV products.

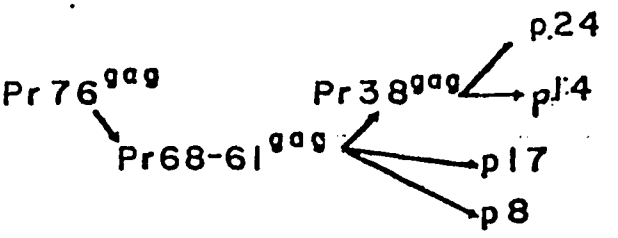
Processing of MMTV gag and env precursors to mature products proceeds via a series of intermediates (Fig. 1). Gag processing was deduced by identifying gag-related proteins using anti-p24 or anti-p8 antisera and then matching corresponding fragments of tryptic peptide maps of precursors, intermediates, and products. Levels of gag precursors $Pr160^{gag/pol}$, $Pr110^{gag}$, and $Pr76^{gag}$ and intermediates $Pr95^{gag}$, $Pr68\text{-}61^{gag}$ $Pr48^{gag}$, and $Pr38^{gag}$ have not been determined, and the rate limiting steps in production of virion p24, p14, and p8 are unknown (Dickson and Atterwill, 1978, 1979). The half-life of $Pr76^{gag}$ has been estimated at 30'-2h; variability has been attributed to inability to detect changes in amount of precursor, and susceptibility of precursors to proteolysis. Intracellular levels of gag products p24, p14, and p8 were low or undetectable, preventing precise kinetic analysis. Dickson and coworkers (1978) detected incorporation of gag precursors $Pr76^{gag}$, $Pr61^{gag}$, and $Pr38^{gag}$ in MMTV-P using a rapid harvest/protease inhibitor technique, and suggested that cleavage of intermediates to mature MMTV gag proteins may occur directly prior to, or after, incorporation into

Fig. 1. Processing scheme of MMTV *env* and *gag* precursors.

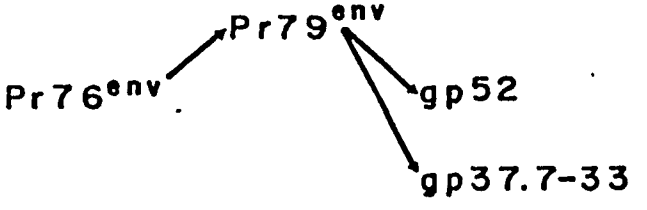
MMTV PROTEIN PROCESSING

gag proteins





env proteins



budding virions. Data of Sen and coworkers (1980) suggested MMTV virions contained a protease which participated in precursor maturation. As shown in Fig. 1, $Pr38^{gag}$ is the direct precursor to p24 and p14, while p8 is derived directly from $Pr76^{gag}$. Differential incorporation of $Pr76^{gag}$ and $Pr38^{gag}$ into MMTV virions provides a mechanism for incorporating different relative amounts of mature gag proteins in virions. Testing of this model by determining relative molarity of p24, p14 and p8 in mature MMTV virions has not been carried out.

Glycoprotein precursor processing of $Pr76^{env}$ to gp52 and gp37.7-33 proceeds via 1-2 intermediates. $Pr79^{env}$ was reported to be the more highly glycosylated product of $Pr76^{env}$, which is cleaved to gp52 and gp37.7-33 (Dickson and Atterwill, 1980; Anderson, et al., 1979). Sarkar and Racevskis (1983) suggested that $Pr79^{env}$ is not cleaved, but is shed into the culture medium as a soluble antigen. Rates of MMTV env pecursor synthesis and processing have not been elucidated. $Pr76^{env}$ has been reported to have a half life of 30 min-2 h (Dickson and Atterwill, 1978; Anderson, et al., 1979; Nusse, et al., 1978; Racevskis and Sarkar et al., 1983). Two problems in these analyses include detection of small amounts of precursor, and susceptibility of precursors to proteolysis. Difficulties encountered in kinetic studies of MMTV polyprotein processing may be alleviated using amino acid analoques and protease inhibitors to suppress precursor breakdown.

Following translation, events in precursor processing include specific glycosylation, phosphorylation, and proteolysis (Dickson and Atterwill, 1981; Nusse, et al., 1978). $Pr76^{env}$ was reported to incorporate glucosamine and fucose (Anderson, et al., 1979; Dickson and Atterwill, 1980; Racevskis and Sarkar 1983), but the level of incorporation was not quantitated, and it is not known how the carbohydrate content or structure of $Pr76^{env}$ compares to that of gp52 or gp37.7-33 in the mature virion. Intracellular sites of processing of MMTV proteins were studied by Dickson (1980) with ambiguous results concerning intracellular location of proteolytic cleavages. Although $Pr76^{env}$ and gp52 were identified in subcellular fractions, no evidence of precursor turnover was presented, and gp37.7-33 was not well visualized. The relative levels of MMTV proteins in different cellular subfractions were not investigated. The carbohydrate structures of MMTV virion glycoproteins are not known, although compositional studies of Yagi et al. (1978c) suggested several oligosaccharide chains were present. Yagi and coworkers (1977, 1978c) demonstrated addition of sialic acid, SO_4, and fucose, thought to be late steps in glycoprotein synthesis, occurred with MMTV virion glycoproteins gp52 (sialic acid, SO_4, fucose) and gp37.7-33 (sialic acid, fucose only).

4. LATE STEPS OF REPLICATION.

Late steps in MMTV replication require appropriate transport of polypeptides and RNA, assembly of components, and budding of particles at the plasma membrane. Investigations of herpes, pox, rhabdo-, and paramyxoviruses have suggested that late steps in virion replication are dependent on an intact cellular cytoskeleton (Stokes, 1976; Wang, et al., 1976; Naito and Matsumoto, 1978; Richardson and Vance, 1978; Hiller et al., 1979; Guiffre, et al., 1982). Electron microscopic observations of MMTV-infected cells revealed close associations between actin-containing microfilaments and intracellular MMTV particles (Damsky, et al., 1977), but the role of the cytoskeleton in MMTV maturation has not been elucidated.

Final events in MMTV replication include budding of virion particles and their release from the host cell surface. Similar to other retroviruses, MMTV buds only from anterolateral plasma membranes in vitro. Boulan-Rodriquez and Sabatini (1978) and Roth and coworkers (1983) demonstrated that other enveloped viruses, such as influenza, bud solely from basolateral membranes, while still others (vesicular stomatitis virus) bud from apical cell membranes. These final steps are virus-dependent, and do not vary with the host cell employed for productive infection. The basis for site-specific virion-budding is unknown, but a knowledge of late steps in replication may facilitate its understanding.

J. SUMMARY

Investigations of MMTV replication have contributed to understanding of viral-induced mouse mammary carcinogenesis. However, detailed information describing the replication of MMTV has not been obtained. Investigations reported in this thesis have addressed host cell involvement in MMTV replication. Studies described here characterize virion polypeptide p44, previously suggested to be cellular actin, and examine the necessity of an intact cellular cytoskeleton for MMTV replication.

Production of MMTV *in vitro* has been achieved in mammary cells cultured on organic and synthetic supports, but the relationship between cellular proliferation and virus production has not been defined. Investigations described here established conditions for growth of MJY-alpha cells in suspension. Stimulation of MMTV production by this adaptation without altering cell growth rate suggested independent controls of MMTV production and cell proliferation.

Glucocorticoids are routinely used to stimulate MMTV production, but knowledge of cellular response to steroid treatment is incomplete. Effects of short-term and prolonged exposure of MJY-alpha cells to hydrocortisone were investigated to further define glucocorticoid-induced responses in MMTV-producing cells. The studies presented here demonstrated a complex interaction between replicating particles and the host cell.

Chapter II

MATERIALS AND METHODS.

A. TISSUE CULTURE METHODS.

1. CULTURES.

The epithelioid MJY-alpha cell line is a chronic producer of MMTV and was derived from spontaneous BALB/cfC3H mammary adenocarcinomas (Yagi, 1973). Cultures released viable cells into the medium after the layers became confluent and subcultures were initiated by harvesting and seeding 5.3 X 10^4 released cells/cm^2 Cells were maintained in RPMI 1640 supplemented with 18% fetal bovine serum (FBS), 1.89 μM bovine insulin, and antibiotics (maintenance medium) in a 37 deg C, 5% CO2 humidified incubator.

Suspension cultures of MJY mammary tumor cells (denoted MJY-beta cells) were initiated in 50 ml sterile translucent conical centrifuge tubes (Corning Science Products Div., Corning, NY) by seeding 1 x 10^7 cells in 20 mls of mainte-

nance medium. Culture tubes were tightly capped, horizontally mounted, and shaken at a rate of one excursion per second on an Eberbach 5850 shaker (Courtesy of Dr. A. Langer, Department of Community Medicine, Mt. Sinai School Medicine) in a 37 deg C room. Medium was changed 48 h after seeding, and daily thereafter by pelleting cells at 800 x g for 10 min and resuspending the pellets in 20 ml of fresh maintenance medium. Suspension cells grew logarithmically with a doubling time of 18- 22 h until a saturation density of 5-7 X 10^6 cells/ml medium was attained. Aliquots of late log or stationary phase cultures were used for subculture.

Observations of MJY-mammary tumor cells were made in situ, or after staining with May-Grunwald-Giemsa. Photomicrographs were taken using an Olympus PM-10AD system with a large format Polaroid camera. Routine screening for mycoplasma was consistently negative (Yagi, 1974).

2. TRYPSINIZATION OF MONOLAYERS.

MJY-alpha cell layers were incubated with a saline-trypsin-EDTA solution and released cells harvested as described (Yagi, 1973).

3. DETERMINATION OF VIABLE CELL NUMBER.

Numbers of viable released, suspension, or trypsinized monolayer MJY-mammary tumor cells were enumerated on a hemocytometer after staining with 0.01% trypan blue vital stain in phosphate-buffered saline (PBS).

4. PLATING EFFICIENCY. Plating efficiency of MJY-mammary tumor cells was determined by seeding petri dishes with 3 X 10^5 released or suspension cells/cm^2 in maintenance medium, and incubating at 37 deg C for 2 h. Monolayers were then washed twice with warm maintenance medium, adherent cells removed by trypsinization and viable cells enumerated.

5. UPTAKE AND INCORPORATION OF RADIOLABELS.

Uptake and incorporation of radiolabeled DNA, RNA, and protein precursors into monolayer and suspension cells were determined using late log cultures of MJY-alpha and beta cells treated in the presence or absence of HC for 24 h. Cultures were then incubated for 6h in labeling medium containing radiolabels with per ml concentrations of 0.5-0.1 μCi ^{14}C-amino acids, 5-15 μCi ^{3}H-uridine, or 10 μCi ^{3}H-thymidine. Cell layers were then washed twice with phosphate-buffered saline at 4 deg C, scraped and uniformly suspended in TE buffer. Cells resuspended in TE buffer were mixed with trichloroacetic acid (TCA; final concentration 10% w/v), and the precipitates washed with cold 5% TCA (w/v) on 0.45 μM Millipore filters. Samples were dried and counted by liquid scintillation using a toluene-Liquifluor cocktail. Soluble radioactivity in the cell/TCA mixture was determined following removal of the precipitated material by centrifugation at 1000 x g for 20 min at 4 deg C.

B. MMTV VIRIONS.

1. UNLABELED MMTV VIRIONS.

MMTV particles were harvested from spent media of MJY-alpha monolayer or beta suspension cultures every 24 h. Comparative analyses of virus production utilized cells grown in maintenance medium in the presence or absence of 14 μM hydrocortisone (HC).

Large scale MMTV production was carried out by growing MJY-alpha cells in maintenance medium in 175 cm^2 flasks or 450 cm^2 roller bottles. When confluent, the medium was changed to RPMI 1640 supplemented with 18% newborn calf serum (NCS), 1.89 μM bovine insulin, and 14 μM HC and antibiotics for the next 2 days, followed by the same medium with NCS reduced to 10% for an additional 3-5 days. Spent culture supernatants were harvested for MMTV purification every 24 h from the confluent cultures.

2. LABELED MMTV VIRIONS.

Labeled MMTV virions were obtained from newly confluent MJY-alpha cultures stimulated for 24-48 h with 14 μM HC in maintenance medium followed by addition of radiolabeled precursors in RPMI 1640 medium supplemented with 2% FBS, 1.89 μM bovine insulin, and 14 μM HC. The concentrations (per ml) of each radiolabel were: 2.5 μCi ^{14}C-amino acids, 10 μCi

^{35}S-methionine, 10 μCi ^{3}H-uridine, 5 μCi ^{3}H-glucosamine, 10 μCi ^{3}H-leucine After a 24 h labeling period, media were harvested daily for an additional 48 h, and MMTV purified from spent culture fluid as described.

3. VIRUS PURIFICATION.

MMTV was harvested from spent culture fluids of MJY-alpha and beta cells by pelleting at 100,000 x g for 50 min. The pellets were resuspended and MMTV concentrated and partially purified by centrifugation on discontinuous 65/32.5/15% (w/v) sucrose gradients at 300,000 x g for 30 min (Yagi, and Compans, 1977). MMTV at the 32.5/65% interface was collected by bottom puncture, and purified by centrifugation on 15-65% (w/v) linear sucrose gradients. Gradients containing radiolabeled MMTV were fractionated by bottom puncture into 0.3 ml fractions. Microliter aliquots of the fractions were counted, and MMTV-containing fractions were pooled, pelleted, and processed for electrophoresis or immune precipitation. Unlabeled MMTV was identified in gradients as a light-scattering band between 1.16 and 1.18 g/cc density markers and harvested by bottom puncture.

C. LABELING AND ISOLATION OF POLYPEPTIDES RELEASED FROM MJY-ALPHA CELLS.

Polypeptides released from MJY-mammary tumor cells were labeled by incubating MJY-alpha or MJY-beta cultures with 10 or 100 μCi ^{35}S-methionine/ml maintenance medium for 6 h. Spent supernatants were then harvested, cleared at 300,000 x g for 30 min to remove debris and MMTV particles. The resultant supernatants were further processed for electrophoresis.

D. IMMUNOLOGIC PROCEDURES.

1. IMMUNOGENS AND ANTISERA.

MMTV particles used as immunogens were isopycnically purified from confluent, HC-treated MJY-alpha cultures. Individual MMTV proteins were isolated by preparative SDS-PAGE (Yagi, et al., 1978b).

Preimmune sera were obtained from New Zealand white rabbits before immunization by subcutaneous injection with 400 μg of gp52, gp37-7-33, p24 or, formalin-fixed MMTV in complete Freund's adjuvant (Yagi and Compans, 1977). A booster immunization of 100-300 μg MMTV proteins in incomplete adjuvant was administered after 2 weeks. Sera were obtained 2 weeks after immunization and weekly thereafter. Immunoelectrophoresis (Yagi and Compans, 1977) detected precipitin

lines of identity using immune sera and corresponding immunogen; no cross-reactivity of individual MMTV polypeptides was identified. Preimmune sera were consistently negative in these assays.

2. Staphlococcus aureus

IMMUNOSORBENT.

Formalin-fixed, heat-killed S. aureus (Cowan I strain) was obtained from Calbiochem (Pansorbin, La Jolla, CA), or was prepared according to the method of Kessler (1975). Seed cultures of bacteria were the generous gift of Dr. E. Ainbinder, Department of Pediatrics, Mount Sinai School of Medicine (MSSM).

3. IMMUNE PRECIPITATION OF VIRIONS.

Metabolically-radiolabeled MMTV was obtained as described previously in section 2B and disrupted in 0.05 M phosphate buffer containing 0.1 M NaCl and 0.1% Triton X-100, pH 7.5 (RIP buffer). Samples were incubated with anti-MMTV antisera (1:3.7 dilution) for 25 min at room temperature (RT) and immunoprecipitates collected by the method of Kessler (1975) using S. aureus immunosorbent. Precipitates were washed 5X in RIP buffer and resuspended in either RIP buffer, for scintillation counting, or in 1% SDS/2-mercaptoethanol for electrophoresis. Samples in SDS/2-mercaptoethanol were heated at 100 deg C for 1 min to disrupt immune complexes, and centrifuged at 10,000 x g for 1 min in a Beckman Microfuge B to remove S. aureus before electrophoresis.

4. IMMUNE PRECIPITATION OF RADIOLABELED INTRACELLULAR MMTV POLYPEPTIDES.

Cell-associated MMTV proteins and glycoproteins were obtained by immune precipitation from newly confluent MJY-alpha cells grown in 100 mm petri dishes. For these studies, MJY mammary tumor cells were maintained and labeled in the presence or absence of 14 μM HC, or were given HC-containing medium just 24-48 h prior to and during labeling. (1) Labeled proteins were precipitated from cells incubated for 15 min-24 h with 100 μCi ^{35}S-methionine/ml labeling medium or for 24 h with 80 μCi ^{3}H-leucine/ml medium. Labeling for 15 min to 4 h was accomplished by adding to each petri dish one ml labeling medium with isotopes, and incubating at 37 deg. C in a humidified, 5% CO2 incubator on a rocker platform (Bellco, NY). Cells labeled for 6-24 h received 2-2.5 ml medium with isotopes and were placed on the rocker. (2) Labeled glycoproteins were precipitated from cells preincubated for 18 h with depleted medium consisting of labeled medium diluted 1:1 with phosphate-buffered saline. Following preincubation, cultures were labeled for 3.5-4 h in depleted medium supplemented with 1.89 μM bovine insulin and either ^{3}H-glucosamine, ^{14}C-glucosamine, or ^{3}H-mannose (100 μCi/ml depleted medium). (3) sulfated glycoproteins were labeled by incubating cells for a total of 48 h with inorganic $^{35}SO_4$ Cells received 1 mCi $^{35}SO_4$/ml labeling medium for 24 h. Spent medium was

then harvested and the cells relabeled with inorganic sulfate for an additional 24 h before immune precipitation.

Immune precipitation of intracellular MMTV polypeptides was carried out using techniques adapted from the method of Kessler (1975). Radiolabeled MJY-alpha cells were washed twice with 5 ml of phosphate-buffered saline at 4 deg C and drained. Protease inhibitors (Sigma Chemical Co., St. Louis, MO) at final concentrations of 10 μM pepstatin, 10 μg soybean trypsin inhibitor/ml, and 1 mM N-ethyl maleimide were added, and cells disrupted in RIP buffer. Lysis was completed by passing scraped cells through a 27 gauge needle 5 times. Nuclei were removed by centrifugation at 1000 x g for 10 min at 4 deg C. Cleared supernatants were preabsorbed by incubation for 15 min at RT with rabbit preimmune sera and 10% (w/v) formalin-fixed, heat-killed Staphlococcus aureus (sample:sera: S. aureus = 100 μl:10 μl:50 μl), then centrifuged at 10,000 x g for 1 min (Beckman microfuge). Preabsorbed supernatants were incubated with rabbit anti-gp52, anti-gp37.7-33, anti-p24, or anti-MMTV antisera for 25 min at RT, followed by 10% S. aureus for 15 min at RT (sample:sera: S. aureus = 100 μl:33 μl:150 μl) before centrifugation at 10,000 x g for 1 min. Precipitates were washed 5 X in RIP buffer, resuspended in 20 μl 1% SDS/2-mercaptoethanol, and heated at 100 deg C for 1 min to disrupt immune complexes. The suspensions were centrifuged at 10,000 x g for 1 min to remove S. aureus, and the resul-

tant supernatants processed for SDS-PAGE. SDS-PAGE of immune precipitates contained large amounts of reduced IgG heavy chain (mw 52,000) which caused alterations in electrophoretic mobility of MMTV gp52. Analysis of immune precipitated MMTV virions indicated gp52 migrated with a mw of 48,000. Immune precipitation with several lots of anti-p24 antisera resulted in degradation of MMTV precursor $Pr76^{gag}$, and this antisera was not used to quantitate MMTV gag polypeptides.

5. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

MMTV production in MJY-alpha cells was quantitated using a direct ELISA adapted from the method of Khan et al. (1981). MMTV for sensitization of ELISA titer plates (Immulon II, Vanguard, VA) was purified by isopycnic centrifugation or obtained from clarified, spent culture medium centrifuged at 100,000 x g for 50 min. Sample wells were coated with dilutions of MMTV preparations or released MJY-alpha cells disrupted in 2% NP-40 in 0.15 M carbonate buffer, pH 8.6 by overnight (18 h) incubation at 4 deg. Sample wells were then washed 3 X with ELISA wash buffer (0.5% Tween-80 in 0.15 M NaCl/ 0.01 M Na phosphate buffer, pH 7.4). To reduce nonspecific adsorption, wells were incubated with 0.5% bovine serum albumin (BSA) in ELISA wash buffer for 1 h at 4 deg C. Adsorbed wells were washed, incubated with rabbit anti-MMTV or preimmune sera (1:50 dilution in ELISA wash buffer) for 1 h at 37 deg in an humidified cham-

ber, and then washed as above. Bound antibody was detected by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Dynatech, Inc., 1:1000 dilution in ELISA wash buffer) for 1 h at 37 deg. Following washing, retained alkaline phosphatase activity was determined by reaction with 100 µl p-nitrophenyl phosphate substrate (1 mg/ml in 0.1 M diethanolamine) at 37 deg; the reactions were stopped with 25 µl 3 N NaOH when the absorbance at 410 nm of the most deeply colored sample was 0.7-1.4. A410 of all samples was then determined using the Dynatech ELISA plate reader (courtesy of Dr. D. Bucher, Department of Microbiology, MSSM). Efficiency of MMTV binding to ELISA plates, estimated by sensitizing wells with detergent-disrupted ^{35}S-labeled MMTV revealed 73% of the total, virion-associated ^{35}S remained bound after 24 h sensitization. Reproducible titrations were obtained in parallel ELISA determinations, with a 6% variation in A410 in corresponding duplicate wells. Background absorbance using preimmune sera was always less than 0.05 at 410 nm. Absence of MMTV as a sensitizing antigen, or use of heterologous protein (e. g. bovine serum albumin) to sensitize plates did not significantly increase the absorbance at 410 nm.

6. DETERMINATION OF MMTV SURFACE ANTIGENS.

Levels of MMTV surface antigens were determined using fluorescent antibody staining. 1 X 10^7 MJY-alpha cells released from confluent monolayers were washed and resus-

pended at 5 X 10^6 cells/ml in Minimal Essential Medium containing 2% newborn calf serum (MEM/2% NCS). Cells were incubated with 1:50 - 1:1000 dilutions of anti-MMTV antiserum for 45 min at 4 deg C with occasional agitation, and then washed twice. Antibody-binding cells were labeled with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 45 min at 4 deg C with occasional agitation. Cells were washed twice more before resuspension in 1% paraformaldehyde in 0.15 M NaCl. Microscopic observations were made with a Leitz fluorescence microscope. Analysis of fluorescence was carried out by M. Zanjani using the prototype Becton-Dickinson FACS analyzer. Cell-associated fluorescence intensity in each sample was determined on 10,000 cells at a rate of 300-600 cells/sec. The logarithm of cell-associated fluorescence intensity was determined, and a photomultiplier tube was used to amplify and convert the signal into log voltage quantities ranging from a low value of 1 to a high value of 256. The FACS was interfaced with an HP-85 calculator/HP82901M flexible disc drive which stored data, generated histograms of log fluorescence intensity, and determined mean and coefficient of variation of log fluorescent intensity.

E. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

1. ONE-DIMENSIONAL SDS-PAGE.

Samples were disrupted under reducing (1% SDS (w/v)/1% 2-mercaptoethanol) or non-reducing (1% SDS) conditions by heating at 100 deg C for 1 min. Gradient (10-20%, 7.5-15%, or 5-12% w/v) polyacrylamide-SDS gel electrophoresis using a discontinuous Tris/glycine buffer system was carried out as previously described (Yagi, et al., 1976c). Molecular weights of polypeptides on SDS-polyacrylamide slab gels were determined by comparison of electrophoretic mobilities of proteins under study with polypeptide standards of predetermined molecular weight: phosphorylase A (94,000 mw), bovine serum albumin (68,000 mw), IgG heavy chain (52,000 mw), ovalbumin (44,000 mw), carbonic anhydrase (30,000 mw), soybean trypsin innibitor (20,000 mw), and alpha-lactalbumin (14,100). (Pharmacia, NJ). ^{14}C-carboxymethylayed polypeptides used as standards included intact gamma-globulin (150,000 mw), phosphorylase A, bovine serum albumin, ovalbumin, and carbonic anhydrase, as above (New England Nuclear, Boston MA). A linear relationship was identified between log molecular weight and migration distance. Gels were fixed, stained with coomassie blue, and densitometrically scanned as described in section H. 3. Protein Determination. Radioactive samples were processed for quantitation of radioactivity as previously described (Yagi, et al., 1976c).

2. TWO-DIMENSIONAL SDS-PAGE.

Second dimension SDS-PAGE was carried out on samples electrophoresed on 10-20% or 7.5-15% gradient polyacrylamide slab gels in the first dimension. Electrophoresed gel lanes were excised and heated at 100 deg C for 1 min in 0.06 M Tris buffer, pH 6.8, containing 1 mM dithiothreitol (10 mls buffer/excised gel lane). Gel lanes were then placed lengthwise across the origin of a second 10-20% or 7.5-15% gradient polyacrylamide gel. A 5% acrylamide stacking gel was cast around the gel lane, with a single sample well for polypeptide molecular weight markers. Electrophoresis was carried out as described, and the gel processed for coomassie blue staining.

3. AUTORADIOGRAPHY. SDS-PAGels (7.5-15% or 5-12% gradient polyacrylamide) were dried onto filter paper using a BIORAD gel dryer (courtesy of Dr D. S. Beattie, Department of Biochemistry, MSSM). Gels were then placed on KODAK X-O-MAT film and sealed in light-exclusive film holders. Film was exposed at -70 deg C for 4-14 days and developed according to Kodak product directions.

F. ACTIN POLYMERIZATION PROCEDURES.

1. DISRUPTION OF MMTV WITH NON-IONIC DETERGENT FOR ACTIN POLYMERIZATION.

Isopycnically purified MMTV was pelleted at 300,000 x g for 30 min, and resuspended at 4 deg C in 20 mM Tris-maleate buffer containing 5 mM EDTA, 10 mM dithiothreitol, 0.34 M sucrose, and 0.5% Triton X-100, pH 7.4 (disruption buffer, Hartwig and Stossel, 1976; Weihing, 1976). Lysis was completed by forcing the suspension through a 27 gauge needle 5-7 times at 4 deg C. Undisrupted virions, were removed by centrifuging the lysates at 100,000 x g for 30 min.

2. ACTIN ASSEMBLY ASSAY.

Polymerization of detergent-disrupted MMTV was monitored in either a Behring nephelometer, or in a Cary 210 recording spectrophotometer using a wavelength of 350 nm. Parallel controls contained rabbit or dog skeletal muscle actin in disruption buffer. Increases in sample turbidity upon addition of a mixture of ATP (final concentration 5 mM), KCl (0.1-0.5 M), and MgCl (2 mM), or ATP alone (5 mM) were measured at 25 deg C. Samples were then incubated overnight at 4 deg C, pelleted at 100,000 x g for 3 h, and the supernatants and pellets individually processed for electrophoresis.

3. DNASE I BINDING ASSAY.

DNase I-Sepharose beads (Worthington Biochemicals, Freehold, NJ; generous gift of Dr. S. Puszkin, Department of

Pathology, MSSM) were washed and hydrated overnight in MES (2[N-morpholino]ethanesulfonic acid) buffer containing 0.5% triton X-100 and 0.1 mM ATP, pH 6.7 (MESTA buffer). Binding was initiated by adding samples in MESTA buffer containing 1 mM CaCl2 directly to DNase I-Sepharose beads (slurry:sample, 1:2). After incubation at 25 deg C for 5-8 h, the mixtures were washed 5 X with MESTA buffer. After the final wash, proteins were eluted from beads by heating at 100 deg C for 1 min in 1% SDS/1% 2-mercaptoethanol (Garrells and Gibson, 1976). The eluted proteins were separated from the beads by centrifugation, and were then processed for electrophoresis.

4. TRYPSIN DIGESTION OF MMTV.

Isopycnically purified MMTV or purified rabbit muscle skeletal actin suspended in 30% sucrose in 0.01 M Tris buffer, containing 5 mM EDTA, pH 7.4 (TE buffer) was incubated with trypsin-TPCK (Worthington Biochemicals, Freehold, NJ; 2 µg MMTV/1 µg trypsin) for 30 min at 25 deg C. Control MMTV samples were incubated without trypsin. Digestion was terminated by adding trypsin inhibitor (1 µg inhibitor/1 µg trypsin) and cooling to 4 deg C. MMTV-containing preparations were then diluted in TE buffer, purified on isopycnic gradients as described above, and the repurified MMTV processed for electrophoresis. Preparations containing rabbit muscle skeletal actin were processed for electrophoresis directly after termination of digestion.

G. TREATMENT WITH CYTODISRUPTIVE DRUGS.

1. DRUGS.

Colcemid (Sigma Chemical Co., St Louis MO) was dissolved in PBS at 0.5 or 1 mg/ml and stored at -20 deg C. Cytochalasin B, from *Helminthosporium dematioideum*, and cytochalasin D, from *Metarrhizeum anisopliae* or *Zygosporium mansonii* (Sigma Chemical Co., St. Louis MO) were reconstituted in 100% ethanol at 2.5 mg/ml and diluted in media before use. Final ethanol concentrations did not exceed 0.4% in our experiments.

2. DRUG TREATMENT.

Petri dish cultures were primed for 24 h with growth medium containing 14 μM HC before labeling for another 24 h with 4 μCi glucosamine and/or 10 μCi ^{35}S-methionine per ml labeling medium. Media containing radioactive labels were removed, and cell cultures were then continuously treated with 2.1 μM CB or CD for 24 h, or with 0.28 μM colcemid for 48 h in isotope-free labeling medium. Culture supernatants were harvested every 6 or 8 h; after each collection, cells received fresh labeling media containing drugs. Following the treatment period, all cultures were incubated with drug-free labeling medium for another 24 h during which time additional viral harvests were made. Parallel control cul-

tures received drug-free labeling medium and were subjected to identical harvest procedures.

3. UPTAKE AND INCORPORATION OF RADIOLABELS DURING DRUG TREATMENT.

The effects of CB, CD, or colcemid on uptake and incorporation of amino acids, amino sugars, and nucleosides were determined using radiolabels with per ml concentrations of: 5-10 μCi ^{3}H-glucosamine, 0.5-10 μCi ^{14}C-amino acids, 5-10 μCi ^{3}H-leucine, or 5-15 μCi uridine. Petri dish cultures were primed with media containing 2.1 μM CB, CD, or 0.28 μM colcemid, or no drug additive. Radiolabeled precursors were added either at the initiation of treatment or for a 1 h pulse after 23 h of drug exposure. Labeled cells were washed, scraped, and TCA-precipitable radioactivity determined as described for monolayer cells above (Section A. 5E.). Soluble radioactivity in the cell/TCA mixture was determined following removal of the precipitated material by centrifugation at 1000 x g for 20 min at 4 deg C.

H. GENERAL METHODS.

1. ELECTRON MICROSCOPY.

Thin sections of cell layers were prepared and processed for electron microscopy and examined as previously described (Yaqi, et al., 1980) in collaboration with Dr. N. W. King, Jr. (New England Regional Primate Ctr., Harvard Univ., MA)

2. MEASUREMENT OF LACTATE PRODUCTION AND CELLULAR REDOX STATE.

The cellular redox state (NAD+/NADH ratio) was measured as described (Krebs and Veech, 1979, Williamson, et al., 1967). Lactate dehydrogenase activity in MJY-alpha cells was assayed as described (Bergmeyer, et al., 1974). Lactate production was measured by the colorimetric procedure of Barker (1957), and pyruvate determined by the method of Bucher et al. (1974).

3. PROTEIN DETERMINATION.

Proteins in solution were quantitated by a modified Lowry procedure (Lowry, et al., 1951; Markwell, et al., 1979). Coomassie blue-stained SDS-polyacrylamide gels were scanned at 590 nm and stained protein bands were quantitated using an EC transmission densitometer (EC Apparatus Corp. St. Petersburg, FL.) using bovine serum albumin or electrophoresis molecular weight marker proteins as standards.

4. STATISTICAL TESTING.

Differences in the relative levels of radioactivity associated with MMTV polypeptides in cytodisruption studies were tested for statistical significance using Dunnett's test for

multiple comparisons. Student's t-test was used to determine statistical significance of differences in means of fluorescence intensity in studies employing fluorescence activated cell sorter. Analysis of variance procedure was employed to determine statistical significance of levels of MMTV polypeptides in coomassie-blue stained preparations (Zar, J., 1974).

5. RADIOISOTOPES.

L-[^{14}C(U)]amino acid mixture (280-305 mCi/mMol, L-[4,5-^{3}H(N)] leucine (52.2 Ci/mMol), D-[6-^{3}H(N)]mannose (6.3 Ci/mMol), and D-[6-^{3}H(N)] glucosamine hydrochloride (6.3 Ci/mMol) were obtained from New England Nuclear (Boston, MA). [5,6-^{3}H]uridine (46 Ci/mMol) was obtained from ICN Pharmaceuticals (Irvine, CA); [^{35}S]O_4 (25-40 Ci/mg) and L-[^{35}S]methionine (600-1100 Ci/mMol) were purchased from Amersham Radiochemicals (Arlington Heights, IL).

Chapter III

RESULTS

A. VIRION COMPOSITION.

1. MMTV POLYPEPTIDE COMPOSITION.

The protein composition of MMTV was established to enable comparison with virions from cells grown under alternate culture conditions or exposed to various drugs. MMTV was routinely purified from supernatants of MJY-alpha cultures at 24 h intervals. Proteins from purified virions were separated by SDS-PAGE, revealing seven polypeptides with apparent molecular weights of 52,000 (gp52), 44,000 (p44), 37,700-33,000 (gp37.7-33), 24,000 (p24), 17,000 (p17), 14,000 (p14), and 8,000 (p8). Scanning densitometry of these gels provided a reproducible measure of the relative levels of MMTV polypeptides (Table 2). MMTV *env* protein gp52 and *gag* pr p24 accounted for >50% of the total detectable protein in virions harvested under these culture conditions.

Table 2. Polypeptide composition of MMTV

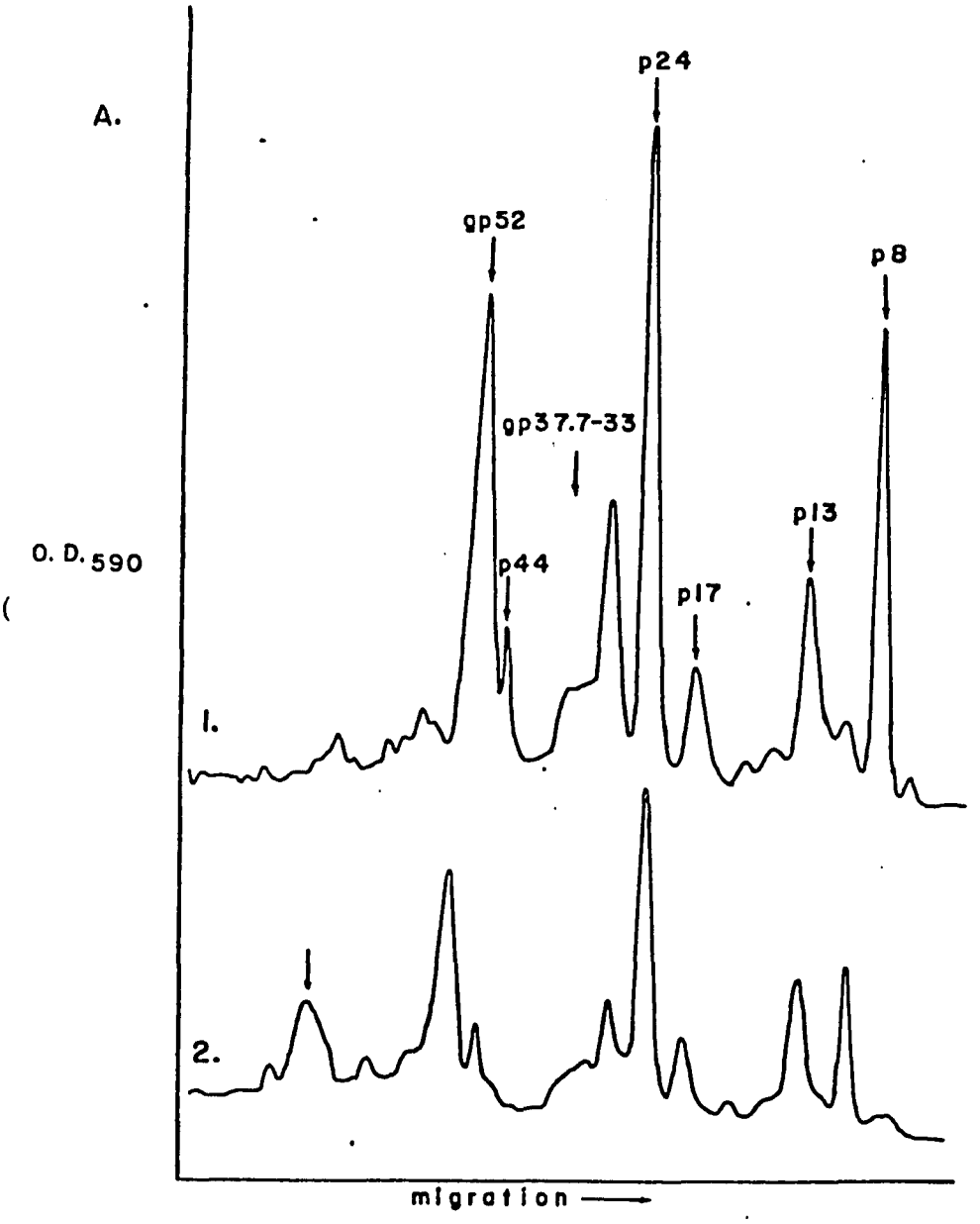
Polypeptide	Percent of Total Polypeptide-associated staining (+/- S. D.)[1]		
	24 h harvest n=9[2]	6-8 h harvest n=14	2 mM Benzamidine n=2
gp52	30.4 +/-5.4	29.9+/-5.7	26.4 +/- 3.3
p44	3.7 +/-2.1[3]	7.2+/-1.9	5.4 +/- 2.1
gp37.7-33	15.8 +/-4.0	20.0+/-5.2	21.6 +/- 2.1
p24	23.3 +/-4.9	21.8+/-4.6	22.0 +/- 0.4
p17	3.8 +/-2.5	5.1+/-3.9	4.0 +/- 2.7
p14	10.6 +/-2.2	10.2+/-2.7	8.6 +/- 0.6
p8	12.7 +/-4.1	6.6+/-2.9[3]	11.4 +/- 3.1

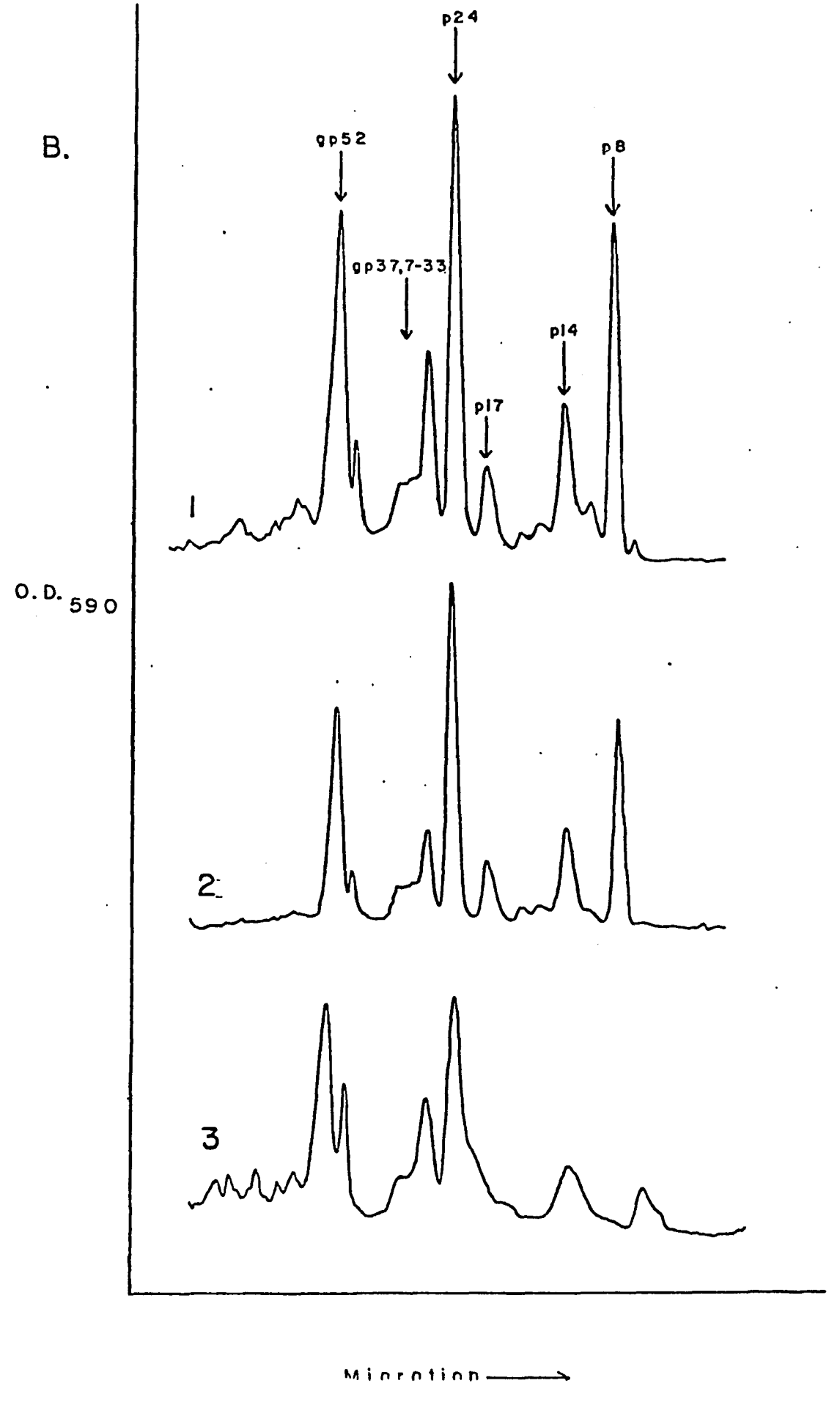
1 MMTV was purified from media incubated with MJY-alpha cultures for 24 h, 6-8 h or after 24 h treatment with benzamidine. Polypeptices were quantitated by scanning densitometry of coomassie blue- stained gels.
2 no. of samples.
3 Relative level of the polypeptide in these virions was significantly different from polypeptide from MMTV produced under other conditions at $p<0.05$ using analysis of variance procedure.

Despite reproducibility of MMTV polypeptide profile, alterations in the proteins were detected by modification of standard procedures. For large scale production of MMTV, cells were grown in medium containing newborn calf serum (NCS) in place of fetal bovine serum (FBS). Polypeptide profiles of virions from these cultures contained an additional polypeptide, p85 (Fig. 2), which accounted for 10-40% of total coomassie blue-staining material. A protein with a similar electrophoretic mobility was reported present in variable amounts in MMTV preparations from milk and tissue culture sources (Teramoto, et al., 1973, Sarkar, et al., 1977), but the origin of this polypeptide is unknown.

Alterations in MMTV polypeptide profile were also detected when virions were harvested from MJY-alpha cells at 8 h intervals or from cells treated for 24 h with benzamidine (2 mM), a serine protease inhibitor (Table 2). A comparison of the standard MMTV profile with virions from these cultures revealed that the relative levels of p44 were increased by either technique, and p8 was decreased in MMTV from short harvest cultures (Table 2). It is not known whether these quantitative differences in MMTV polypeptides exerts any effect on the stability or the infectivity of the virions. Treatment with the protease inhibitor or frequent harvesting also did not yield MMTV particles containing detectable levels of polypeptide precursors (Fig. 2B).

Fig. 2. SDS-PAGE of MMTV. A. MMTV harvested from MJY-alpha cultures grown in maintenance medium supplemented with 14 μM HC for 24 h (1) or in maintenance medium supplemented with 14 μM HC with 18% NCS in place of 18% FBS (2). B. MMTV harvested from MJY-alpha cultures grown in labeling medium alone for 24 h (1) or 8 h (3), or in labeling medium medium supplemented with 2 mM benzamidine for 24 h (2).


A.
O. D. 590
p24
gp52
p8
gp37.7-33
p13
p44
p17
1.
2.
migration


B.
55
p24
gp52
p8
gp37,7-33
p14
p17
1
O.D. 590
2
3
Migration

2. INCORPORATION OF RADIOLABELED PROTEINS INTO MMTV.

MMTV polypeptide composition was investigated further by analyses of MMTV purified from MJY-alpha cells labeled for 24 h with ^{3}H-leucine, ^{35}S-methionine, or ^{14}C-amino acids. Seven principal MMTV polypeptides incorporated ^{3}H, ^{14}C, and ^{35}S-radioactivity (Table 3). Radiolabeled MMTV was also obtained 24-48 h following removal of the labels indicating that cellular pools of radiolabeled amino acids and/or virion polypeptides were relatively large, and had not completely turned over after 24 h. The 85,000 mw protein observed in MMTV from MJY-alpha cells grown in 18% NCS was not detected following ^{35}S-methionine labeling, suggesting it is a serum contaminant. It is possible this polypeptide is synthesized de novo and contains few methionine residues. An additional polypeptide, gp60, which did not stain with coomassie blue, was identified by the three radiolabels (Table 3).

As expected from investigations of MMTV polypeptide composition, gp52 and p24 accounted for 46% or more of ^{3}H, ^{14}C, or ^{35}S radioactivity. Comparison of ^{3}H, ^{14}C, or ^{35}S incorporation revealed variations in the relative levels

Table 3. Incorporation of radiolabeled proteins into MMTV.

MMTV protein	Percent of total polypeptide-associated MMTV radioactivity[1]		
	^{14}C-amino acids	^{35}S-methionine	^{3}H-leucine
gp60	6.8	2.0	2.0
gp52	29.1	22.8	28.5
p44	4.8	**[2]	**
gp37.7-33	24.0	24.6	26.2
p24	17.7	41.2	23.7
p17	5.7	**	5.2
p14	6.7	6.6	7.1
p6	5.3	4.1	9.3

[1] MMTV was purified from spent culture medium harvested 24 h following addition of ^{35}S-methionine, ^{3}H-leucine, or ^{14}C-amino acids. MMTV samples were subjected to SDS-PAGE, and polypeptide-associated radioactivity determined as described.
[2] Incorporation into these polypeptides is negligible during labeling period.

Table 4. Molar levels of radiolabeled-MMTV polypeptides in MMTV[1]

	Labeling period[2] fmol radiolabeled protein		Chase period fmol ^{35}S-polypeptide	
	^{35}S methionine	^{3}H leucine		
Protein	label	label	0-24 h[3]	24-48 h[4]
qp52	1.00	1.50	0.50	0.39
qp37.7-33	0.86	1.60	0.70	0.36
p24	1.30	2.20	0.75	0.22
p17	*****[5]	0.79	*****	*****
p14	1.02	1.30	*****	*****
p8	0.81	3.07	*****	*****

[1] femtomolar levels of ^{35}S-labeled MMTV proteins were determined by the formula:

$$\frac{\text{dpm incoporated into MMTV polypeptide}}{\text{specific activity of label}} \Big/ \text{no. of residues of amino acid per protein}$$

[2] MMTV was obtained from MJY-alpha cells labeled for 24 h with ^{3}H-leucine or ^{35}S-methionine, and polypeptide-associated radioactivity was determined after SDS-PAGE separation. Experiment was performed 3 times and a representative sample is presented.

[3] MJY-alpha cells were chased with fresh medium at 6 or 8 h intervals following labeling. Representative sample was obtained from medium harvested from the 16-24 h chase.

[4] MJY-alpha cells were chased with fresh medium at 8 or 12 h intervals during the 24-48 h after labeling. Representative sample was obtained from medium harvested from the 40-48 h chase.

[5] quantity not sufficient for analysis.

of MMTV polypeptides, possibly due to differential incorporation of the radiolabeled amino acid residues. To enable direct comparison of radiolabeled MMTV polypeptides, the relative molarities of the proteins were determined. Isotopic incorporation into MMTV proteins was analyzed in conjunction with previously published amino acid compositions of MMTV polypeptides (Yagi, et al., 1978c). For each MMTV protein, the quotient of incorporated radioactivity and numbers of corresponding amino acid residues present yielded the relative molar amount of polypeptide (Table 4). Comparison of ratios of ^{35}S-labeled polypeptides or ratios of ^{3}H-labeled polypeptides suggested gp52 and gp37.7-33, which are synthesized as a single polypeptide precursor, were present in equimolar amounts in virions. However, gag gene products, also synthesized as a polyprotein precursor, were not found in equimolar amounts. Although molar levels of p24 were approximately equivalent to p14 and to p8, level of p17 (calculated from a single determination of ^{3}H leucine incorporation) was 2.7-fold lower than p24.

To investigate incorporation of prelabeled proteins into MMTV, molarity of virion-associated ^{35}S-labeled gp52, gp37.7-33, and p24 was measured in MMTV harvests obtained during a 48 h chase period following uniform

labeling of MMTV proteins. Only ^{35}S-labeled MMTV proteins were investigated because of higher specific activity of labeled protein compared to ^{3}H-labeled polypeptides (Table 4). As shown in Table 5, ratios of ^{35}S-labeled gp52:gp37.7 and gp52:p24 varied, but were not significantly different from those measured during the labeling period, and remained relatively constant throughout the 48 h chase period. These data suggest pool sizes of ^{35}S-methionine or ^{35}S-labeled MMTV gag or env proteins were similar.

Determination of relative ^{14}C-radioactivity in polypeptides after ^{14}C-amino acid labeling has been used to calculate numbers of individual protein molecules in each virion particle (Adolph and Haselkorn, 1972). Similar calculations (Appendix A, Table 6), revealed MMTV particles contain 1800-2400 of each principal MMTV env and gag polypeptide. These findings are 30 - 60% greater than values reported by Sarkar and Moore (1974) who employed polypeptide-associated coomassie blue staining to estimate numbers of polypeptides per MMTV virion.

3. PRESENCE OF INTER-PROTEIN DISULFIDE BONDS.

The presence of protein-protein disulfide bonds among MMTV polypeptides was also investigated. Previous findings by Dion and coworkers (1979b) indicated interprotein disulfide bonds were present between gp52 and gp37.7-33, and between gp37.7-33 and p24 in MMTV-P; the

Table 5. Comparison of relative molarity of MMTV polypeptides.

Time period	n[2]	Ratio of molarity of MMTV proteins[1]				
		gp52/gp37.7-33	gp52/p24	p24/p17[3]	p24/p14	p24/p8
Labeling period	2	1.02±0.17	1.35±0.11	2.73	1.45±0.30	1.15±0.62
Chase I 0-24 h	8	1.16±0.32	1.08±0.39	--	--	--
Chase II 24-48 h	3	1.53±0.40	0.99±0.38	--	--	--

[1]molar levels were determined as described in Table 4 for each MMTV profile obtained; mean of n ratio determinations presented ± S.D.
[2]number of samples.
[3]single determination.

Table 6. Numbers of protein molecules in MMTV particles.

Polypeptide	Number molecules/virion[1]
qp52	1805.73
p44	351.24
qp37.7-33	2180.45
p24	2382.91
p17	1079.64
p14	1558.00
p8	2128.00

[1] Numbers of molecules per virion were calculated using the formula:

$$N(i) = \frac{w \times r(i)}{m(i)}$$

where N(i) = number of molecules of protein i in particle,
w = protein mass in particle (2.66×10^{-8}, Lyons, M., and Moore, D. H., 1962)
r(i) = proportion of total ^{14}C-amino acid incorporation into protein i
m(i) = molecular weight of protein i.

authors suqqested disulfide bonds were involved in generating the eccentric orientation of the core in the B-type particle. However, the aggregated proteins were not identified, and the abundance of these inter-protein bonds was not determined. To identify the presence of similar disulfide bonds between MMTV-S polypeptides, virions were dissociated in the presence or absence of sulfhydryl reducing agents, and analyzed by one- and two-dimensional SDS-PAGE. With one exception, no significant decreases in the relative levels of MMTV gag or env proteins or the appearance of new protein bonds were observed between the two viral preparations (Table 7). The one protein which was decreased under non-reducing conditions was p44, a minor protein reported to be cellular in origin. Since p44 may not be of viral origin, the relative levels of MMTV proteins were quantitated independently; again no significant decreases were observed among the known polypeptides (Table 7). Additional high molecular weight proteins were not detected in non-reduced MMTV samples, although an increase in coomassie-blue staining material at the origin of the standard 10-20% gradient polyacrylamide gel was noted. A 7.5-15% gradient gel was employed to better resolve proteins in the higher molecular weight region. Under these conditions, proteins with lower molecular

Table 7. Polypeptide composition of reduced and nonreduced MMTV

	Relative level of MMTV polypeptide[1]			
	Reduced		Non-reduced	
Polypeptide	with p44	without p44	with p44	without p44
gp52	24.0	26.4	21.6	21.7
p44	9.4	-	-	-
gp37.7-33	20.3	22.4	18.9	19.0
p24	29.0	32.0	38.1	38.3
p17	0.6	0.7	1.4	1.4
p14	12.3	13.6	16.2	16.3
p8	4.3	4.7	2.9	2.9

[1] Isopycnically-purified MMTV was disrupted in 1% SDS, and heated at 100 deg C in the presence or absence of 1% 2-mercaptoethanol for 1 min, and subjected to SDS-PAGE (10-20% acrylamide gradient). Protein bands were stained with coomassie blue and quantitated by densitometry as described. Three replicate samples were electrophoresed for each group, and a representative sample is presented as percent of total polypeptide staining; relative levels were calculated with or without p44.

weights (<35,000) were not identified or quantitated with precision, and the gp52 peak was widened and occasionally split (Fig. 3). Analysis of MMTV obtained from MJY-alpha cells cultured in NCS revealed a decrease in relative amount of 85,000 mw contaminant in non-reduced samples, with the appearance of a polypeptide with electrophoretic mobility corresponding to a molecular weight of 165,000, suggesting disulfide linked dimers of the 85,000 contaminant.

A two-dimensional electrophoresis system was employed to identify disulfide-linked proteins without ambiguity. Gel lanes of single dimension 7.5-15% gradient SDS-PAGE of MMTV which had been disrupted and run in the presence or absence of 2-mercaptoethanol were next heated in the presence of reducing agent 1 mM dithiothreitol, oriented horizontally, and subjected to 7.5-15% gradient SDS-PAGE. Polypeptides not linked to other proteins by disulfide bonds would exhibit similar electrophoretic mobilities in both gels, and migrate along a single diagonal in the second dimension. Disulfide-linked polypeptides which migrate as aggregates in the first dimension are disrupted by DTT, and do not migrate along the predicted diagonal in the second dimension. These analyses revealed that all principal MMTV proteins migrated along a single diagonal without additional polypeptides (Fig. 4), suggesting

Fig. 3. Densitometry scans of MMTV disrupted under reducing or non reducing conditions. MMTV purified from MJY-alpha cells maintained in medium with 18% NCS was disrupted at 100 deg C with 1% SDS in the presence (A) or absence (B) of 1% 2-mercaptoethanol. Preparations were subjected to SDS-PAGE on 7.5-15% gradient polyacrylamide gels. Protein bands were stained with coomassie blue, and samples scanned by densitometry as described.

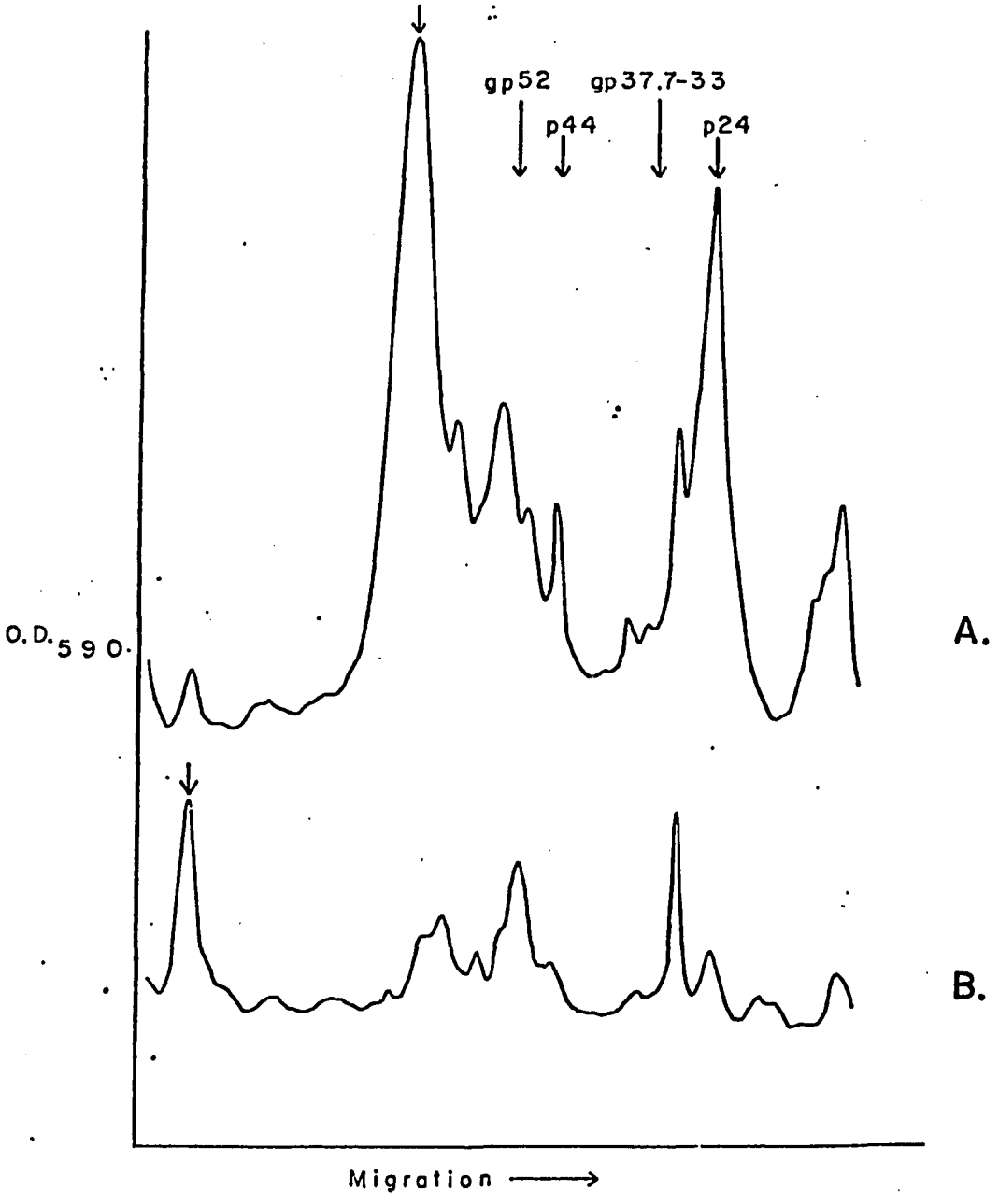

gp52
gp37.7-33
p44
p24
O.D.590
A.
B.
Migration

Fig. 4. Two-Dimensional SDS-PAGE separation of MMTV polypeptides. Cut gel lanes of MMTV, disrupted and electrophoresed in presence or absence (B) of 2-mercaptoethanol were heated at 100 deg C in the presence of 1 mM dithiothreitol, electrophoresed as described, and protein bands were stained with comassie blue.

A.

B.

the absence of disulfide bonds between MMTV proteins. The 165,000 mw polypeptide identified in one dimensional non-reduced samples was resolved as a dimer of the 85,000 contaminant. These findings indicate that principal MMTV-S polypeptides are not linked by disulfide bonds, suggesting that the characteristic B-type particle appearance does not require protein-protein disulfide linkages.

B. MMTV VIRION PROTEIN P44.

1. MMTV-ASSOCIATED POLYMERIZATION ACTIVITY.

Although MMTV virion proteins gp52, gp37.7-33, p24, p17, p14, and p8 have been demonstrated to be products of the MMTV genome, several other proteins are apparently not viral in origin. The 44,000 dalton virion protein, p44, was suggested to be cellular actin due to similar electrophoretic mobility to actin purified from rabbit muscle. Characterization of p44 was undertaken by assessing whether actin-like activities were demonstrable in MMTV particles using the polymerization reaction specific for actin. In this assay, conversion of G-actin monomers into F-actin polymers requires a minimal concentra-

tion of 0.1-0.2 mg actin protein/ml and occurs at 25 deg C following addition of the effector molecules ATP, K+, and Mg++ (Korn, 1978). Actin polymerization is then detected by monitoring increases in sample turbidity as a function of time. Addition of effector molecules to intact MMTV particles failed to initiate actin polymerization (Fig. 5). However, polymerization occurred when MMTV virions were first lysed in Triton X-100-containing disruption buffer prior to the addition of K+, Mg++, and ATP. Lysis of the MMTV particles was required for exposure of the G-actin-like species. Incubation of Triton X-100-disrupted MMTV with ATP alone also resulted in increases in the turbidity suggestive of actin crosslinking (Fig. 5). Similar ATP-dependent reactions have been observed in Dictylostelium extracts, which mediated crosslinking of actin-containing fibers (Hartwig and Stossel, 1976).

Separation of polypeptides in cleared extracts polymerized with K+, Mg++, and ATP by SDS-PAGE revealed that 93% of solubilized MMTV p44 was present in the precipitated complex (Fig. 6). Several other MMTV proteins were also detected and included an 85,000 mw protein, and approximately 6% of the solubilized internal core protein, p24 (Fig. 6). Their presence may be attributed to specific interaction with p44, or to nonspecific trapping during polymerization. Undisrupted

Fig. 5. Polymerization activity in MMTV. A. Isopycnically-purified MMTV was incubated in the presence or absence of 0.5 M KCl, 2 mM MgCl2, and 5 mM ATP, and polymerization monitored by nephelometry. Undisrupted virions, effector molecules added at time 0 (Δ). Virions disrupted in 0.5% Triton X-100, no additions (O). Detergent-disrupted MMTV, effector molecules added at time 0 (●). Incubation buffer without MMTV, effector molecules added at time 0 (□). b. MMTV was incubated in the presence or absence of 5 mM ATP, and turbidity increases were monitored spectrophotometrically against a buffer blank. Undisrupted virions, ATP added at time 0 (Δ). MMTV disrupted in 0.5% Triton X-100, no additions (O). Detergent-disrupted MMTV, ATP added at time 0 (●).

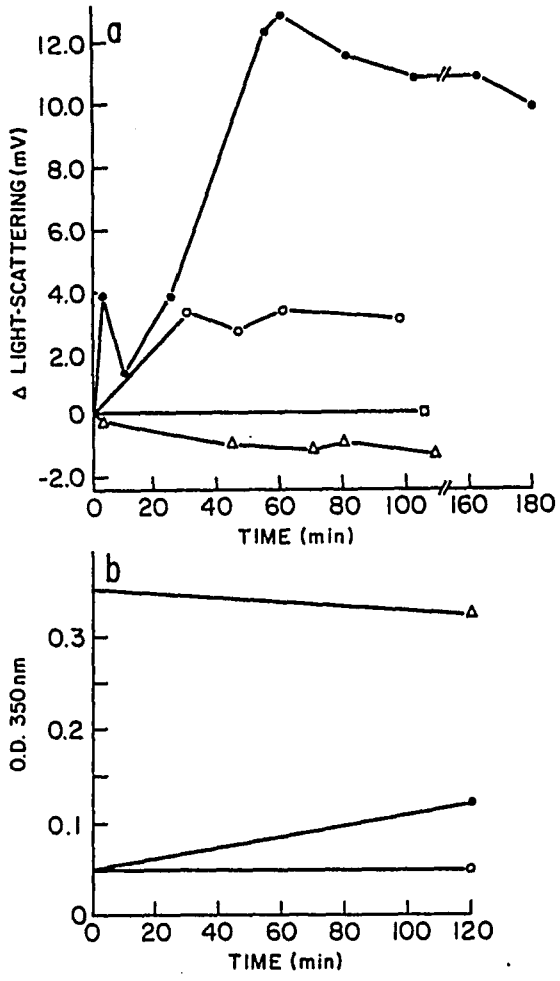
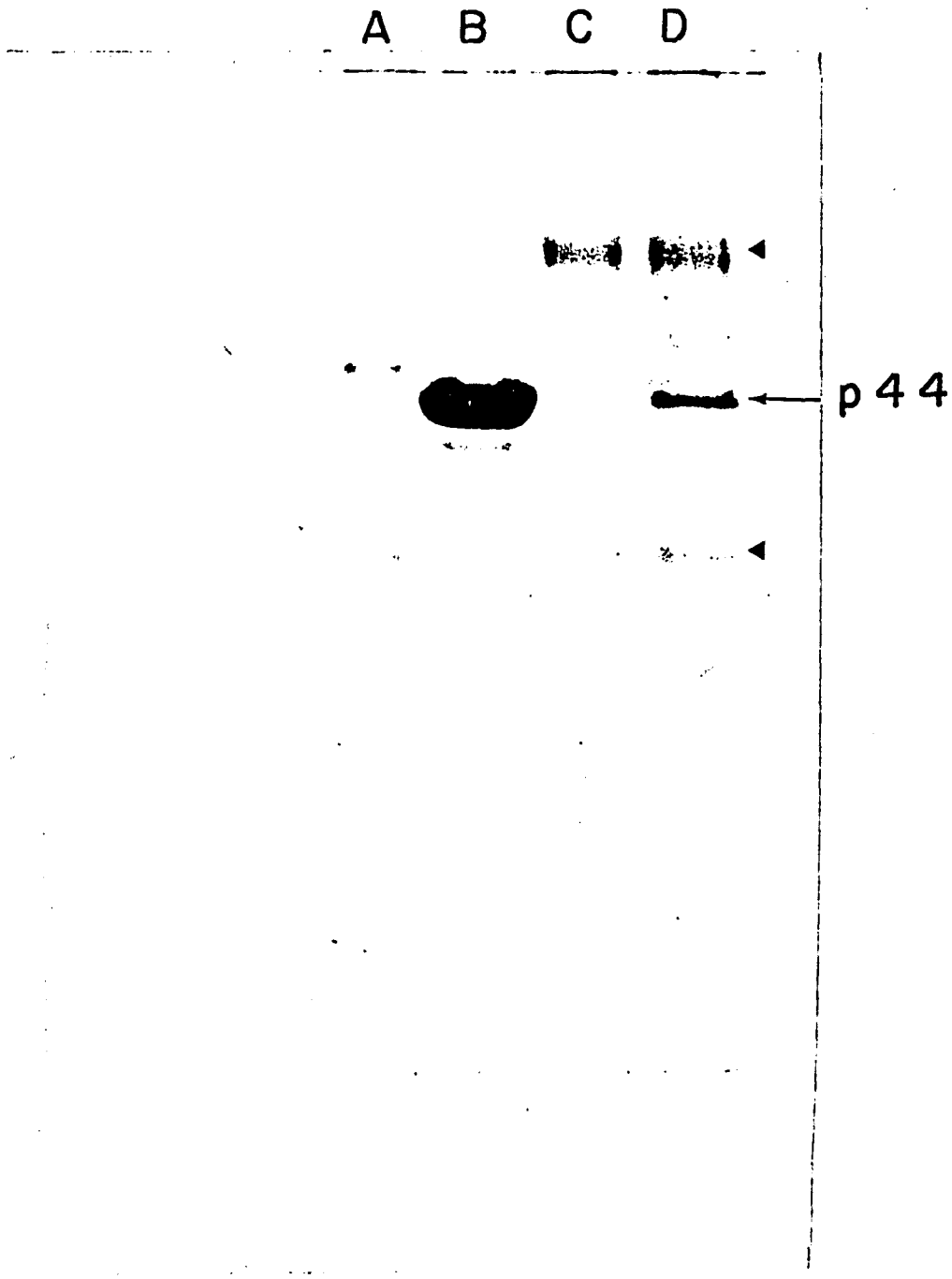

a
12.0
10.0
8.0
6.0
4.0
2.0
0
-2.0
Δ LIGHT-SCATTERING (mV)
0 20 40 60 80 100 160 180
TIME (min)
b
0.3
0.2
0.1
0
O.D. 350nm
0 20 40 60 80 100 120
TIME (min)

Fig. 6. Proteins in polymerized MMTV lysates.
Cleared lysates of isopycnically purified MMIV were polymerized with K+, Mg++, and ATP as described in Materials and Methods, and electrophoresed in SDS, 10-20% gradient polyacrylamide gels (A). Rabbit muscle skeletal actin (B) and MMTV (C) were electrophoresed for comparison. A sample of MMTV lysate containing < 0.1 mg actin/ml threshold for polymerization demonstrates presence of 85,000 mw contaminant and p24 (C).

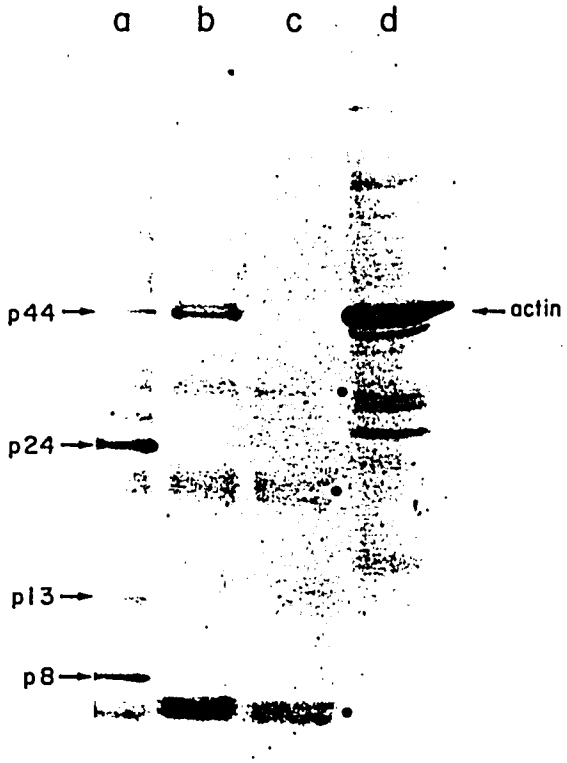

A
B
C
D
p44

cores remaining after preclearing may sediment with actin polymers and could also account for the low levels of p24. The 85,000 mw protein was also detected in the pellets when effector molecules were added to MMTV lysates containing a concentration of p44 below the threshold for polymerization (less than 0.1 mg/ml), suggesting that its presence may have been due to nonspecific salting out of this protein.

2. BINDING OF P44 TO DNASE I.

The identity of p44 was further assessed by examining if p44, like actin, possessed the ability to stochiometrically complex with DNase I (Lazarides and Lindberg, 1974). DNase I-Sepharose was incubated with either detergent-disrupted MMTV or purified dog skeletal muscle actin. Complexes were washed and bound polypeptides released by heating the mixtures in electrophoresis disruption buffer for 1 min. SDS-PAGE analysis revealed 3 proteins released from the DNase I reagent alone (Fig. 7). Analyses of the bound proteins in both the MMTV and skeletal muscle actin preparations demonstrated that virion p44 complexed to immobilized DNase I as did control actin protein (Fig. 7). Quantification of MMTV polypeptides by scanning densitometry revealed that approximately 100% of MMTV p44 was bound after 5-8 h incubation. MMTV internal core proteins were also bound to DNase I-Sepharose; elu-

Fig. 7. SDS-PAGE analysis of binding of MMTV p44 to DNase I. SDS-PAGE analysis of polypeptides bound to DNase I-Sepharose incubated with isopycnically purified, detergent-disrupted MMTV (A), dog actin (B), or incubation buffer (C). Bound polypeptides were eluted as described and electrophoresed on a 10-20% acrylamide gradient gel. A sample of purified actin prepared without incubation with DNase I was included (□) for molecular weight comparison. Periods denote polypeptides eluted from DNase I-Sepharose in the absence of protein addition.


a
b
c
d
p44
p24
p13
p8
actin

ates contained 74% of total virion p24, 59% of p8, and 40% of p13. In contrast, p85, gp52, and gp37.7-33 did not exhibit any affinity for the bound enzyme. Presence of core proteins may result from binding of intact cores to p44, with subsequent binding of the entire complex to DNase I via p44.

3. INCORPORATION OF ^{35}S-LABELED P44 IN MMTV.

As demonstrated in Section A-3, levels of virion-associated p44 were significantly lower than levels of principal MMTV proteins. To investigate whether low incorporation was due to a relatively small intracellular pool of p44, incorporation of radiolabeled p44 into MMTV was determined during and following 24 h ^{35}S-methionine labeling. Determination of molar levels of ^{35}S-labeled protein revealed levels of ^{35}S-p44 were 2.3-3.5 fold lower than other ^{35}S-labeled MMTV gag or env proteins in MMTV obtained during labeling period (Table 8). After a 48 h chase period, molar amounts of ^{35}S-labeled gag or env proteins decreased 37-65 fold as pools of cell-associated ^{35}S-labeled MMTV polypeptides were depleted. Molar levels of MMTV-associated ^{35}S-p44 also decreased after labeling period, but the magnitude of this decrease (6-7 fold) was less than that observed for MMTV gag or env proteins; no differences in relative levels of ^{35}S-p44 in MMTV harvested early (0-8 h) or late (40-48 h) during the chase period were observed. During

Table 8. Incorporation of ^{35}S-labeled polypeptides into MMTV

MMTV protein	Level of ^{35}S-MMTV polypeptide: Labeling period 0 - 24 h ^{35}S(dpm)[1]	(fmol)[2]	Chase periods: 0 - 8 h ^{35}S(dpm)	(fmol)	40 - 48 h ^{35}S(dpm)	(fmol)
gp52	16,928	6.90	1418	0.58	533	0.22
p44	7,090	2.89	1072	0.44	1147	0.47
gp37.7-33	22,222	9.10	1499	0.61	597	0.24
p24	31,545	12.90	1481	0.60	489	0.20

[1] MMTV was obtained from MJY-alpha cells labeled for 24 h with ^{35}S-methionine and polypeptide-associated radioactivity was determined after SDS-PAGE separation. Three replicate experiments were performed and typical trial is presented.

[2] Molar levels of MMTV polypeptides were determined using the formula:

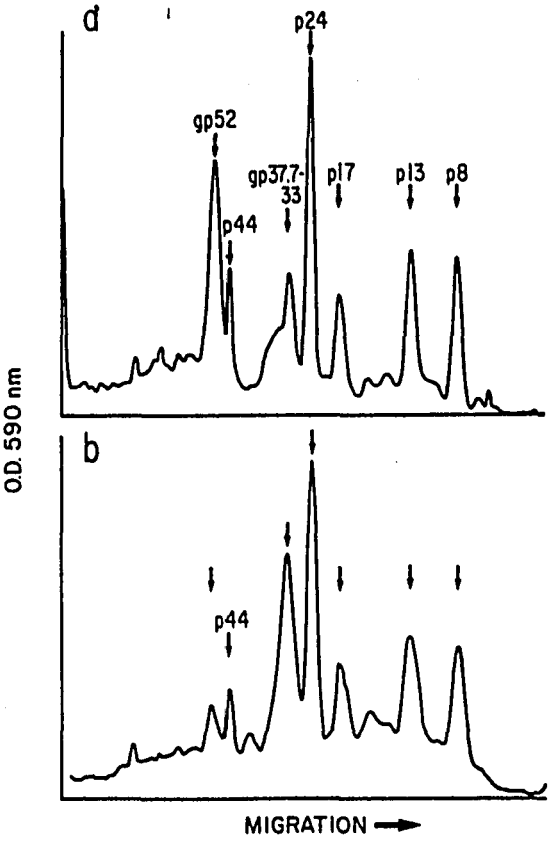
$$\frac{\text{(dpm incorporated into MMTV polypeptide)}}{\text{specific activity of } ^{35}S\text{-methionine}} \Big/ \text{no. of residues of labeling amino acid per polypeptide}$$

the final chase period (40-48 h), ^{35}S-p44 was the principal radiolabeled polypeptide. These data indicated a larger pool size existed for ^{35}S-p44 than for labeled MMTV env or gag proteins suggesting that synthesis and/or processing of p44 was independent of other virion proteins.

4. LOCATION OF P44 IN MMTV VIRIONS.

The inability of intact MMTV virions to participate in polymerization reactions suggested that the p44-associated actin-like activity was not exposed on the virion surface. To further investigate the internal location of p44, intact MMTV virions were subjected to limited trypsin digestion, followed by repurification and SDS-PAGE analysis. As previously reported (Yagi and Compans 1977), trypsin treatment decreased the levels of the external spike glycoprotein gp52 by 50% due to enzymatic cleavage (Fig. 8). The levels of nonglycosylated polypeptides not exposed on the virion surface, including the internal core protein, p24, were unaffected by trypsin treatment. Likewise, levels of p44 in MMTV were not decreased by trypsin treatment; 92% of p44 remained after proteolytic digestion (Fig. 8). Exposure of purified actin to trypsin under identical conditions resulted in complete degradation, with an absence of coomassie blue-staining material in the 44,000 mw region of the SDS-gel.

Fig. 8. Trypsin treatment of MMTV. Densitometry profiles of isopycnically purified MMTV treated with buffer or trypsin, repurified, and subjected to electrophoresis as described. A. control MMTV. B. trypsin-treated MMTV.


d
p24
gp52
gp37,7-
33
p17
p13
p8
p44
O.D. 590 nm
b
p44
MIGRATION

Prolonged trypsin exposure (2 h) resulted in disruption of MMTV virions and degradation of all proteins. Inaccessibility of p44 to proteolytic degradation indicated that p44, like nonglycosylated MMTV polypeptides, occupied an internal location in the virion particle.

Association of p44 with MMTV cores was also detected in detergent-disruption studies. Intact MMTV virions were exposed to Triton-X-100-containing disruption buffer and immediately centrifuged at 100,000 X g for 30 min; SDS-PAGE analyses of supernatants and pellets on 10-20% gradient polyacrylamide gels revealed lysis of viral outer membrane, with loss of gp52 and gp37.7-33 from pelleted, p24-containing cores. The majority of p44 remained core-associated (Table 9). Resuspension and incubation of the cores in disruption buffer for 72 h in the absence of K^+, Mg^{++}, or ATP to favor depolymerization of actin, followed by repelleting of the cores resulted in decreased levels of p44 compared to p24. These data suggest that p44 is bound to the MMTV core, and that only a portion may be dissociated in the presence of depolymerizing buffer.

Table 9. Presence of actin in MMTV cores.

Sample	percent of level of protein in intact virion[3]			
	gp52	p44	p24	ratio p44/p24
Intact MMTV	100	100	100	0.375
Disrupted virions:				
Pelleted core preparations[1]	0	67	100	0.249
Cores incubated with depolymerization buffer[2]	0	48	100	0.180

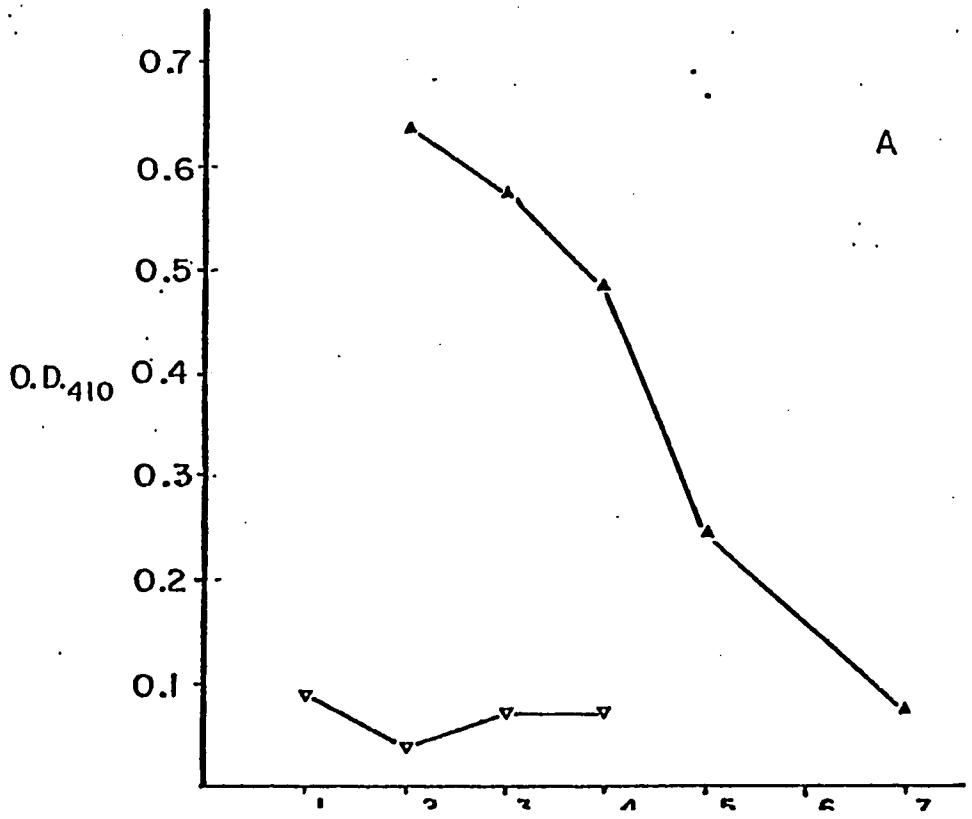
[1] Isopycnically purified MMTV was lysed in disruption buffer, and pelleted at 100,000 X g
[2] Pellets containing viral cores were disociated for 72 h as described and repelleted.
[3] Levels of gp52, p44, and p24 were determined by scanning densitometry.

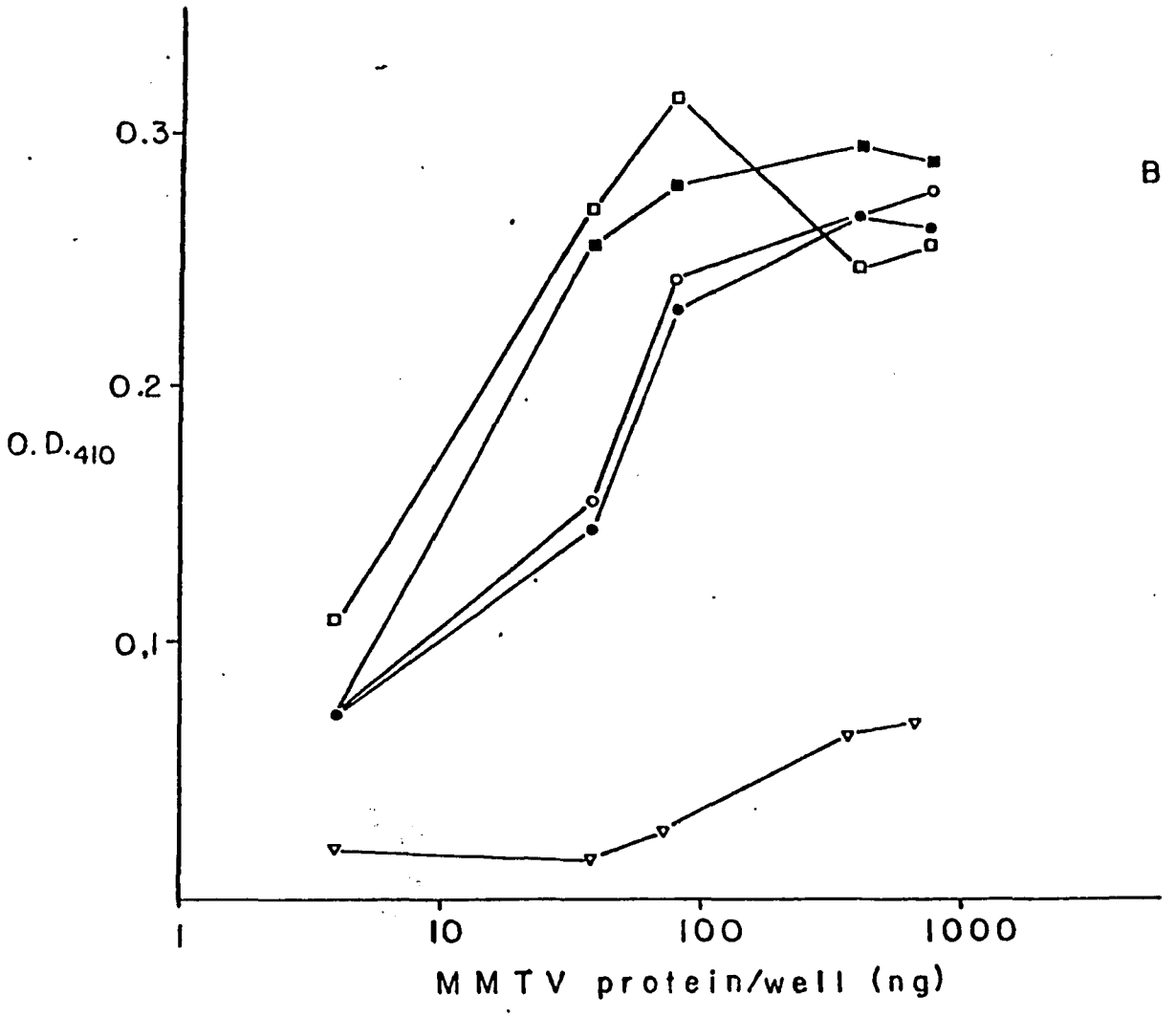
C. DETECTION OF MMTV PROTEINS IN MJY-ALPHA CELLS.

1. ELISA DETECTION OF MMTV PROTEINS.

Studies of MMTV replication required accurate assays to determine levels of MMTV antigens. The feasibility of a direct, enzyme-linked immunosorbent assay (ELISA) as a rapid, sensitive measure of MMTV polypeptides was investigated. As described in Materials and Methods, a reproducible one-step protocol was employed to bind test samples directly to ELISA plates for assay, eliminating requirements for a second specific "bridging" antibody. Dilutions of anti-MMTV antiserum (1:25-1:5000) detected MMTV antigens bound to ELISA plates as evidenced by increases in absorbance at 410 nm. Corresponding dilutions of pre-immune serum resulted in 10-fold lower A410 values, which constituted background absorbance (Fig. 9 A). At relatively high protein levels (500-1000 ng/well), no differences in absorbance yield were obtained with 1:5-1:100 anti-MMTV antisera; the presence of this plateau region suggested either rabbit anti-MMTV or goat anti-rabbit IgG (Fab fragment) was limiting reagent with an excess of MMTV protein. A minimum of 3-5 ng MMTV protein per well was required to obtain significant elevations in A410 using anti-

Fig. 9. Enzyme-linked assay of MMTV. A. Detection of MMTV antigens by ELISA. ELISA wells were sensitized with isopycnically purified, detergent-disrupted MMTV (3.8 µg MMTV protein/well). Wells were then incubated rabbit-anti-MMTV (▲) or preimmune rabbit sera (▽), and the ELISA completed as described. B. Sensitivity of ELISA. ELISA wells were sensitized with serial dilutions of MMTV proteins, and incubated with various dilutions of rabbit antisera: 1:5 (□), 1:10 (■), 1:50 (○), 1:100 (●), no antisera (▽).

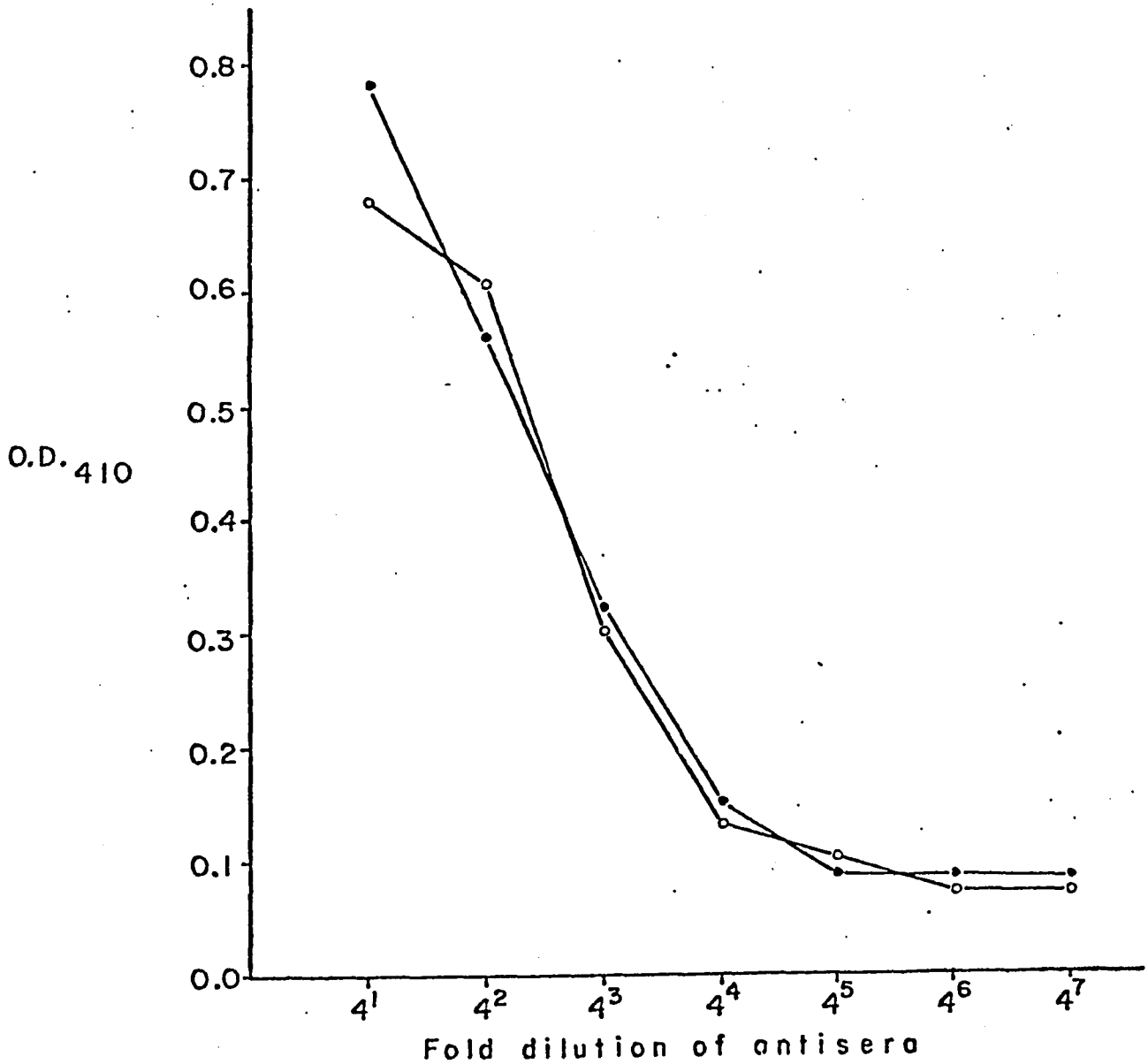

0.7
0.6
0.5
0.4
0.3
0.2
0.1
O.D.410
A


0.3
0.2
0.1
O. D.410
1
10
100
1000
MMTV protein/well (ng)
B

MMTV antiserum dilutions of 1:5-1:100 (Fig. 9 B); dilution of 1:50 anti-MMTV antiserum was employed for routine quantitation of MMTV polypeptides.

The ELISA procedure was applied to quantitate levels of MMTV in partially purified preparations of MMTV. A crude MMTV preparation was obtained from clarified, spent culture medium by centrifugation at 100,000 x g for 50 min. Viral pellets were resuspended and aliquots further purified on isopycnic gradients. As shown in Fig. 10, titration curves of serially diluted crude and purified MMTV were nearly identical, indicating use of partially purified virions did not result in interference or high background absorbance. Significant reactivity was not observed when preimmune sera was employed in place of anti-MMTV antiserum, suggesting contaminants in partially purified MMTV preparations did not contribute excess background. Routine use of ELISA to quantitate MMTV production in crude MMTV preparations using an independently generated isopycnically purified MMTV sample as a standard confirmed this demonstration. ELISA determinations of daily MMTV yields from confluent, HC-treated, MJY-alpha cells were compared to values obtained using scanning densitometry of coomassie blue-

Fig. 10. Detection of MMTV in partially purified MMTV preparations by ELISA. Aliquots of MMTV pelletted from culture supernatants or isopycnically purified by sucrose gradients (approximately 100 µg MMTV/ml) were serially diluted for sensitization of ELISA wells. Wells were then incubated with 1:50 dilution of anti-MMTV or 1:50 dilution of pre-immune sera, and bound antibody detected as described. Pelleted MMTV (●), anti-MMTV antisera; purified MMTV (○), anti-MMTV antisera;


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
O.D.410
4¹
4²
4³
4⁴
4⁵
4⁶
4⁷
Fold dilution of antisera

stained proteins of SDS-PAGE separations of purified MMTV. As shown in Table 10, comparable values were obtained with these methods.

2. RADIOLABELING INTRACELLULAR MMTV POLYPEPTIDES.

Metabolic radiolabeling of cellular proteins was accomplished by incubation of MJY-alpha cells in media containing protein or glycoprotein precursors. Proteins were labeled by incubation with 80-100 μCi ^{3}H-leucine or ^{35}S-methionine/ml medium for periods of 15 min-24 h. Short pulse labeling of MMTV glycoproteins by directly incubating cells with ^{14}C- or ^{3}H-glucosamine or ^{3}H-mannose was not possible due to the relatively large intracellular glycose pools, and to the low specific activity of the available radiolabeled monosaccharides. Labeling was accomplished by preincubating cells for 12-18 h in glucose-depleted medium, followed by addition of 100 μCi ^{3}H- or ^{14}C-labeled glycoses/ml depleted medium for 3-3.5 h. This modification resulted in a two-fold increase in uptake of ^{3}H radioactivity. Prolonged incubation in depleted media (>30 h) resulted in cell death; consequently long (24 h) labeling periods, similar to those used for protein labeling were not possible. Sulfated glycoproteins were labeled by incubation with $^{35}SO_4$; SO_4 uptake was proportional to the concentration

Table 10. MMTV production in MJY-alpha cells.

Method	μg MMTV/1X 10^8 cells
Scanning densitometry[1]	18.2
ELISA[2]	14.5

[1] MMTV was isopycnically purified from confluent, HC-treated MJY-alpha cells and quantitated by the SDS-PAGE scanning densitometry protocol as described.

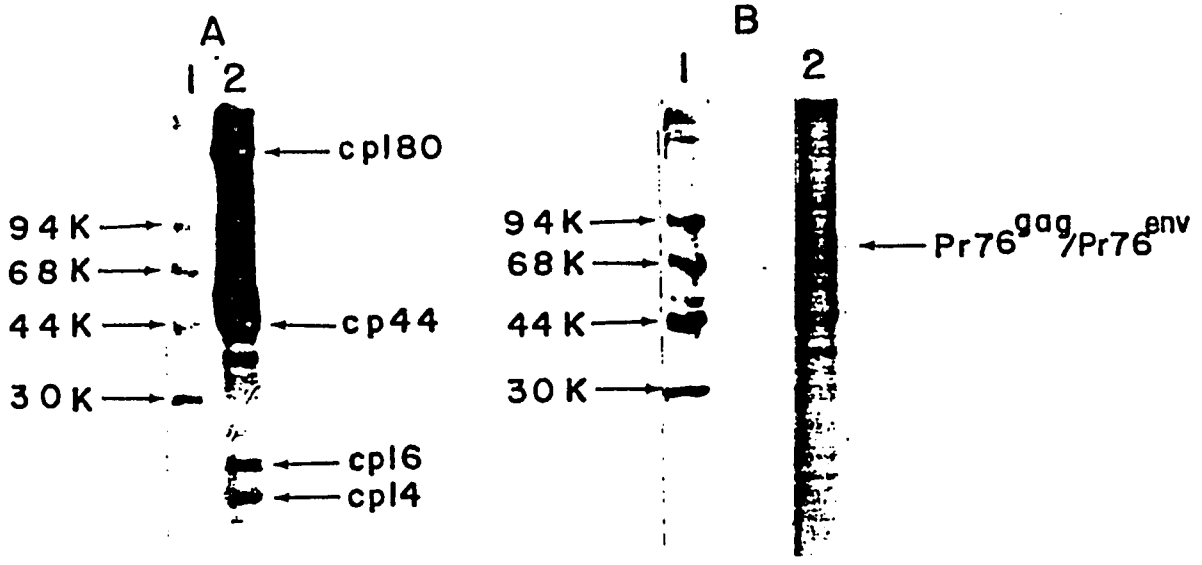
[2] MMTV was pelleted from clarified media of confluent, HC-stimulated MJY-alpha cells, and quantitated using ELISA as described. Samples were quantitated 4-10 times and a representative sample is presented.

of SO_4 in culture medium, precluding use of SO_4 depleted medium. Although uptake of $^{35}SO_4$ into MJY-alpha cells was low relative to other radiolabeled precursors, incubation of cells for 24-48 h with medium containing 10^{-5} M SO_4 and $^{35}SO_4$ at a final specific activity of 66 mCi-100 mCi ^{35}S/mMol SO_4 did result in measurable incorporation into immune precipitated MMTV proteins.

3. RADIOIMMUNOPRECIPITATION PROCEDURES.

Radioimmunoprecipitation procedures using anti-MMTV immune sera and the immunoabsorbent S. aureus were developed to detect MMTV proteins in MJY-alpha cells. To obtain MMTV proteins, MJY-alpha cells were first lysed in 0.1% Triton X-100 which disrupted plasma and cytoplasmic membranes. Intact nuclei were removed by centrifugation; the resulting cytosol-containing supernatant (denoted S1) was further cleared of radiolabeled contaminants by incubation with preimmune sera and the S. aureus immunosorbent. Nonspecifically bound ^{35}S-labeled polypeptides were eluted from S. aureus cells as described, and analyzed by 7.5-15% gradient acrylamide SDS-PAGE (Fig. 11); 8-10 polypeptides with mw >40,000, and 6-7 polypeptides with mw 14-40,000 were detected with all lots of rabbit preimmune sera tested. Two polypeptides, with mw of 180,000 and 44,000 were also identified by SDS-PAGE after incubation of labeled cell lysates with S. aureus immunosorbent without

Fig. 11. Autoradioagram of SDS-PAGE of precipitations
A.Preimmune precipitation. MJY-alpha cells were labeled with ^{35}S-methionine, lysed, incubated with rabbit preimmune serum and S. aureus, then pelleted as described. Solubilized pellets (2) were subjected to SDS-PAGE on 7.5-15% gradient gels adjacent to molecular weight markers (1) and autoradiographed.
B. Immune precipitation. Cell supernatants cleared of debris were immune precipitated by incubating with anti-MMTV and S. aureus. Immune complexes were washed, solubilized, and subjected to SDS-PAGE and autoradiography (4) adjacent to molecular weight markers (3).


A
1
2
cp180
94K
68K
44K
cp44
30K
cp16
cp14
B
1
2
94K
68K
44K
30K
Pr76gag/Pr76env

antisera, suqqestinq these polypeptides contaminated immune precipitations by binding directly to the immunosorbent. Preabsorption was incomplete, as SDS-PAGE analysis of MMTV polypeptides isolated from cleared supernatants usinq specific anti-MMTV antisera and S. aureus cells revealed variable amounts of persisting contaminants of 180,000 daltons, 44,000 daltons, 16,000 daltons, and 14,000 daltons (Fiq. 11). Contaminants were present reqardless of specificity (gp52, gp37.7-33, p24), or deqree of dilution (1:3-1:30) of rabbit antisera employed, suqqestinq nonspecific binding was responsible for their presence. Althouqh these contaminants did not prevent specific precipitation of MMTV proteins, the 180,000, 16,000, and 14,000 dalton proteins did obscure detection of MMTV proteins which exhibit similar electrophoretic miqration. Addition of ionic detergents (0.05% SDS) or chaotropic cholate derivatives (0.5% deoxycholate, 0.5% CHAPS) throughout preclearing and immune precipitation steps did not remove these proteins; use of S. aureus cells preincubated with unlabeled cell lysates, as suggested by Racevskis and Sarkar (1978), was also ineffective in preventinq contamination by these proteins in immune precipitates. Acetone extraction of immune precipitates prior to electrophoresis to remove bound, lipid-soluble

contaminants, also did not reduce nonspecific background. Although it was possible to reduce levels of cp-180 and cp-44 contaminants by centrifuging S1 supernatants at 100,000 x g for 30 min prior to immune precipitation, high speed centrifugation also reduced levels of MMTV polypeptides, and this step was not incorporated into the immune precipitation protocol.

Since radioimmunoprecipitation was to be employed to study MMTV precursors and their cleavage, the stability of MMTV precursors was investigated. Lysates of pulse-labeled confluent, HC-stimulated MJY-alpha cells were immune precipitated immediately after lysis, or after incubation at 22 deg C for 3 h. Quantitation of MMTV precursor-associated ^{35}S identified by SDS-PAGE revealed that 95% of precursor-associated radioactivity remained, indicating that incubation at RT did not significantly affect precursor breakdown.

4. MMTV POLYPEPTIDES IMMUNE PRECIPITATED FROM MJY-ALPHA CELLS.

MMTV is composed of 7 polypeptides derived from high molecular weight polypeptide precursor products of the env and gag genes (Dickson and Atterwill, 1978, 1980). Studies were carried out to identify these precursors and their processing sequences in MJY-alpha cells. MMTV polypeptides in lysates of ^{35}S-methionine- or ^{3}H-leucine-labeled MJY-alpha cells were separated by immune precipitation

using anti-MMTV antisera. SDS-PAGE analysis of immunoprecipitates identified several precursors and virion polypeptides transcribed from the MMTV gag gene. P24, the core protein, was the only virion polypeptide of gag origin that was identified (Fig. 12). The other gag components, p17, p14, and p8 were not typically detected by anti-MMTV antisera due to interference from the cellular contaminants, cp-16, cp-14. Several polypeptide precursors and intermediates of mature gag gene products, were also detected using anti-MMTV antisera, including $Pr110^{gag}$ (110,000 mw), $Pr95^{gag}$ (95,000 mw), and $Pr38^{gag}$. (38,000 mw, Fig. 12). Previously reported gag intermediates with mw 68,000-61,000 were not clearly resolved. A 76,000 mw polypeptide was also detected, but it was not possible to determine whether this polypeptide was $Pr76^{gag}$ or $Pr76^{env}$. Altering the gradient of the polycrylamide gel to 5-12% and carrying out electrophoresis for longer periods did resolve these two species (Fig. 13), although anti-MMTV antisera could not distinguish between the peaks for the env and gag precursors. In addition, the $Pr68,000\text{-}61,000^{gag}$ intermediates were more clearly resolved in the 5-12.5% gradient gels. The number and electrophoretic mobility of MMTV gag precursors in the MJY-alpha cells were consistent with previous findings, although the relative amounts of ^{35}S-labeled $Pr110^{gag}$ and $Pr95^{gag}$ were low

Fig. 12. Cell-associated MMTV polypeptides. Lysates of confluent HC-stimulated MJY-alpha cells labeled for 4 h with ^{35}S-methionine were immune precipitated with anti-MMTV antisera as described; precipitates were subjected to SDS-PAGE on 7.5-15% gradient polyacrylamide gels and radioactivity determined as described.

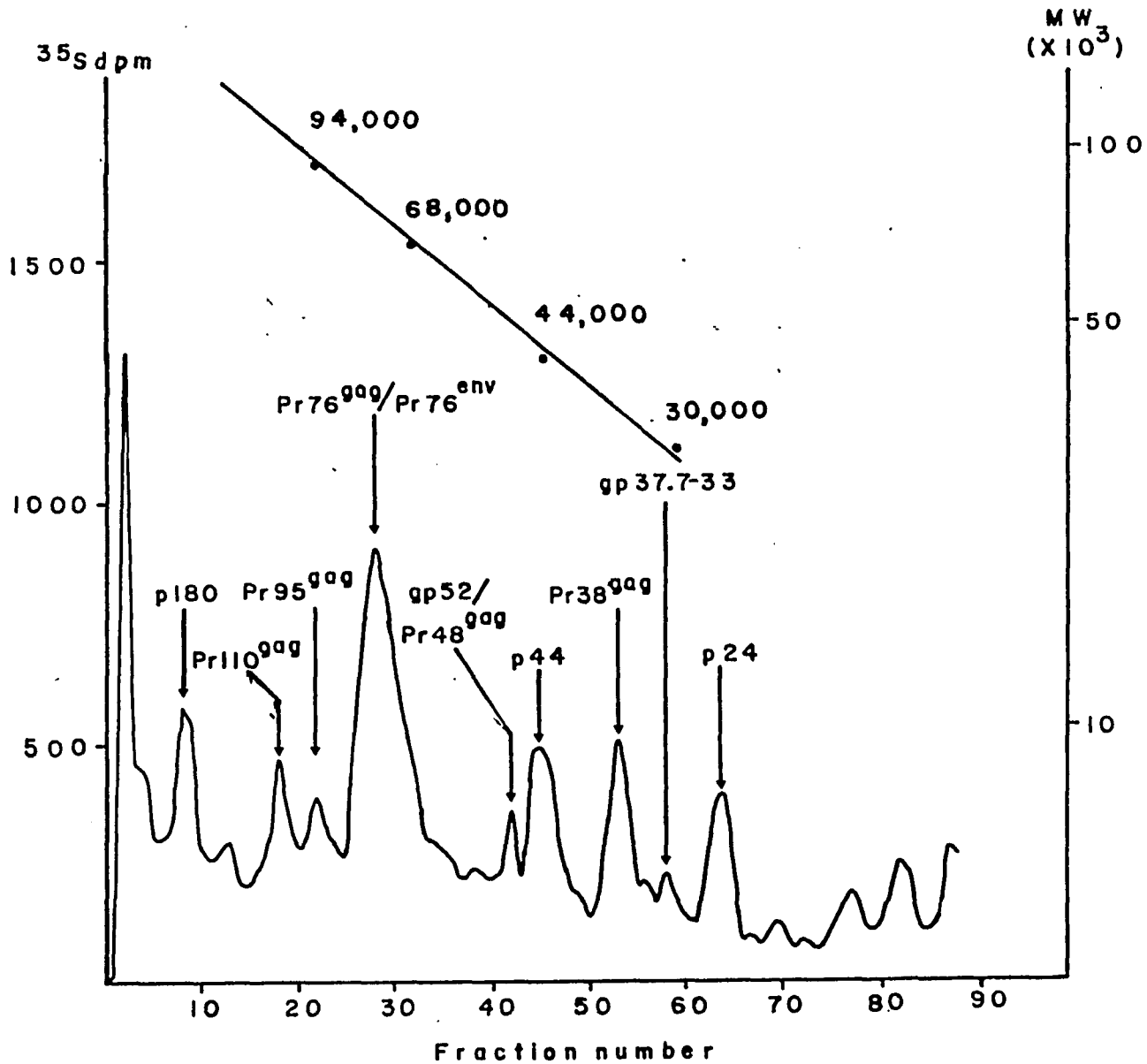
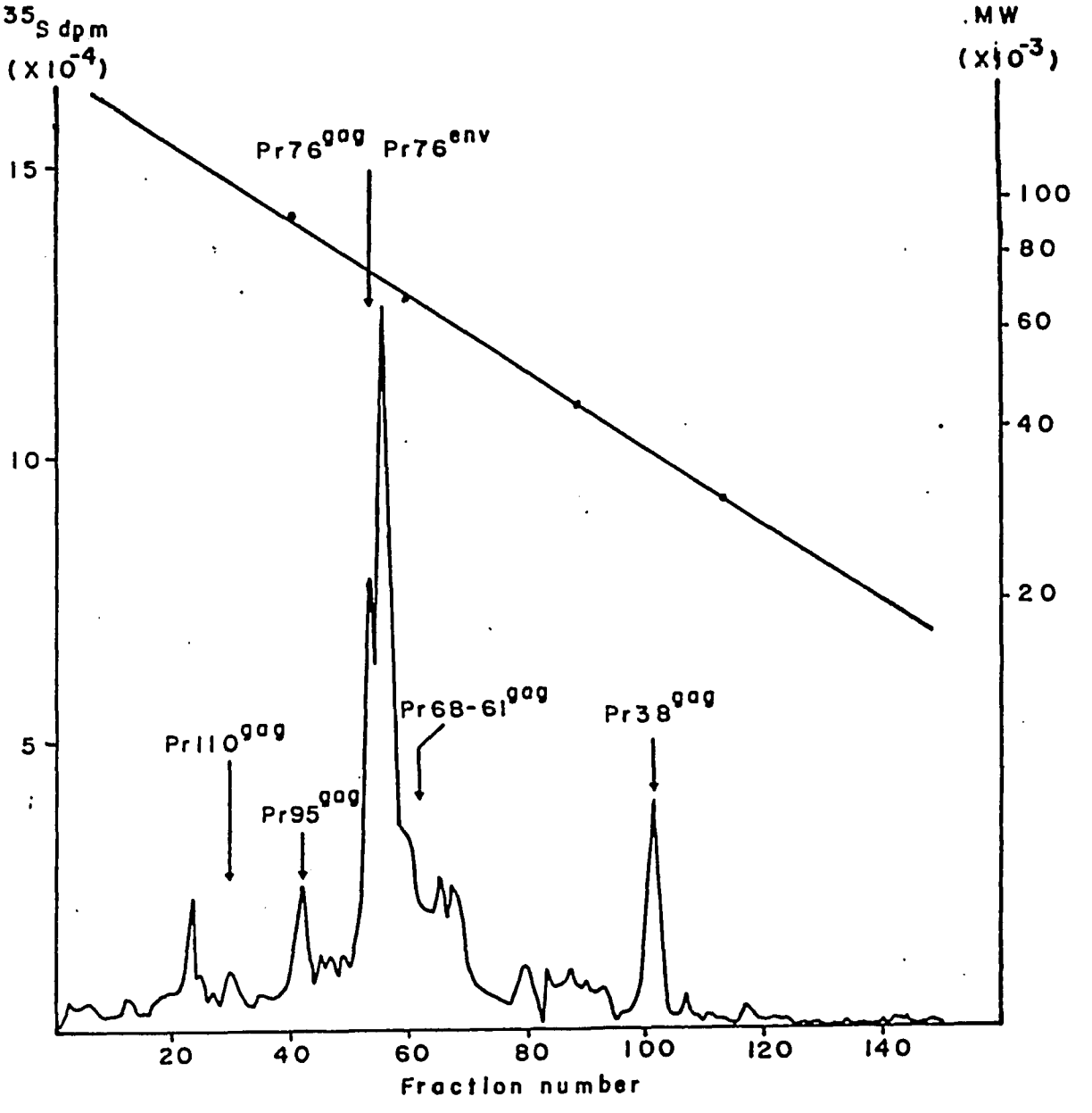

35S dpm
MW (X10^3)
94,000
68,000
44,000
30,000
1500
1000
500
100
50
10
p180
Pr110gag
Pr95gag
Pr76gag/Pr76env
gp52/
Pr48gag
p44
Pr38gag
gp37.7-33
p24
10
20
30
40
50
60
70
80
90
Fraction number

Fig. 13. Separation of $Pr76^{gag}$ and $Pr76^{env}$. Lysates of MJY-alpha cells pulse-labeled with 100 μCi ^{35}S-methionine/ml medium for 15 min were immune precipitated with anti-MMTV antisera, subjected to SDS-PAGE on 5-12.5% qradient polyacrylamide gels and radioactivity determined as described.

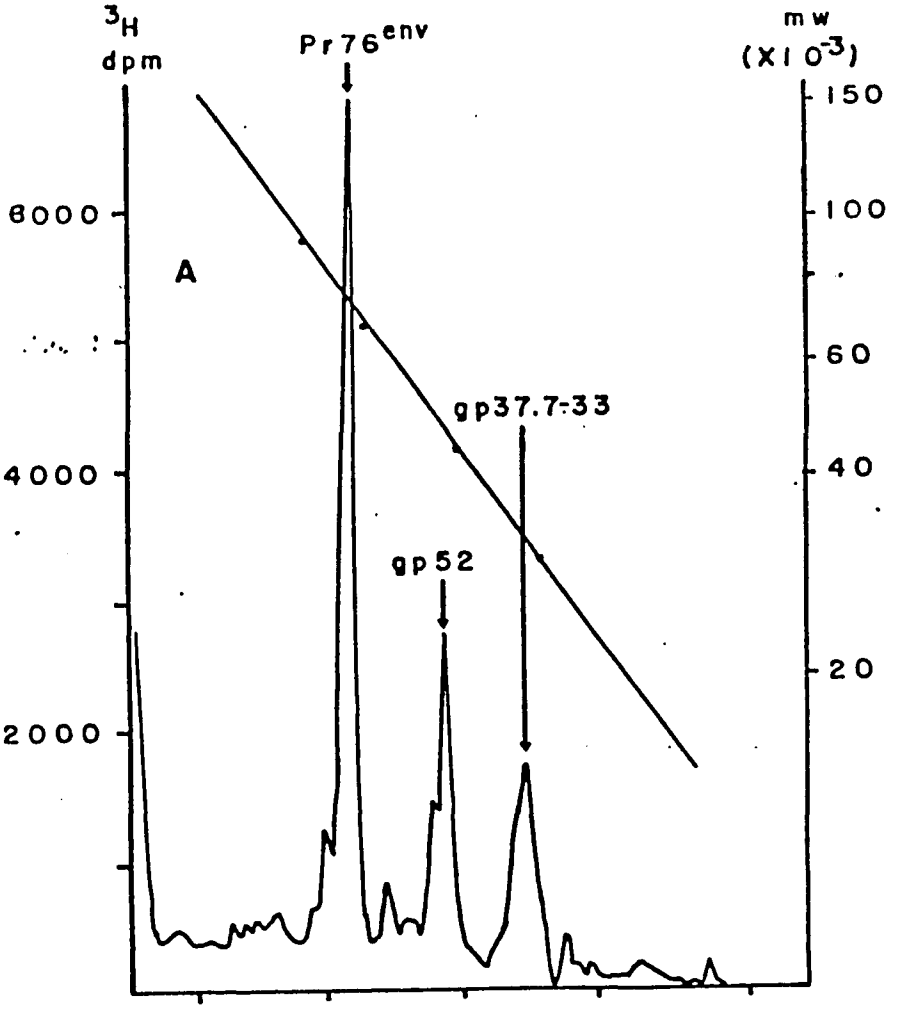

35S dpm
(X10-4)
MW
(X10-3)
15
10
5
100
80
60
40
20
Pr76gag
Pr76env
Pr110gag
Pr95gag
Pr68-61gag
Pr38gag
20
40
60
80
100
120
140
Fraction number

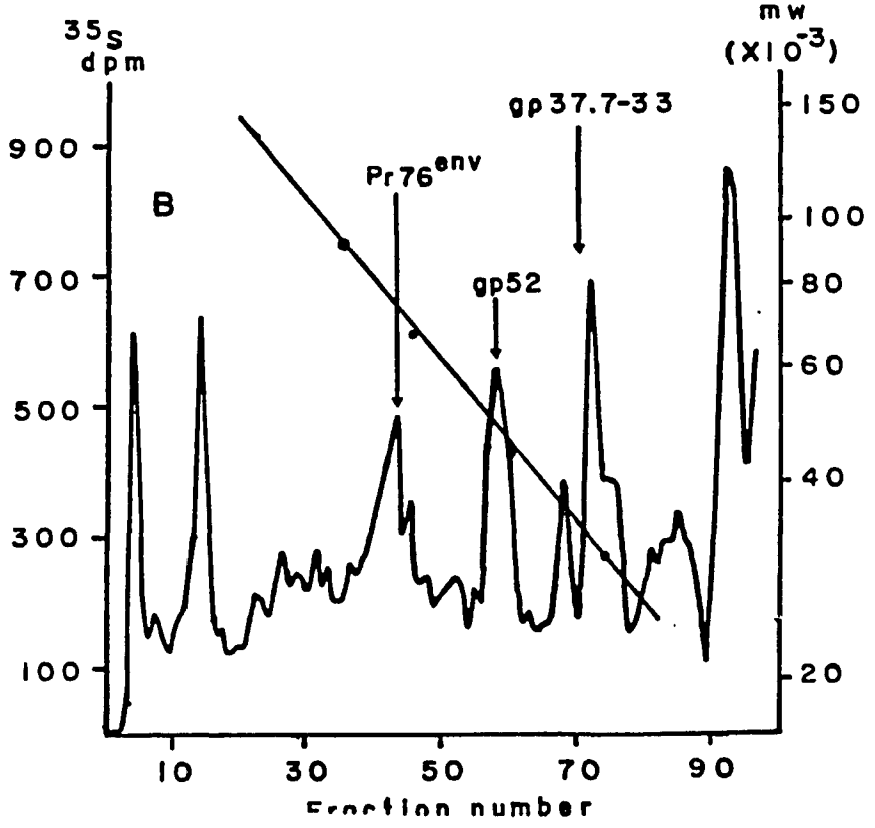
compared to that obtained by Dickson (1979). $Pr160^{gag/pol}$, reported to be synthesized at low levels (Dickson and Atterwill, 1979) was inconsistently present, and not usually resolved from the cp-180 contaminant.

Antisera elicited to detergent-disrupted MMTV particles also detected major virion polypeptide products of the MMTV env gene. The two glycoproteins, ^{35}S-gp52, and ^{35}S-gp37,7-33 were identified (Fig. 12). As previously described (Dickson and Atterwill, 1980), large amount of immunoglobulin heavy chain (mw 52,000) altered the electrophoretic mobility of immune precipitated gp52, which subsequently comigrated with a minor gag intermediate Pr48 ; electrophoresis on 5-12% gradient polyacrylamide gels did not resolve these species (Fig. 13). Immune precipitation studies using anti-p24 and anti-gp52 both identified a 48,000 mw component (see below, Fig. 15), indicating both proteins are found in MJY-alpha cells, and that the 48,000 mw peak obtained with anti-MMTV sera contains gp52 and $Pr48^{gag}$. Immune precipitation detected the principal MMTV env precursor, $Pr76^{env}$ (Fig. 12). However, MMTV protein $Pr79^{env}$, a cell-associated glycoprotein reported to be an intermediate in env precursor processing, and gp60, a virion-associated glycoprotein of uncertain origin, were not readily detected. The relative levels of these polypeptides were possibly too low to detect, and/or these polypeptides were obscured on the gels by the more abundant ^{35}S-labeled species.

Since env proteins gp52 and gp37.7-33 of the virion are highly glycosylated, experiments were carried out to identify the intracellular glycosylated polypeptides using radiolabeled carbohydrates. MJY-alpha cells were labeled with ^{3}H-glucosamine or ^{3}H-mannose for 3.5-4 h, lysed, and MMTV components immune precipitated with anti-MMTV antisera. SDS-PAGE (Fig. 14) revealed $Pr76^{env}$ was the principal labeled species, accounting for 40% of the total MMTV-associated ^{3}H radioactivity. In addition, $Pr79^{env}$ which was not clearly identified following ^{35}S-methionine labeling, was consistently detected after ^{3}H-glucosamine labeling and contained 15-20% of total ^{3}H-radioactivity Previous studies with other MMTV-producing cell lines suggested intracellular $Pr79^{env}$ and gp37.7-33 did not contain mannose (Sarkar and Racevskis, 1983), although carbohydrate analysis determined virion-associated gp37.7-33 from MJY-alpha cells did contain this moiety (Yagi, M. et al., 1978c). SDS-PAGE analysis of immune precipitates of ^{3}H-mannose labeled MJY-alpha cells resulted in ^{3}H-incorporation into MMTV glycoproteins (see below, section D. 3., Fig. 26). It is not known whether previous findings resulted from alternative processing of env glycoproteins in different cell lines, or reflect different sensitivities of the immune precipitation assay. A ^{3}H-labeled 60,000 mw glycoprotein was detected by immunoprecipitation with anti-MMTV antisera after labeling

Fig. 14. MMTV glycoproteins in MJY-alpha cells. Confluent, MJY-alpha cells were labeled with ^{3}H-glucosamine, or $^{35}SO_4$, and immune precipitated with anti-MMTV antisera as described. Immune precipitates were subjected to SDS-PAGE on 7.5-15% gradient polyacrylamide gels, and radioactivity determined as described. Samples presented here were electrophoresed on different slab gels, accounting for variation in polypeptide mobility. A. ^{3}H-glucosamine labeling, 3.5 h. B. B. $^{35}SO_4$ labeling, 48 h.


^{3}H dpm
Pr76^{env}
mw (X10^{-3})
8000
4000
2000
150
100
60
40
20
A
gp37.7-33
gp52


^{35}S dpm
mw (X10^{-3})
900
700
500
300
100
150
100
80
60
40
20
B
gp37.7-33
Pr76^{env}
gp52
10
30
50
70
90
Fraction number

with ^{3}H-glucosamine, but not ^{3}H-mannose (Fig. 14) This glycoprotein may correspond to gp60.

Previous reports documented that virion-associated gp52 was a sulfated glycoprotein, whereas, gp37.7-33 did not contain sulfated glycose residues (Yagi and Compans, 1977). To determine whether intracellular sulfated env proteins occurred, MJY-alpha cells were labeled with inorganic $^{35}SO_4$ as described, and immune precipitated with anti-MMTV antisera. SDS-PAGE analyses (Fig. 14B) revealed MMTV precursors $Pr76^{env}$, as well as, intracellular gp52 incorporated $^{35}SO_4$ Radioactivity was detected throughout the gp37.7-33 mw region, suggesting all forms of this heterogeneous protein were sulfated. Because of prolonged $^{35}SO_4$ labeling periods (36-48 h) required to obtain sufficient radiolabeled material for immune precipitation, relatively large amounts of cell contaminants cp-180, cp-16, cp-14 were also detected (Fig. 14B). Previous reports demonstrated extracellular MMTV particles purified from $^{35}SO_4$-labeled MJY-alpha cells contained ^{35}S-labeled gp60, gp52, and an 85,000 mw component (Yagi and Compans, 1977), but no ^{35}S- radioactivity was detected in virion gp37.7-33. Lack of gp37.7-33-associated ^{35}S radioactivity in MMTV purified from $^{35}SO_4$-labeled MJY-alpha cells was confirmed in a repetition of this experiment. It is possi-

ble gp37.7-33-associated $^{35}SO_4$ was removed during processing steps prior to its incorporation into virions.

To selectively identify MMTV gag or env proteins in MJY-alpha cell lysates, antisera elicited against specific MMTV proteins were employed in immune precipitation studies. Anti-p24 antisera detected principal gag precursors, $Pr76^{gag}$, $Pr48^{gag}$, and $Pr38^{gag}$, as well as, mature p24 (Fig. 15). Both anti-gp52 and anti-gp37.7-33 identified principal MMTV precursor $Pr76^{env}$; neither antisera identified ^{35}S-$Pr79^{env}$ or ^{35}S-gp60 from lysates of ^{35}S-methionine-labeled MJY-alpha cells, similar to results obtained using anti-MMTV antisera. It is possible these polypeptides were not present in sufficient levels for consistent detection by immune precipitation. Although gp52 and gp37.7-33 isolated from virus particles were reported to be immunologically distinct (Yagi and Compans, 1977), anti-$gp37$ precipitated a 52,000 mw protein (Fig. 16). It is possible anti-$gp37$ recognizes common antigenic determinants on carbohydrate moieties of gp52 and gp37.7-33. No gag proteins were identified using anti-gp52 or anti-gp37.7-33 antisera, and no env proteins were present in immune precipitates using anti-p24 antisera, demonstrating that common antigenic determinants were not detected between intracellular MMTV env and gag polypeptides.

6. QUANTITATION OF MMTV PROTEINS.

Fig. 15. Discrimination of MMTV env and gag precursors. Lysates of ^{35}S-methionine pulse labeled MJY-alpha cells were immune precipitated with anti-p24 (C), anti-gp52 (B), or anti-MMTV (A), subjected to SDS-PAGE on 7.5-15% gradient polyacrylamide gels and autoradiographed as described.

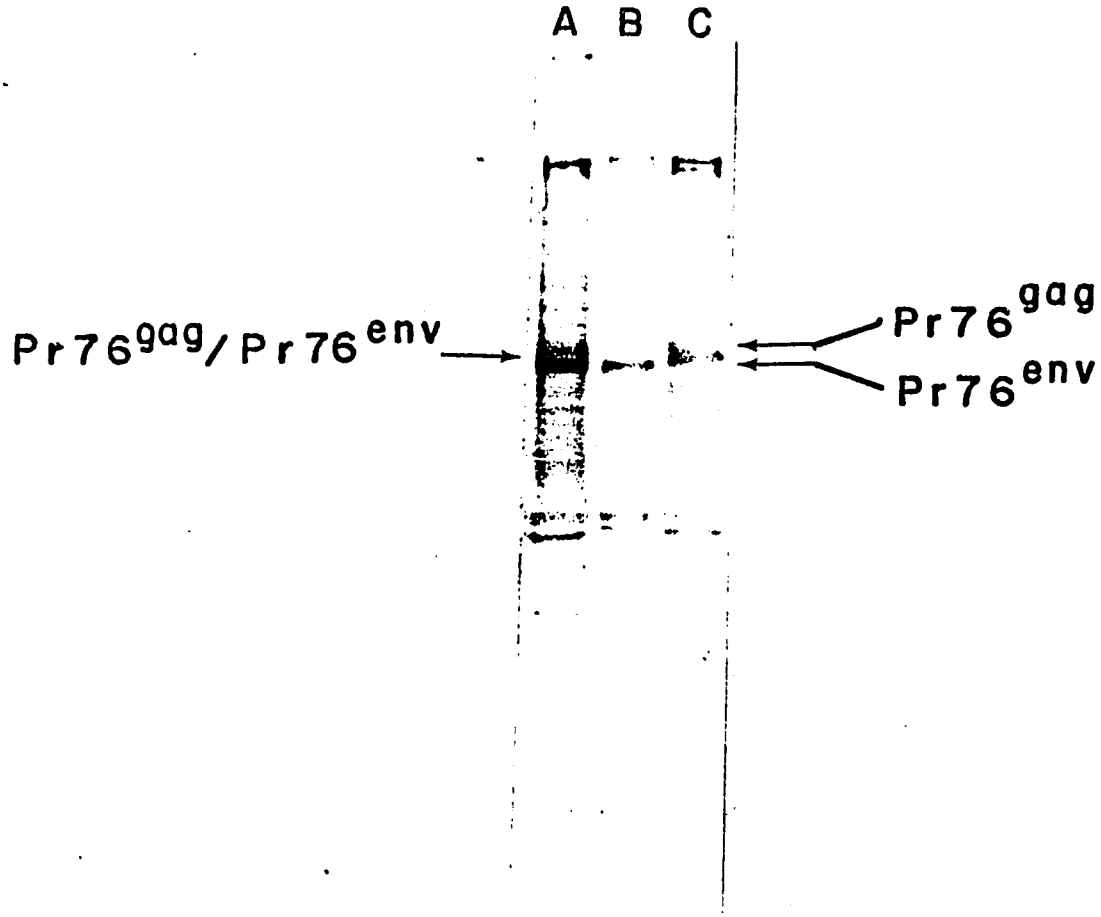
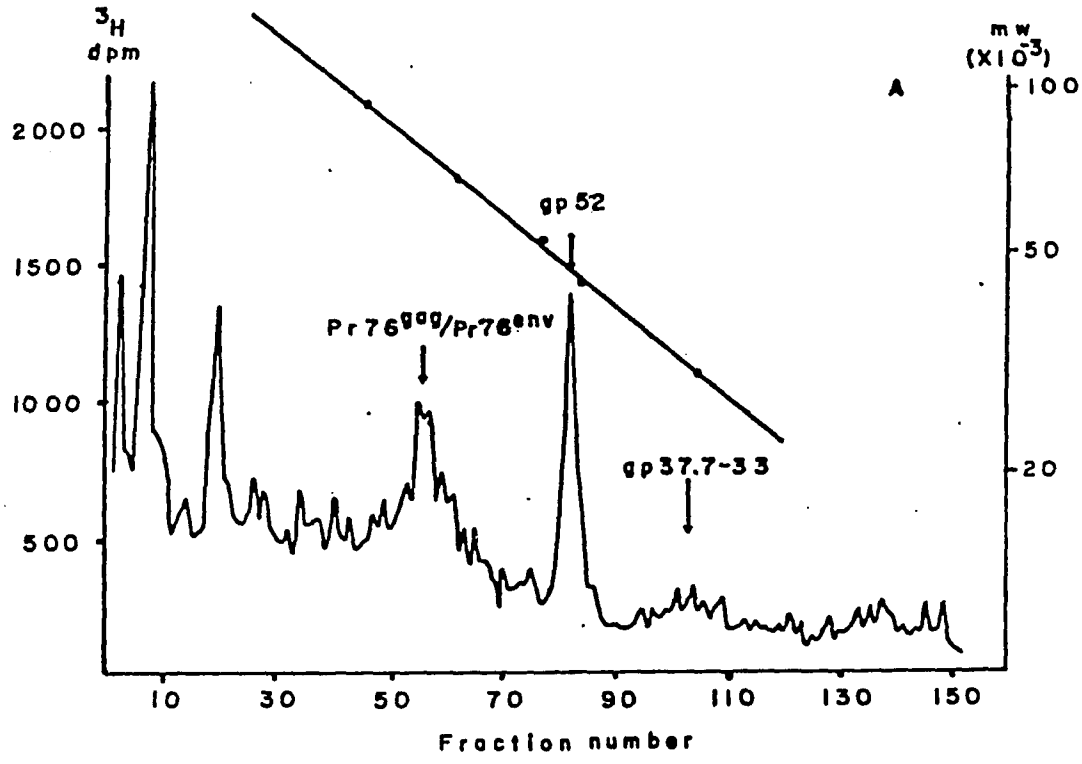
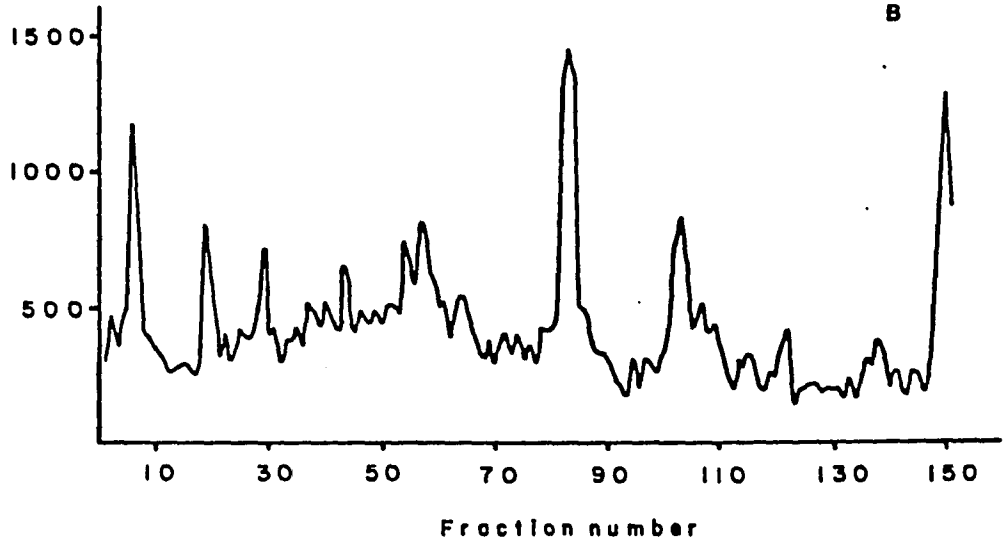

A B C
Pr76gag/Pr76env
Pr76gag
Pr76env

Fig. 16. MMTV env proteins in MJY-alpha cells. Lysates of MJY-alpha cells labeled 20 h with ^{3}H-leucine (80 µCi/ml medium) were immune precipitated with anti-gp52 antisera (A) or anti-gp37.7 antisera (B), subjected to SDS-PAGE on 5-12.5% gradient polyacrylamide gels and radioactivity determined as described.


^{3}H
dpm
mw
(X10^3)
A
2000
1500
1000
500
100
50
20
gp52
Pr76^{gag}/Pr76^{env}
gp37.7-33
10
30
50
70
90
110
130
150
Fraction number


B
1500
1000
500
10
30
50
70
90
110
130
150
Fraction number

Although MMTV antigens have been detected in lysates of infected cells, the relative levels of cell-associated MMTV polypeptides and MMTV particles released from the cells have not been compared. Development of ELISA and immune precipitation techniques permitted quantitation of cell-associated MMTV in MJY-alpha cells. Femtomolar/nanomolar levels of radiolabeled amino acid incorporation into TCA-precipitable material and MMTV proteins were achieved by incubating cells with 100 μCi ^{35}S-methionine and 80 μCi ^{3}H-leucine/ml medium for 18-24 h (Table 11). ^{35}S incorporation was lower than ^{3}H, and the magnitude of this reduction was greater for MMTV proteins (25-fold less than ^{3}H) than for the ^{35}S-TCA precipitated (4-fold less than ^{3}H, Table 11). Relative differences are partially attributable to lower methionine content of MMTV proteins compared with total cellular proteins. Prior amino acid analyses (Yagi, et al., 1978c) demonstrated that the methionine content of MMTV proteins was 6-fold lower than that of leucine. As shown in Table 11, incorporation of ^{35}S or ^{3}H into MMTV proteins accounted for less than 1% of corresponding total cell-incorporated radioactivity. The level of cell-associated MMTV protein was calculated assuming the fraction of total TCA-precipitable radioactivity associated with MMTV proteins was equivalent to the fraction of the total cell protein associated with MMTV. Determination of the total

Table 11. Levels of cell-associated MMTV proteins in MJY-alpha cells.

	Incorporation[1]				
			TCA-precipitable		
Label	MMTV radioactivity[2]		radioactivity[3]		μg protein/10^8 cells
	dpm (x 10^5)	fmol	dpm (x 10^5)	fmol	
^3H	2.37	2.05	250	216.0	95
^{35}S	1.79	0.08	760	34.2	24

[1] HC-stimulated MJY-alpha cells were uniformly labeled by incubating with ^{35}S-methionine (100 μCi/ml medium) and ^3H-leucine (80 μCi/ml medium) as described. Cells were lysed, and clarified cytosolic supernatants were prepared for immune precipitation. Experiment was performed 2-3 x using incubations of 15 min-24 h, and a representative sample from 21 h time period is presented.
[2] Determined by immune precipitation of labeled MJY-alpha cells with anti-gp52 and anti-p24. Data reported for entire 100 mm petri dish.
[3] Clarified supernatants were TCA-precipitated, and radioactivity determined as described in Materials and Methods.
[4] MJY-alpha cells were enumerated by hemocytometer, and protein content was determined by Lowry assay as described in Materials and Methods. MMTV content was determined using formula:
MMTV protein in 1 X 10^8 cells =

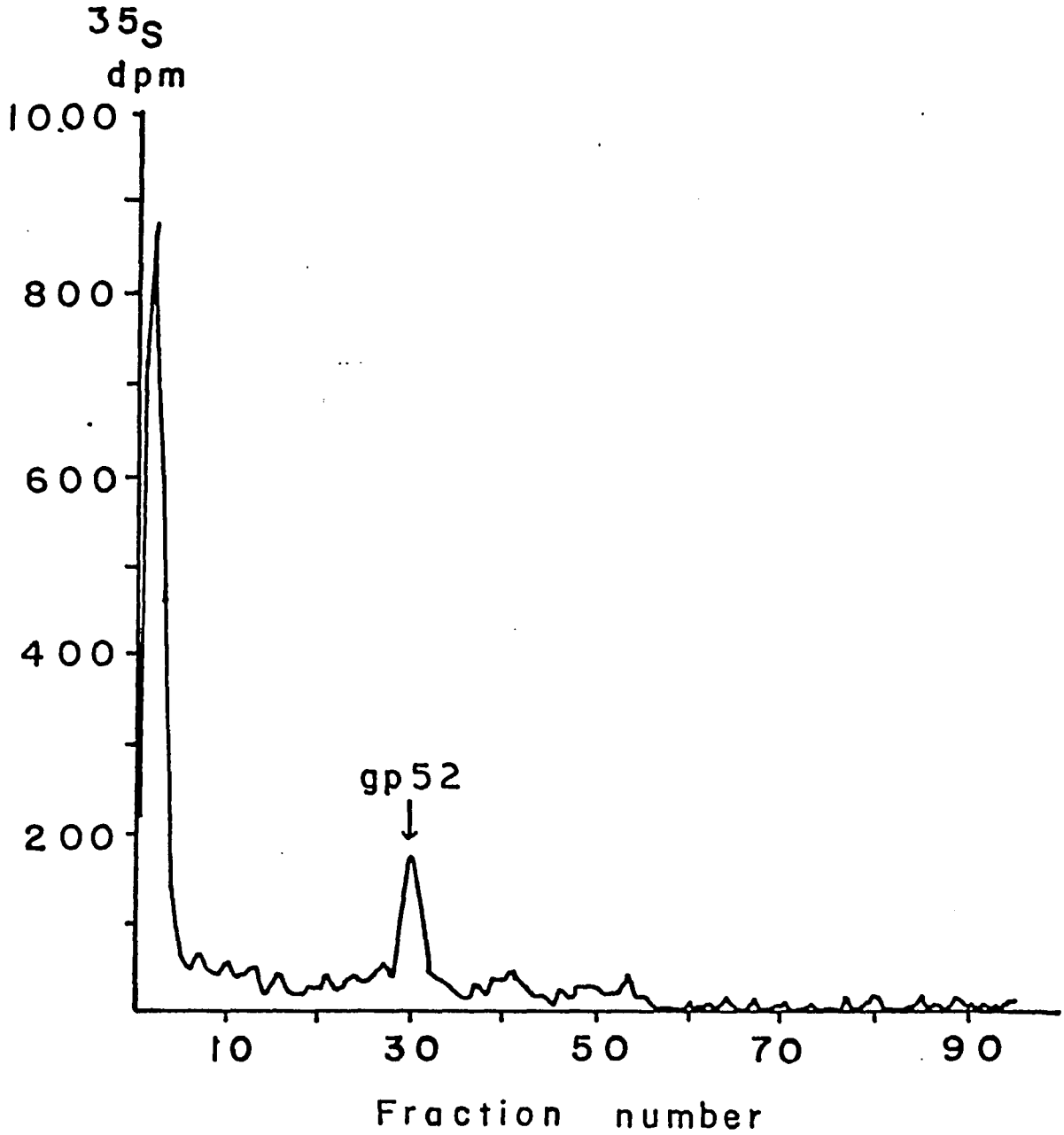
$$\frac{\text{MMTV-associated radioactivity (dpm)}}{\text{TCA-precipitable radioactivity (dpm)}} \times \text{protein content of } 1 \times 10^8 \text{ MJY-alpha cells}$$

cell protein by Lowry assay revealed MJY-alpha cells contained 1 x 10^{-10} μg protein/cell. Consequently, the level of viral protein in MJY-alpha cells was 2.0-9.5 x 10^{-13} μg MMTV protein/cell or 20-95 μg MMTV protein/1 x 10^8 cells when calculated using either ^3H or ^{35}S radioactivity.

The utility of a one-step ELISA assay to determine levels of MMTV polypeptides in MJY-alpha cells was evaluated using serial dilutions of detergent-disrupted MJY-alpha cells as the sensitizing antigen. However, relatively high protein concentrations were required to detect cell-associated MMTV protein, and protein overloaded wells resulted in excessive background, preventing routine use in quantitating cell-associated protein.

Comparison of determinations of levels of cell-associated MMTV protein (Table 11) and yield of extracellular MMTV-associated protein (Table 10) revealed daily production of MMTV was lower than levels of MMTV protein synthesized. To determine whether this difference was due to shedding of soluble MMTV antigens, spent culture medium of MJY-alpha cells labeled 24 h with ^{35}S-methionine were cleared of virions and immune precipitated with anti-MMTV antisera. SDS-PAGE analysis did not consistently identify any MMTV protein, although gp52 was occasionally detected (Fig. 17). Parallel experiments using ELISA to quantitate MMTV antigens in cleared media

Fig. 17. MMTV proteins shed from MJY-alpha cells. MJY-alpha cells were labeled with 100 μCi ^{35}S-methionine/ml labeling medium for 24 h; spent labeling medium was cleared of debris and virions by centrifugation at 300,000 x g for 30 min, and immune precipitated with anti-MMTV antisera as described. Immune precipitates were subjected to SDS-PAGE on 10-20% gradient polyacrylamide gels, and radioactivity determined as described. Molecular weight of peaks was estimated by comparison with non-radioactive markers in an adjacent lane.

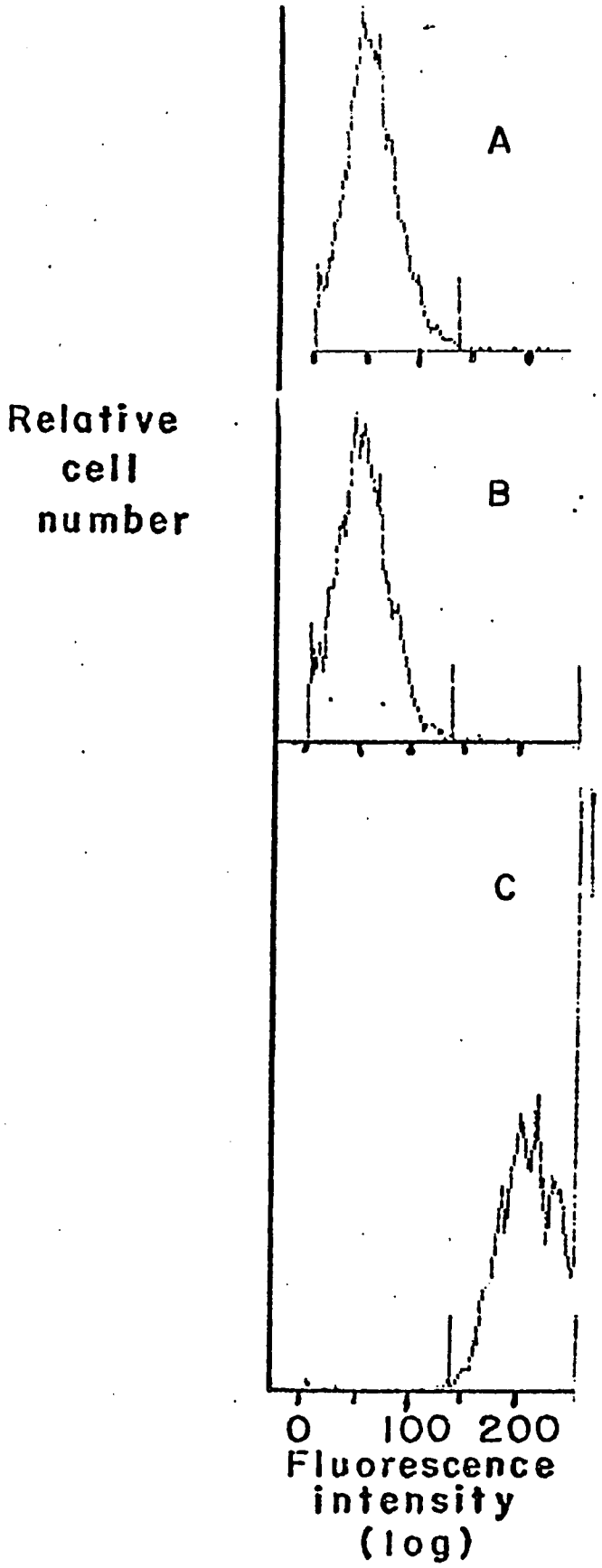

35S
dpm
1000
800
600
400
200
gp52
10
30
50
70
90
Fraction number

supernatants revealed levels of MMTV antigens were at the lower limit of ELISA sensitivity (3-4 ng MMTV protein/50 µl spent media). These data suggest shedding of MMTV protein from cells amounted to 100 ng MMTV protein per day per 1 x 10^8 cells, and represented a minor pathway for release of MMTV proteins from MJY-alpha cells.

7. MMTV ANTIGENS ON MJY-ALPHA CELL SURFACES.

Indirect immunofluorescence using rabbit anti-MMTV antisera and FITC-conjugated goat anti-rabbit IgG was used to identify MMTV antigens expressed on MJY-alpha cell surfaces. Viable MJY-alpha cells stained by this procedure had bright green punctate fluorescence uniformly distributed on cell surfaces; intracellular staining, or fluorescence capping was not observed. Unstained cells or cells stained with preimmune sera had a low level of diffuse, dull grey cytoplasmic fluorescence. Further enumeration of cells with surface bound fluorescence and quantitation of the fluorescence intensity was carried out using a fluorescence-activated cell analyzer. Analysis of unstained samples revealed all MJY-alpha cells exhibited a relatively low level of autofluorescence (Fig. 18A). Staining with nonspecific, preimmune rabbit serum as the first antibody did not increase autofluorescence (Fig. 18B). However, all MJY-alpha cells were stained by anti-MMTV antisera, resulting in

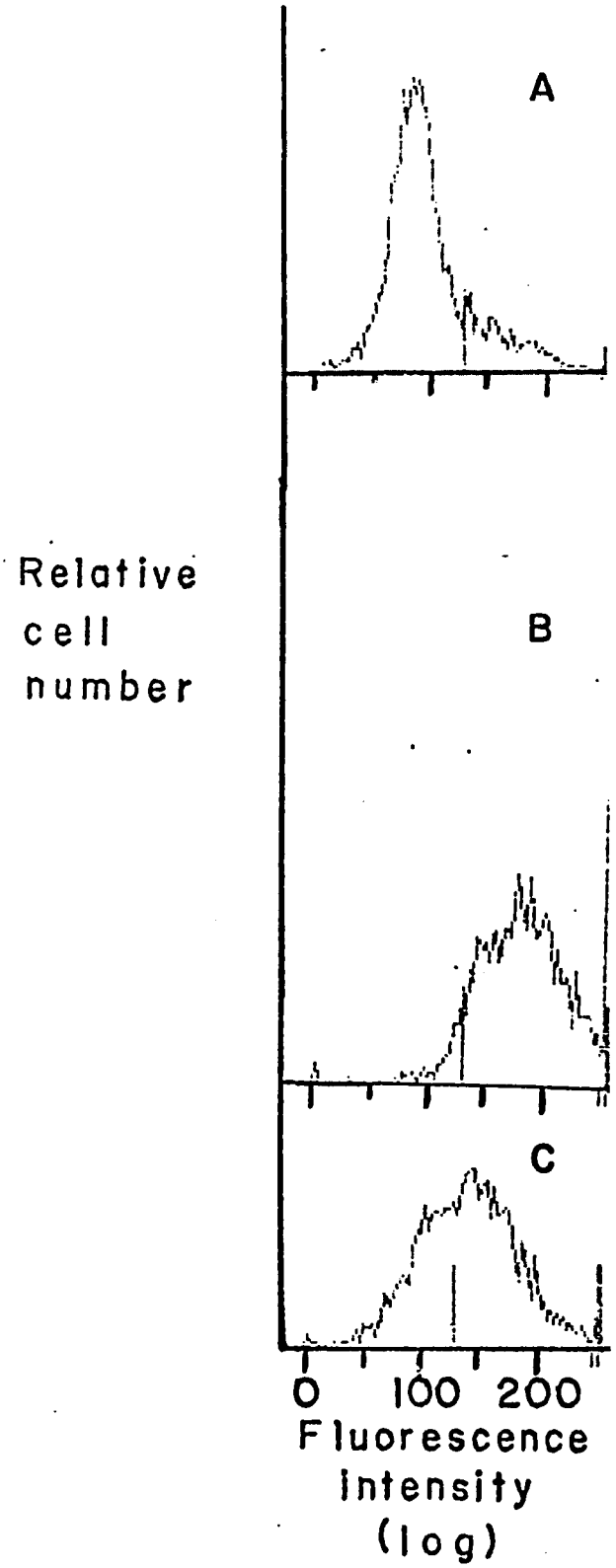
Fig. 18. Histogram analysis of fluorescent labeling of MJY-alpha cells. Viable MJY-alpha cells released from confluent, HC-stimulated MJY-alpha cells were stained with anti-MMTV (C), preimmune sera (B), or buffer (A) as the first antibody, and FITC-conjugated goat anti-rabbit antisera as the second antibody as described in Materials and Methods.


A
B
C
Relative cell number
0
100
200
Fluorescence intensity (log)

increased fluorescence intensity of the entire cell population (Fig. 18C). Fluorescence intensity of a significant proportion of cells was greater than maximum recordable value of the analyzer using 1:4 - 1:100 fold dilutions of anti-MMTV antisera (Fig. 19B), which precluded discrimination among subgroups of cells with different levels of cell surface MMTV antigens. Staining MJY-alpha cells with 1:500 dilutions of anti-MMTV antisera resulted in less than 1% of cells with fluorescence intensity greater than maximum. A single, broad, symmetrical peak of fluorescence was resolved, with intensity range of 50-250 (Fig. 19C). The standard deviation of fluorescence intensity, a measure of variability in degree of cell-associated fluorescence, was 28% greater for cells stained with anti-MMTV antiserum than unstained cells, or cells stained with pre-immune serum. These data suggested levels of MMTV expression varied on MJY-alpha cell surfaces, although distinct cell populations exhibiting high and low levels of MMTV were not detectable.

These initial studies demonstrated the practicality of identifying anti-MMTV antigens on cell surfaces using a polyclonal antisera to MMTV proteins. This procedure can be used to quantitate and compare MMTV expression on surfaces of infected normal and tumor cells from in

Fig. 19. Histogram analysis of MMTV antigens on MJY-alpha cell surfaces. MJY-alpha cells were fluorescently labeled using anti-MMTV or preimmune sera as described. A. Stained with 1:10 dilution preimmune serum. B. Stained with 1:100 dilution anti-MMTV. C. Stained with 1:500 dilution anti-MMTV.


A
B
C
Relative cell number
0
100
200
Fluorescence intensity (log)

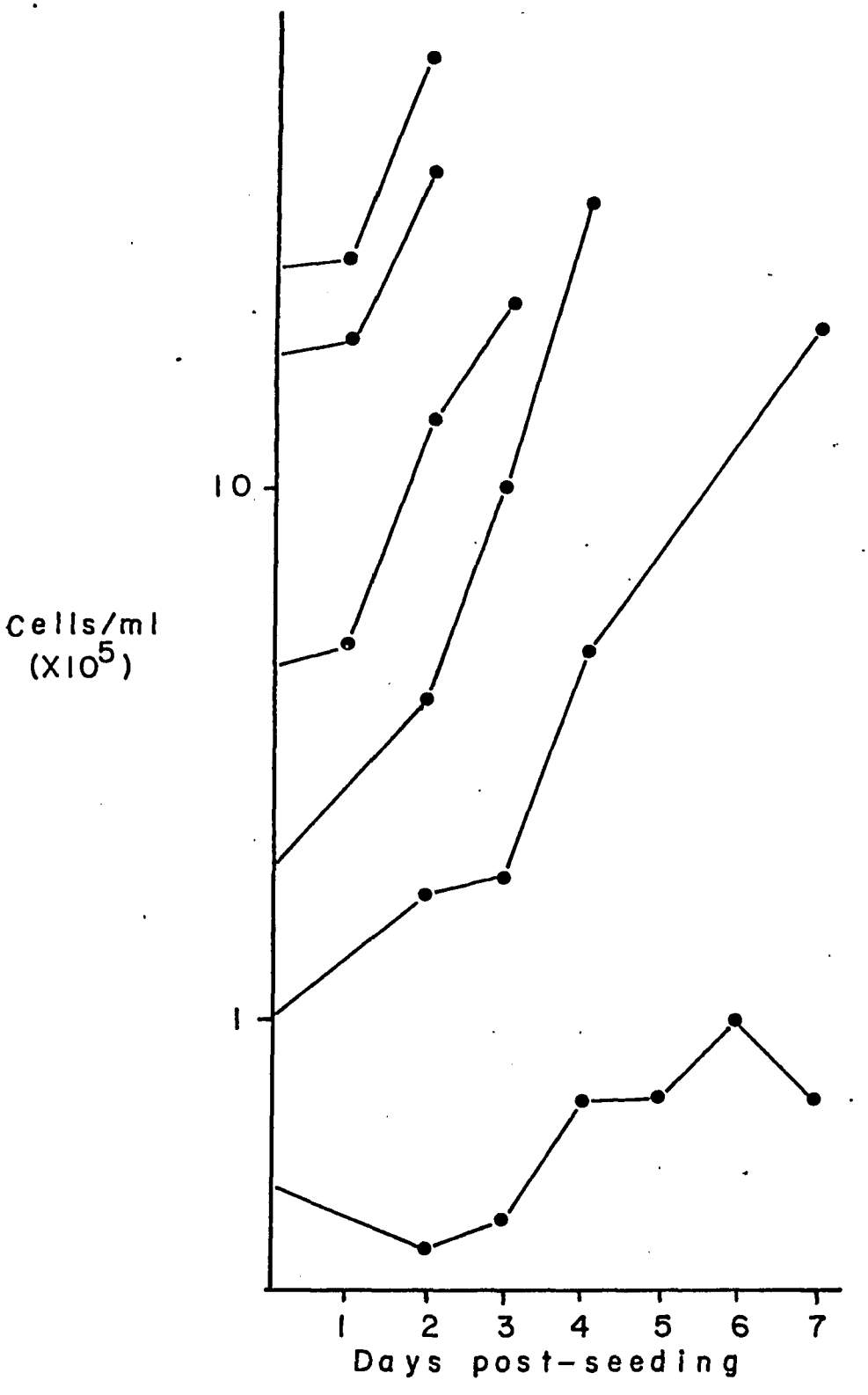
vivo and *in vitro* sources. Inclusion of monospecific or monoclonal antibodies to MMTV components in this assay would enable detection of specific viral polypeptides and could be developed for the quantitation of these proteins.

D. ALTERNATE CULTURE OF MAMMARY EPITHELIAL CELLS.

1. GROWTH OF MJY-MAMMARY CELLS IN SUSPENSION.

Requirement of a solid support for *in vitro* cultivation of epithelial-like cells was evaluated by investigating growth of MJY-alpha cells in suspension. The MJY-alpha adenocarcinoma cell line was routinely grown in tissue culture as an epithelial monolayer. When confluent, the monolayer released viable cells into the culture medium. MJY-alpha cells released from monolayers were used to initiate suspension cultures using maintenance medium and a shaking protocol described in Materials and Methods. Cultivation in suspension required a minimum seeding density of 1-2 X 10^5 cells/ml medium for growth (Fig. 20); log increases in cell number were detected using seeding densities of 1 X 10^5 - 1 X 10^6 cells/ml after variable initial lag periods. The

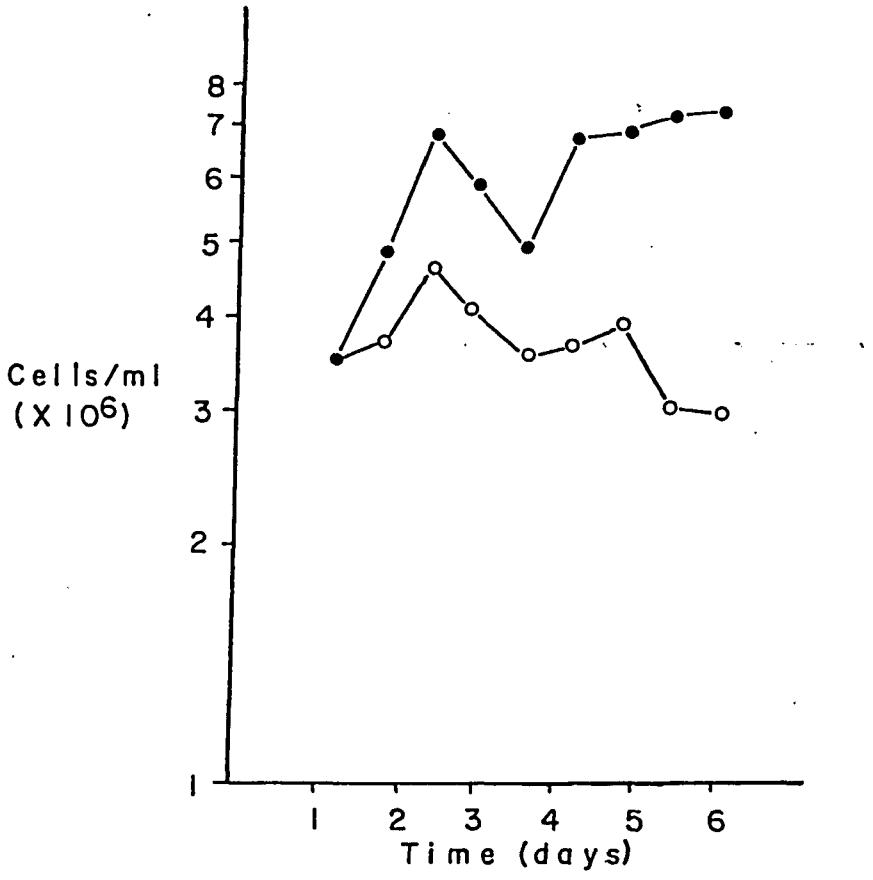
Fig. 20. Logarithmic growth of MJY mammary tumor cells in suspension. MJY-beta cells were seeded at 1 X 10^4 - 3 X 10^6 cells/ml maintenance medium as indicated, and numbers of viable cells determined over the next 6 days. Standard deviation of cell counts was 10-12%.


Cells/ml
(X10^5)
10
1
1
2
3
4
5
6
7
Days post-seeding

doubling time of suspension cells (denoted MJY-beta) in log growth phase ranged from 16-24 h (Fig. 20); variation was related to seeding density: shortest doubling times were detected with highest seeding densities (1-2 x 10^6 MJY-beta cells/ml medium) while longest doubling times were observed after initiating cultures with 1 x 10^5 MJY-beta cells/ml medium (Fig. 20). Regardless of seeding density, suspension cultures attained saturation densities of 5-7 x 10^6 MJY-beta cells/ml medium within 3-5 d after seeding. Similar lag periods, growth rates, and time to saturation density have been observed in MJY mammary tumor cells in monolayer culture (Yagi, 1973, 1974).

To determine whether the saturation density achieved by MJY-beta cells was limited by levels of available nutrients or growth factors, medium of stationary MJY-beta cultures was replenished either on the standard 24 h schedule, or on a 12 h schedule, and cell proliferation compared. As shown in Fig. 21, more frequent medium replenishment resulted in 2-fold increases in cell number compared to cultures refed on a 24 h basis. These findings indicated saturation density attained by MJY-beta cells was not an intrinsic cell parameter, but a variable dependent upon cultivation conditions and/or nutrient requirements. For routine culture maintenance, MJY-beta cells were seeded at 5 X 10^5 cells/ml medium, and were subcultured at 3-4 X10^6 cells/ml medium.

Fig. 21. Saturation density of MJY mammary tumor cells in suspension. MJY-beta cells were grown until confluent as medium was then replenished on a 24 h (○) or 12 h (●) schedule; numbers of viable cells were determined as described at 12 h intervals. Standard deviation of cell counts was 10-12%.


Cells/ml
(X 10^6)
8
7
6
5
4
3
2
1
1
2
3
4
5
6
Time (days)

Cultivation of MJY-beta cells in maintenance medium containing 14 μM HC did not alter cell doubling time (Table 12), and only minor fluctuations were observed in growth curves (Fig. 22). Continuous culture of MJY-beta cells in HC-containing maintenance medium for >10 passages did not result in alterations in cell doubling time or saturation density.

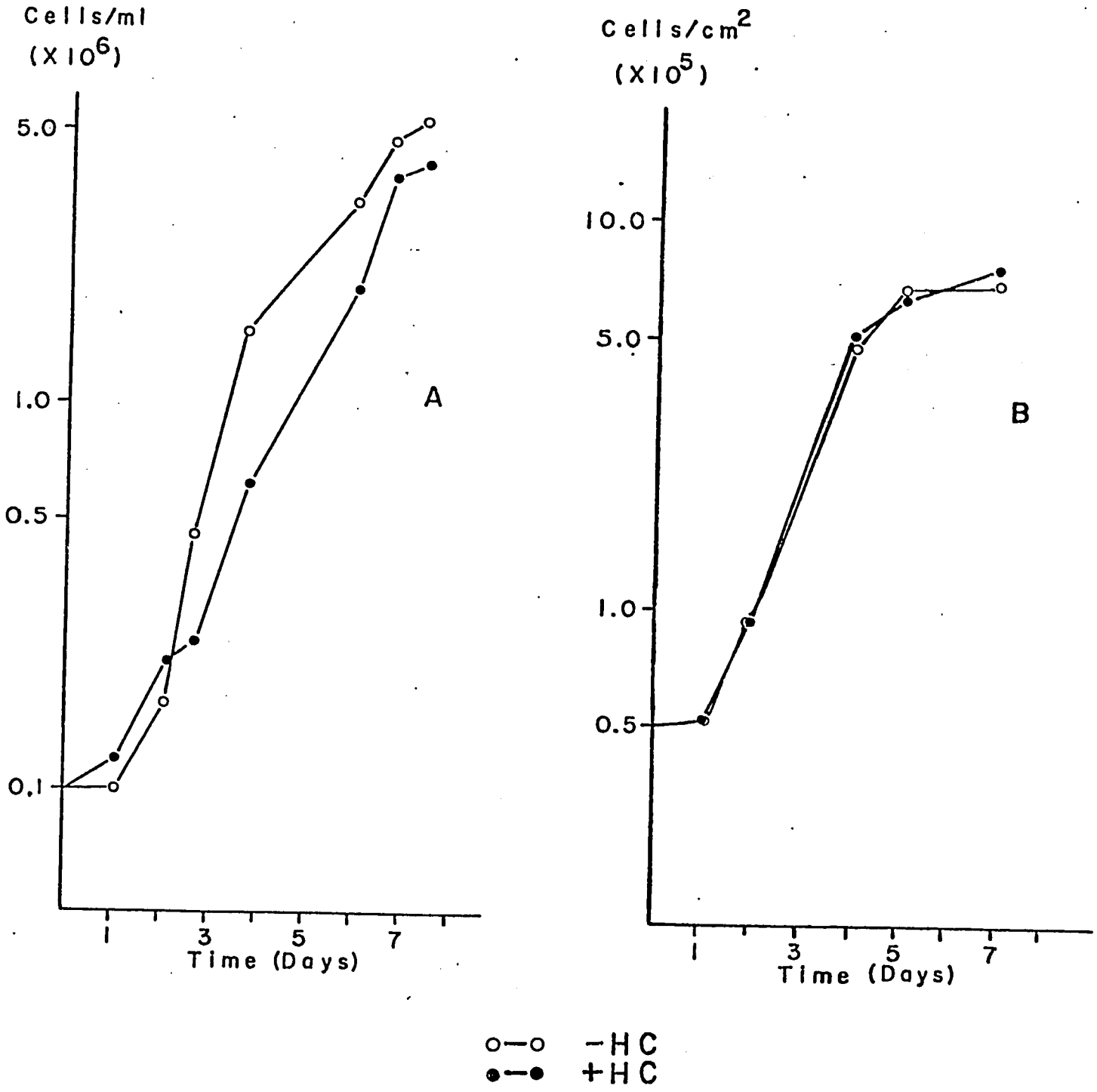
As previously determined (Yagi, 1973), MJY-alpha monolayers require relatively high (18%) concentrations of FBS for optimal cell growth. To determine whether similar serum concentrations were required for proliferation of MJY-beta cells, released cells from confluent MJY-alpha monolayers were seeded in monolayer or suspension in RPMI 1640 medium supplemented with 2, 6, 12, or 18% FBS (v/v), and 1.89 μM bovine insulin/ml medium. As shown in Fig. 23, MJY-beta cells exhibited stringent serum requirements; medium supplemented with 2% or 6% FBS did not support cell growth. Growth of MJY-beta cells in 12% FBS was characterized by prolonged log phase doubling times and reduced stationary phase saturation densities compared to corresponding parameters in MJY-beta cells maintained in 18% FBS. In contrast, monolayer cultures grown in 2, 6, or 12% FBS all exhibited log growth (Fig. 23B); doubling times and saturation densities did not vary more than 10% from corresponding parameters measured in MJY-alpha cells cultured in 18% FBS. However, release of viable cells from confluent monolayers into

Table 12. Growth of MJY-alpha and -beta cells.

	Doubling time (h)	
Culture[1]	-HC	+HC
MJY-alpha	22+/-2	22+/-2
MJY-beta	25+/-2	21+/-2

[1] MJY-alpha cells were seeded at 5 x 10^4 cells/cm^2 and MJY-beta cells were seeded at 1 x 10^5cells/ml medium; doubling times were determined from daily cell counts during expoential growth phase as described. Experiment was replicated 2-5 times, and a typical experiment is presented.

Fig. 22. Effects of HC on proliferation of MJY mammary tumor cells. MJY-beta (B) or alpha (A) cells were seeded in the presence or absence of 14 μM HC, and cell numbers were determined daily as described.

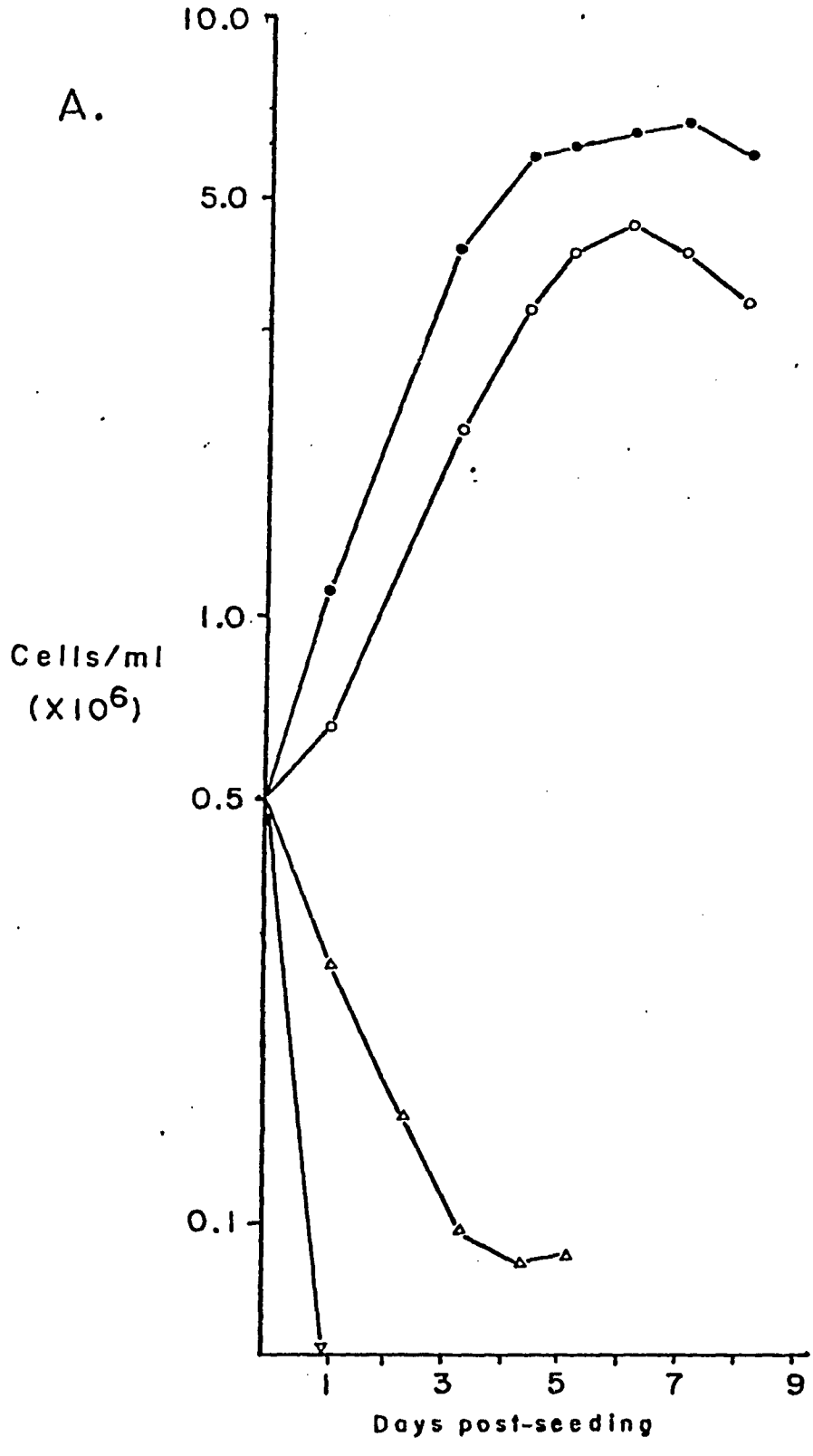

Cells/ml
(X 10^6)
5.0
1.0
0.5
0.1
A
1
3
5
7
Time (Days)
Cells/cm^2
(X 10^5)
10.0
5.0
1.0
0.5
B
1
3
5
7
Time (Days)
o—o -HC
●—● +HC

culture medium, which represented 1% of the total cell number, was significantly decreased in cultures maintained in serum-depleted medium (Fig. 23B). These data suggested MJY-beta cells were more similar in serum requirements to MJY-alpha released cells than to monolayer cells. The more stringent serum requirements exhibited by MJY-beta cells newly initiated in suspension were also observed in cells maintained in suspension for prolonged (>3 passages) periods, suggesting altered serum sensitivity was an immediate and persistent characteristic of MJY-beta cells.

To determine whether cultivation in suspension prevented subsequent monolayer growth, stationary phase MJY-beta cells were seeded in petri dishes after more than 5 passages in suspension. Plating efficiency of MJY-beta cells was equivalent to that measured for newly seeded cells released from confluent MJY-alpha monolayers (Table 13). Observations of newly seeded MJY-beta cells revealed predominantly rounded cells with relatively high nuclear/cytoplasmic ratio, similar to newly plated MJY-alpha cells (Fig. 24); discernible cytoplasmic processes were evident within 3 h of plating (Fig. 24), demonstrating growth in suspension did not prevent attachment or spreading on solid substrates. Subsequent observation for 3-5 d revealed continued epithelial-like appearance indistinguishable from MJY-alpha cells.

The above studies suggested MJY-alpha and beta cells exhibited similarities in appearance and growth rate,

Fig. 23. Effects of fetal bovine serum on growth of MJY mammary tumor cells. MJY-alpha cells released from confluent monolayers were seeded in suspension (A) or monolayer (B) culture in RPMI 1640 medium supplemented with 1.89 μM bovine insulin and 18% (●), 12% (○), 6% (Δ), or 2% (▽) fetal bovine serum; numbers of viable cells were determined as described.


A.
10.0
5.0
1.0
0.5
0.1
Cells/ml
(X10^6)
1
3
5
7
9
Days post-seeding

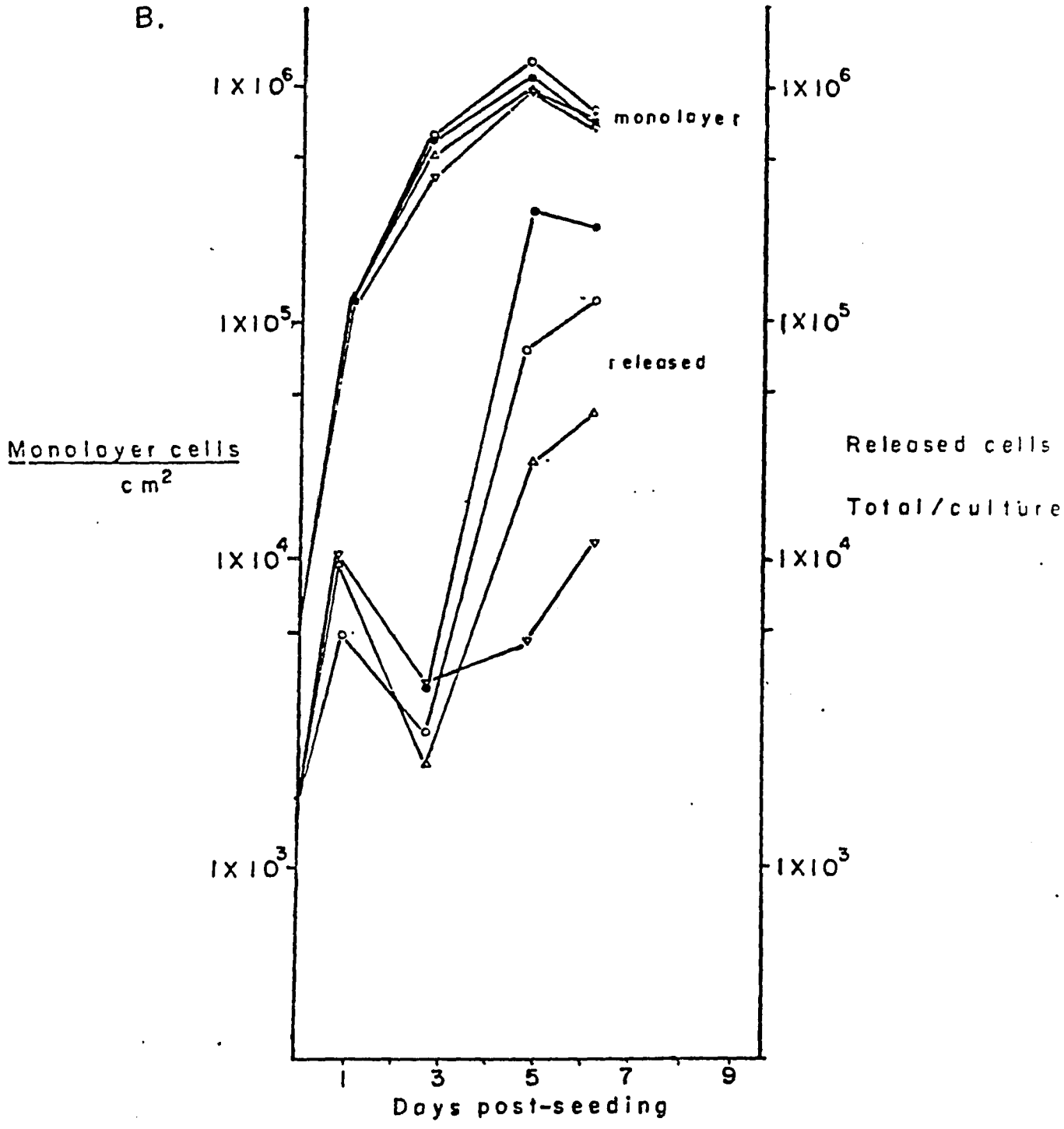
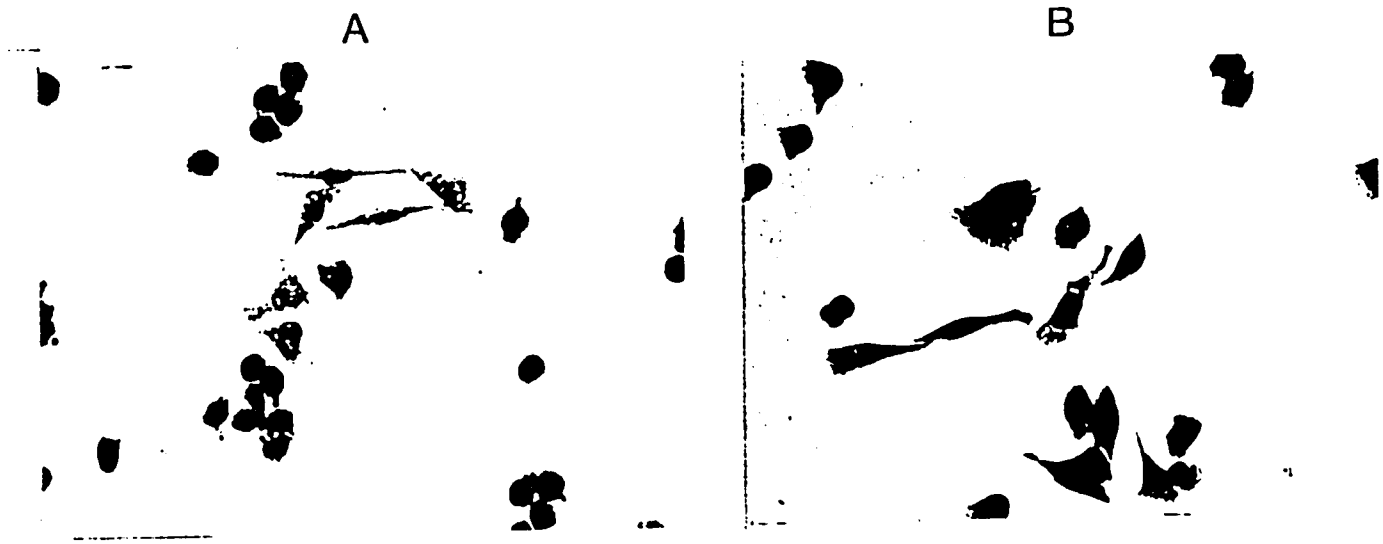

B.
1X10^6
1X10^5
1X10^4
1X10^3
monolayer
released
Monolayer cells
c m^2
Released cells
Total/culture
1
3
5
7
9
Days post-seeding

Table 13. Plating efficiency of MJY-mammary tumor cells.

	Percent cells adhering[1]
MJY-alpha cells	79
MJY-beta cells	84

[1] MJY-alpha or -beta cells were seeded at 1.5×10^5 cells/cm^2 in petri dishes, incubated for 2 h at 37 deg C, washed 2 X with warmed maintenance medium; adherent cells were trypsinized, and viable cells enumerated as described. Experiment was replicated 2-4 x, and representative data are presented.

Fig. 24. Newly seeded MJY mammary tumor cells. MJY-alpha (A) and beta (B) cells were seeded in maintenance media on cover slips, allowed to adhere for 3 h at 37 deg C, then stained with May-Grünwald-Giemsa. X 400.


A
B

although differences in serum sensitivity were noted. To investigate whether cultivation in suspension also altered metabolic functions of mammary tumor cells, rates of DNA, RNA, and protein synthesis, and lactate production in MJY-alpha and beta cells were compared. MJY-alpha and beta cells were labeled with ^{3}H-uridine, ^{3}H-thymidine, or ^{14}C-amino acids for 15 min to 6 h, and levels of cell-associated, TCA-precipitable radioactivity determined. For these experiments, MJY-beta or alpha cells were labeled while in late log phase, and monolayers were not yet releasing cells into the medium. The rates of ^{3}H-incorporation after ^{3}H-thymidine labeling were similar in MJY-alpha and -beta cells, suggesting there were no alterations in DNA synthesis following cultivation in suspension (Table 14). These data are consistent with similar doubling times observed for MJY-alpha and beta cells. Greater differences were observed in the rates of incorporation of ^{3}H-uridine and ^{14}C-amino acids; these were 33-50% lower in MJY-beta cells than MJY-alpha cells (Table 14). Whether these changes reflect alterations in the uptake, incorporation, pool size, or metabolism of RNA and protein precursors must be established. Spent medium from stationary phase MJY-beta cultures contained lactate levels twice as high as that obtained from confluent MJY-alpha cultures consisting of

Table 14. Metabolic parameters of MJY-mammary tumor cells.

	dpm incorporated/μg protein/h[1]			
Sample	^{3}H-uridine	^{3}H-thymidine	^{14}C-amino acids	Lactate[2]
Monolayer	315.0	614.4	72.6	2.5 +/- 0.19
Suspension	212.4	721.2	37.6	5.2 +/- 0.54

[1] MJY mammary tumor cells were labeled with 5-10 μCi radiolabeled precursor as described. Cell-associated, TCA- precipitable radioactivity was determined at 1, 2, 4, and 6 h, and rate of incorporation determined. Standard deviation of determinations was 10-12%. Protein was determined by Markwell modification of Lowry assay. Data are from a representative trial of an experiment done in duplicate.

[2] Spent media was collected daily from MJY-alpha and -beta cells, and levels of extracellular lactate determined. Levels were determined in duplicate for cultures treated for 24 h in the presence or absence of 14 μM HC, and the data pooled. Data expressed as μM lactate produced per 1 x 10^{6} cells per 24 h.

monolayer and released cells. This suggested that metabolism of carbohydrates or lactate in MJY-alpha and beta cultures was not identical (Table 14). Parallel studies in which MJY-alpha and beta cells were exposed to 14 μM HC for 24-48 h prior to initiation of experiments did not alter the rate of radiolabel incorporation or lactate production compared to corresponding untreated samples (Table 14). Alterations detected in incorporation rates of RNA and protein precursors and lactate production suggest there are differences in metabolic characteristics due to growth of mammary tumor cells in suspension vs. monolayers. However, these preliminary studies did not evaluate relative contributions of released and adherent cells in monolayer cultures, and it is not known whether similar alterations occur in other phases of the growth cycle, or under conditions of reduced serum.

2. MMTV PRODUCTION IN MJY-BETA CELLS.

Cultivation of mammary tumor cells in suspension represents a unique adaptation for MJY-alpha epithelial-like cells, which typically exhibit polarized organization of organelles on a solid substratum. Under these conditions, MMTV buds only from anterolateral plasma membranes of infected cells; growth in suspension eliminates this orientation and a potential restriction on MMTV replication.

MMTV production was investigated in MJY-beta cultures grown to a density of 1-2 x 10^6 cells/ml medium, and treated for 48 h with HC in maintenance medium. Spent culture fluid harvested every 24 h and subjected to standard purification procedures for MMTV yielded a light-scattering band with a buoyant density of 1.17 g/cc in final isopycnic gradients. SDS-PAGE of the light-scattering material revealed the polypeptide pattern for MMTV; seven principal MMTV proteins corresponding to gp52, p44, gp37.7-33, p24, p17, p13 and p8 were identified. The relative levels of MMTV polypeptides from MMTV from MJY-alpha and -beta cells were similar (Table 15) and only minor differences in gp52, gp37.7-33, and p24 were noted. These findings demonstrated that MMTV was synthesized by MJY-beta cells, indicating <u>in vitro</u> production of MMTV does not require cellular attachment or polarized cellular organization.

To determine whether MJY-beta cultures produced similar levels of MMTV compared to MJY-alpha cells, yields of MMTV from late log cultures of monolayer and suspension cells were quantitated. Significant increases in MMTV production by MJY-beta cells were detected (Table 16). Suspension cells routinely produced 10-200 fold more MMTV than comparable MJY-alpha cultures. Increases in MMTV yield were observed within 48 h of initiating MJY-beta cultures and

Table 15. MMTV from MJY-alpha and beta cells.

Polypeptide	Relative level of polypeptide-associated staining[1]		
	MJY-alpha		MJY-beta
	A	B	
qp52	30.4 +/- 5.4		26.0 +/- 5.4
p44	3.7 +/- 2.1		6.5 +/- 2.7
qp37.7-33	15.8 +/- 4.0		19.1 +/- 0.6
p24	23.3 +/- 4.9		17.5 +/- 1.5
p17	3.6 +/- 2.5		8.8 +/- 1.4
p14	10.6 +/- 2.2		12.3 +/- 2.6
p8	12.7 +/- 4.1		9.7 +/- 1.7

[1] MMTV was purified from MJY-alpha and beta cells, and processed for electrophoresis as described. Representative SDS-PA gels with coomassie blue-stained proteins are presented from MJY-alpha (A, 2.7 µg) and beta (B, 93 µg) are shown. Relative levels of coomassie blue stained polypeptides were determined from 5-9 MMTV samples, mean +/- standard deviation are presented.

Table 16. Production of MMTV in MJY mammary tumor cells.

	MMTV yield (μg MMTV protein/10^8 cells)[1]	
Sample	Control	HC-Stimulated
MJY-alpha cells	0.2	2.7
MJY-beta cells	93.8	194.2

[1] MMTV was purified from spent cultures of MJY-alpha or beta cells, subjected to SDS-PAGE, and MMTV yield determined by scanning densitometry as described.
Comparisons were performed 8 times and a representative experiment is included.

were consistently elevated throughout the 16 wk cultivation period. These findings indicated that suspension cultures may represent a viable alternative to monolayers for obtaining large quantities of virions for analysis.

Exposure of MJY-beta cells to 14 μM HC resulted in 2-5 fold increases in extracellular MMTV compared to that obtained in untreated suspensions. Similar increases in MMTV production were detected after steroid stimulation of MJY-alpha cells (Table 16), suggesting growth in suspension did not alter steroid-induced increases in MMTV production.

3. PROLONGED HC EXPOSURE OF MJY-ALPHA AND -BETA CELLS.

Although production of MMTV is stimulated by glucocorticoids, the duration of steroid responsiveness by mammary tumor cells in vitro has not been determined. The effects of prolonged glucocorticoid exposure on MMTV production were ascertained by culturing MJY-beta cells in the presence or absence of 14 μM HC. As shown in Table 17, exposure of MJY-beta cells to 14 μM HC for 11-13 days resulted in the expected 3-5 fold increase in MMTV production. Treatment for >14 d, however, resulted in decreased MMTV production compared to levels obtained in cultures without HC. Attenuation of MMTV production was consistently observed during 16 subsequent cell passages in HC-supplemented medium. Similar reductions in MMTV production were detected after prolonged exposure of MJY-alpha cells to HC

Table 17. Production of MMTV in MJY-beta cells following exposure to HC.

	MMTV yield (μg protein/1 X 10^8 cells)[1]	
Culture duration	Control	HC-treated
Days 11-13	58.6	205.1
Days 14-21	40.2	42.7

[1] MMTV was obtained from MJY-beta cells maintained in the presence or absence of HC for 21 d (3 passages); MMTV yield was determined daily by quantitative SDS-PAGE, and typical values obtained during the periods indicated are presented.

To investigate whether MMTV production in cells grown in the presence of HC were permanently refractory to glucocorticoid stimulation, MJY-beta cells maintained in 14 μM HC for greater than 3 passages (denoted HC-LT) were stimulated with 28 or 60 μM HC. As shown in Table 18, MMTV production increased 2-3 fold within 72 h of exposure to elevated concentrations of HC. It was also possible to restore HC responsiveness to 14 μM HC by subculturing HC-LT MJY-alpha cells for 7 days in HC-free maintenance medium. Exposure of these cultures to 14 μM HC again resulted in 2-3 fold increases in MMTV yield within 24 h (Table 18). These studies demonstrated that the depression of MMTV production in HC-LT cells was reversible by either increasing the HC concentration or by brief subculture in HC-free medium.

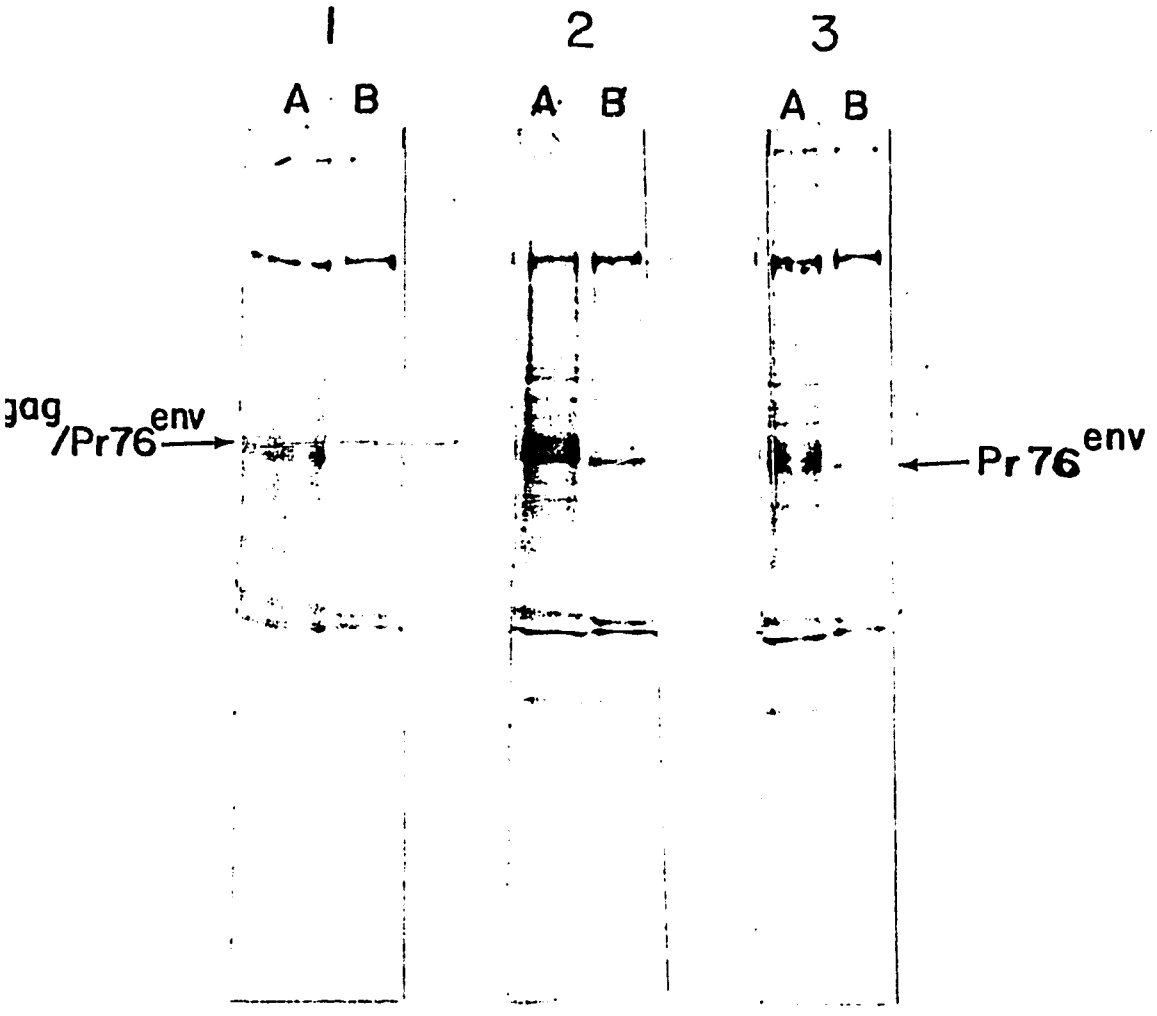
The effects of prolonged glucocorticoid exposure on MJY-alpha cell-associated MMTV proteins were examined by immune precipitation. As demonstrated above, 10 cell-associated MMTV <u>env</u> and <u>gag</u> precursors and products were identified by immune precipitation. Synthesis of principal MMTV polypeptide precursors $Pr76^{gag}$ and $Pr76^{env}$ was identified using short, 15 min ^{35}S-methionine pulse-labeling, and anti-MMTV antisera; $Pr76^{env}$ was selectively identified using anti-gp52 antisera (Fig. 25). Quantitation of polypeptide-associated

Table 18. MMTV Production in MJY-beta cells after prolonged HC. exposure[1]

Culture	Treatment	Fold stimulation
Control	---	1.0
Control	14 μM HC	3.5
HC-LT cells[2]	14 μM HC	1.1
HC-LT cells[3]	---	1.4
Subcultured HC-LT[4]	14 μM HC	2.9
HC-LT cells	28 μM HC	1.9
HC-LT cells	60 μM HC	2.4

[1] Averaqe daily MMTV yield was determined for 1 or 3 d, and presented as a proportion af corresponding control culture. Experiments were performed 2 X and a representative experiment is presented.
[2] HC-LT cells were continuously exposed to 14 μM HC for 3-5 passages prior to these experiments.
[3] HC-LT cells subcultured in absence of added HC for 7 d.
[4] HC-LI cells subcultured in the absence of HC for 7 d prior to restimulation with 14 μM HC for 3 d.

Fig. 25. MMTV proteins synthesized in MJY-alpha cells. Lysates of MJY-alpha cells pulse-labeled with 100 μCi ^{35}S-methionine/ml medium for 15 min were immune precipitated with anti-MMTV (A) or anti-gp52 (B) antisera, and immune precipitates were subjected to SDS-PAGE on 7.5-15% polyacrylamide gels as described. Control cells (panel 1), HC-treated cells (panel 2), and HC-LI cells (panel 3) are presented. Experiment was performed 2-5 times and a representative trial is shown.


1
2
3
A B
A B
A B
gag/Pr76env
Pr76env

radioactivity by scanning densitometry of autoradiograph exposures revealed ^{35}S-incorporation into MMTV precursors $Pr76^{gag}$ and $Pr76^{env}$ was 2.5-3.2 fold greater in MJY-alpha cells incubated in HC-containing medium (HC-treated cells) than in control cells. Prolonged exposure to HC resulted in little or no increases in these MMTV precursors using either anti-MMTV or anti-gp52 antisera. Since only low levels of other MMTV polypeptides were detected after 15 min pulse-labeling; a longer labeling period of 4 h was employed to enable identification of other MMTV intermediates and products. Under this condition, the relative level of ^{35}S-incorporated into MMTV intermediates and products was consistently higher in HC-treated cells than in controls (Table 19). Prolonged exposure of MJY-alpha cells to 14 μM HC did not result in increases in any ^{35}S-labeled MMTV polypeptide above levels detected in control cells. Comparison of steady state levels of cell-associated MMTV proteins using a 24 h labeling period detected only minimal (1.1-1.2 fold) elevations in cell-associated MMTV proteins in HC-treated cells compared to control or HC-LT cells.

Similar alterations in the synthesis of MMTV <u>env</u> precursors were detected when the effects of glucocorticoid treatment on post-translational modification of MMTV polypeptides were investigated by comparing glycosylation of intracellular $Pr76^{env}$, $Pr79^{env}$, and gp52. Although no differences in

Table 19. Effects of HC on cell-associated MMTV precursors and products.

MJY-alpha culture	Polypepetide-associated radioactivity (dpm $^{35}S/\mu g$ protein)[1]							
	$Pr110^{gag}$ + $Pr95^{gag}$	$Pr76^{gag}$ + $Pr76^{env}$	$Pr61^{gag}$	gp52	$Pr38^{gag}$	gp37.7-33	p24	Total
Control	0.83	4.21	0.86	0.83	1.17	1.17	0.34	9.41
HC-treated	1.86	5.09	0.86	0.79	2.01	0.79	0.19	11.59
HC-LT	1.32	2.00	0.89	1.16	0.48	0.48	0.48	6.81

[1]Lysates of confluent cultures lebeled for 4 h with ^{35}S-methionine were immune precipitated with anti-MMTV and subjected to SDS-PAGE; polypeptide-associated ^{35}S was quantitated for each MMTV precursor or product. Level of cell protein per sample was determined by Lowry protein assay, and data presented as:

$$\frac{\text{dpm incorporated into MMTV polypeptide}}{\mu g \text{ total cell protein}}$$

Results of two experiments were similar, and data from one trial is presented.

total cell-associated radioactivity were detected after incubation of control, HC-treated, and HC-LT MJY-alpha cells with ^{3}H-mannose or ^{14}C-glucosamine, levels of ^{3}H and ^{14}C incorporation into MMTV env proteins were increased 1.8-1.9 fold in HC-treated cells compared to control MJY-alpha cells (Table 20). No increases in polypeptide-associated radioactivity were detected in lysates of HC-LT cells (Table 20).

SDS-PAGE analysis of immune precipitates from HC-treated and control MJY-alpha cells revealed significant differences in the relative levels of radiolabeled MMTV glycoproteins (Fig. 26). $Pr76^{env}$ was principal radiolabeled MMTV glycoprotein, containing 45% of total MMTV-associated radioactivity in MJY-alpha cells stimulated with HC for 24 h. Control or HC-LT cells contained increased levels of $Pr79^{env}$ compared to $Pr76^{env}$ (Fig. 26); $Pr79^{env}$ accounted for 40-50% of the total MMTV-associated radioactivity. These findings suggest that short-term, but not prolonged HC treatment alters processing of the MMTV env polyprotein precursor.

To investigate whether HC-induced alterations occurred at cell membranes, MMTV antigens on MJY-alpha cell surfaces were identified and quantitated by immunofluorescent labeling with anti-MMTV and analysis by cell sorter as described. As shown in Table 21, MMTV antigens were detected on surfaces of control, HC-treated, and HC-LT cells. However, the magnitude of fluorescence intensity, a measure of the quantity of surface-bound antibody, was significantly greater in

Table 20. Glycosylation of cell-associated MMTV proteins.

	Immune precipitated radioactivity (dpm)[1]	
MJY-alpha culture	anti-gp52 antisera (^3H)	anti-MMTV antisera (^{14}C
Control[2]	10,634.0	8,330.0
HC-treated[3]	19,036.8	15,591.4
HC-LT[4]	10,936.0	7,847.8

[1] MJY-alpha cultures were labeled with ^3H-mannose or ^{14}C-glucosamine as described. Lysates of labeled cultures were immune precipitated and MMTV-associated radioactivity quantitated. Data for each entire lysate (5.5×10^7 cells) is presented.
[2] Untreated MJY-alpha cultures.
[3] MJY-alpha cells treated with 14 μM HC for 24 h prior to labeling.
[4] MJY-alpha cells maintained in 14 μM HC for >5 passages prior to labeling.

Fig. 26. Effects of HC exposure on glycosylation of MMTV polypeptides. Lysates of MJY-alpha cells labeled with ^{3}H-mannose were immune precipitated with anti-gp52 antisera, subjected to SDS-PAGE on 7.5-15% gradient polyacrylamide gels, and radioactivity determined as described. A. Control MJY-alpha cells. B. MJY-alpha cells stimulated with 14 μM HC prior to labeling. C. HC-LT MJY-alpha cells.

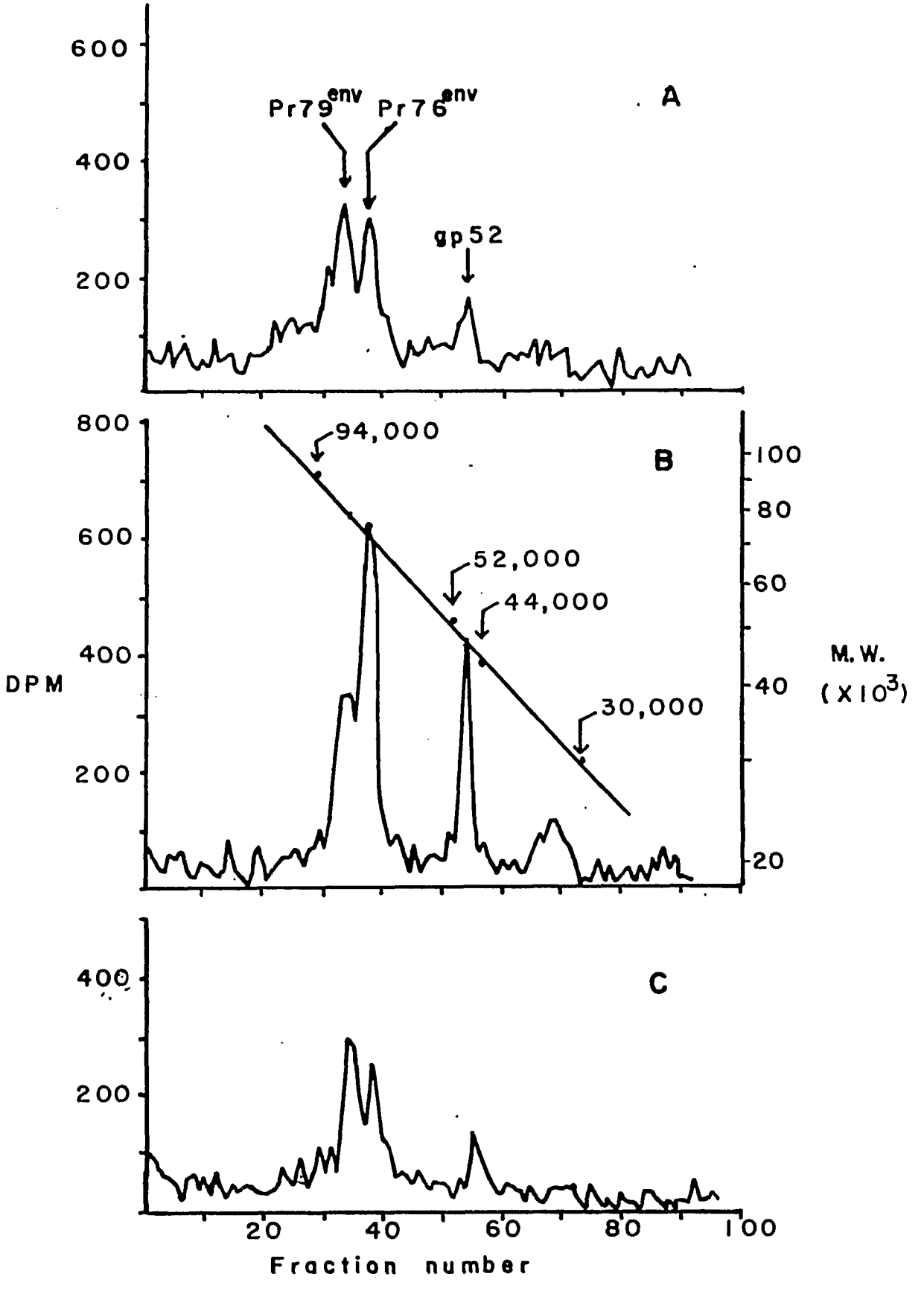

600
400
200
Pr79env
Pr76env
A
gp52
800
600
400
200
94,000
B
52,000
44,000
30,000
DPM
100
80
60
40
20
M.W.
(X10³)
400
200
C
20
40
60
80
100
Fraction number

Table 21. Effects of HC on expression of MMTV antigens on MJY-alpha cell surfaces[1]

	Dilution of anti-MMTV antisera							
	1:50		1:100		1:250		1:500	
Sample	Mean[2]	%[3]	Mean	%	Mean	%	Mean	%
Control	205.9	98.5	199.3	95.9	187.5	85.6	185.2	80.4
HC-treated	231.7	98.9	225.4	98.7	209.7	97.6	200.2	94.9
HC-LT	205.1	98.9	193.0	96.8	185.6	83.4	181.8	70.9

[1] MMTV proteins on MJY-alpha cells surfaces were fluorescently labeled and analyzed by FACS as described. Use of pre-immune sera resulted in 16% fluorescing cells with a mean log fluorescent intensity of 172. Staining studies were repeated 3-4 times and a representative sample is presented. Differences in means for HC-treated or HC-LT and control were statistically significant in two-way student t tests with $p<<0.0001$.
[2] Average log fluorescent intensity.
[3] Proportion of cells fluorescing above background.

HC-treated cells compared to controls at each corresponding dilution of anti-MMTV antisera (Table 21). A five fold higher dilution of anti-MMTV antisera was required to obtain an equivalent fluorescent magnitude of HC-treated MJY-alpha cells compared to unstimulated controls. Prolonged exposure of MJY-alpha cells to HC did not result in increases in MMTV surface antigens and the magnitude of fluorescence intensity of these cells was similar to that measured on surfaces of control cells (Table 21). A 1:500 dilution of anti-MMTV resulted in only 5% decrease in the proportion of fluorescing HC-treated cells, whereas, the proportion of cells fluorescing above background was decreased 18-28% in control or HC-LT cells.

It is possible short-term HC exposure resulted in accumulation of MMTV antigens on cell surfaces. Similar increases were not detected by immune precipitation of lysates of whole cells, perhaps due to relatively low contribution of cell membrane to the total quantity of cell protein. Alternatively, differences in fluorescence magnitude may represent differences in reactivity of MMTV antigens on surfaces of HC-treated and control cells with anti-MMTV antisera. Studies reported above detected differences in glycosylation of MMTV <u>env</u> proteins detected after short-term HC exposure which may have altered antigenicity of these polypeptides. Anti-MMTV antisera used in these experiments was obtained by

immunizing rabbits with disrupted preparations of MMTV obtained from HC-stimulated MJY-alpha cells; greater avidity of anti-MMTV antisera for MMTV antigens in HC-treated cells may have resulted in increases in fluorescence intensity. In addition, reduced temperatures at which reactions took place (4 deg C) may have prevented binding of low affinity antibodies.

4. TURNOVER OF MMTV PRECURSORS.

The studies reported above indicated glucocorticoid exposure resulted in alterations in MMTV production, in addition to, short-term stimulation of virion production. To determine whether rate of processing of MMTV precursors was affected by HC, turnover of principal env precursor $Pr76^{env}$ was investigated. HC-treated and control were pulse-labeled with ^{35}S-methionine and chased in the absence of label for 0-12 h. Lysates of labeled cells were immune precipitated with anti-gp52 and ^{35}S-incorporation into MMTV polypeptides determined. Half-life determinations revealed $Pr76^{env}$ turnover was relatively rapid (190 min); treatment with 14 μM HC for 24 h resulted in a 5-fold increase in turnover rate of $Pr76^{env}$ (Table 22); suggesting glucocorticoid exposure did alter MMTV polypeptide processing rate.

Despite relatively rapid turnover of MMTV env precursor, low levels of ^{35}S-$Pr76^{env}$ persisted throughout prolonged chase periods; 5% of initial $Pr76^{env}$ remained after 12 h chase,

Table 22. Effects of HC on turnover of $Pr76^{env}$.

	^{35}S-labeled $Pr76^{env}$ remaining			
	Pulse label	Chase period		t(1/2)
Sample	15 min	1 h	2h	min
Control	1.00	1.04+/-0.56	0.37+/-0.02	182
HC-stimulated	1.00	0.27+/-0.18	0.13+/-0.04	36

1 MJY-alpha cells were labeled for 15 min with ^{35}S-methionine, washed, chased for 1 or 2 h, and immune precipitated with anti gp52 antiserum; levels of ^{35}S-$Pr76^{env}$ were determined and presented as fraction of total incorporated radioactivity at time 0.
2 Half life determined using the formula:

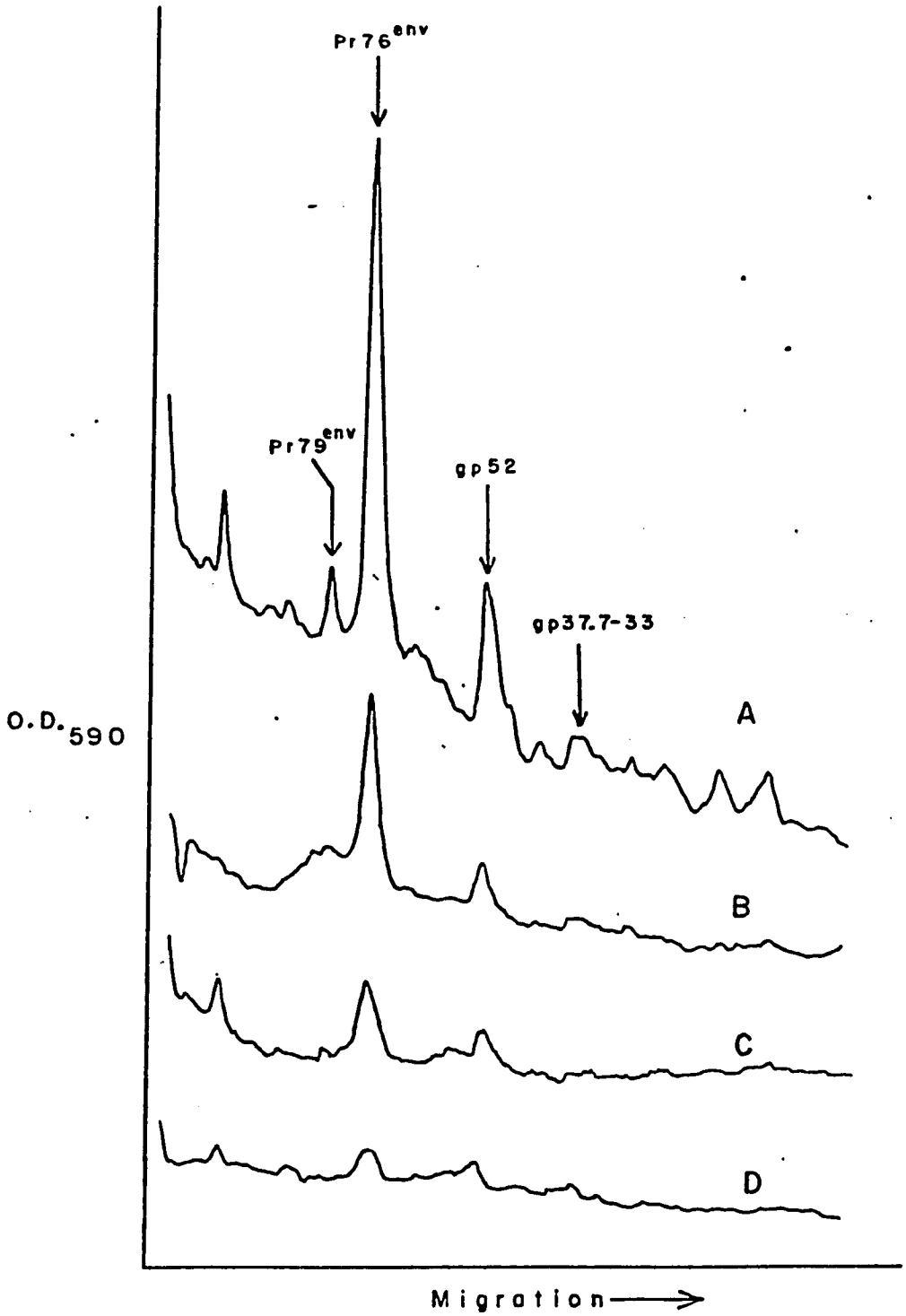
$$A = A0e^{-kt}$$

where A = ^{35}S-labeled $Pr76^{env}$ at time t
A0 = ^{35}S-labeled $Pr76^{env}$ at time t0
k = constant
t = time

while less than 0.001% of labeled precursor would be predicted by first order kinetics (Fig. 27). To investigate whether residual ^{35}S-$Pr76^{env}$ was due to incorporation of free intracellular ^{35}S-methionine remaining at the end of labeling period, pulse labeled cells were chased for 2 h in medium containing high (50 μM) or low (10 μM) levels of unlabeled methionine SDS-PAGE analysis of MMTV env proteins precipitated with anti-gp52 revealed no differences in ^{35}S-methionine incorporation into $Pr76^{env}$ between cultures chased with 10 or 50 μM methionine. This suggested that residual ^{35}S-labeled methionine pool did not contribute to labeling MMTV precursor during the chase period.

Decreases in levels of ^{35}S-labeled precursor were not accompanied by corresponding increases in intracellular ^{35}S-labeled gp52 or gp37.7-33 (Fig. 27) in HC-treated or control cells. ^{35}S-labeled polypeptides were not detected by immune precipitation in extracellular MMTV obtained during chase periods. These data suggest that proteolytic degradation may have contributed to turnover of MMTV precursor, with a relatively small proportion of MMTV precursor processed into the appropriate product.

Fig. 27. Turnover of ^{35}S-$Pr76^{env}$ in MJY-alpha cells. Confluent, HC-stimulated MJY-alpha cells were labeled with 100 μCi ^{35}S-methionine/ml medium for 15 min, or pulse-labeled and chased for 1 h, 2 h, or 12 h, then immune precipitated with anti-gp52 antisera, and subjected to SDS-PAGE on 7.5-15% gradient polyacrylamide gels. A. 15 min labeling with ^{35}S-methionine B. 15 min pulse-labeling and 1 h chase. C. 15 min pulse-labeling and 2 h chase. D. 15 min pulse-labeling and 12 h chase.


Pr76env
Pr79env
gp52
gp37.7-33
O.D.590
A
B
C
D
Migration

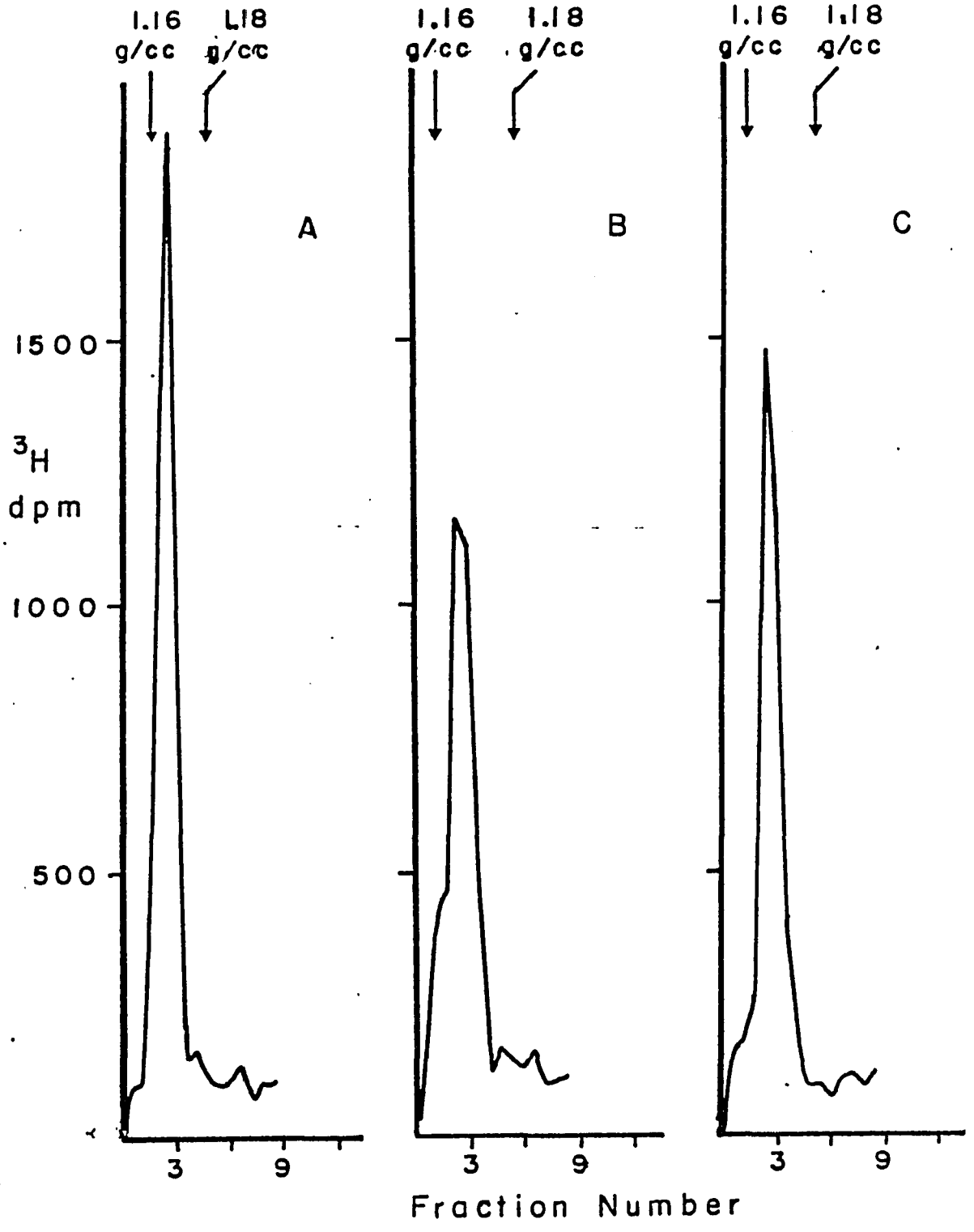
E. Effects of cytodisruption on MMTV.

1. MMTV PRODUCTION.

Studies on the role of the cellular cytoskeleton in MMTV replication were initiated by examining MMTV production during 2.1 μM cytochalasin B (CB), cytochalasin D (CD), and 0.14 μM colcemid treatment. MMTV purified from MJY-alpha cultures labeled with ^{3}H-uridine during the 24 h treatment with CB and colcemid appeared as single bands of ^{3}H radioactivity with bouyant densities of 1.17 g/cc in the final isopycnic gradients (Fig. 28). Although MMTV production was not abolished, differences in levels of ^{3}H-incorporation into the MMTV-associated band were evident, suggesting alterations in MMTV production or in metabolism of radiolabeled uridine occurred.

MMTV production during and following exposure to 2.1 μM CB or CD or to 0.28 μM colcemid was quantitated by immune precipitation of radiolabeled preparations. Multiple harvests obtained throughout the treatment period differentiated between immediate and delayed drug effects. ^{35}S-methionine with high specific activity (600-1200 Ci/mMole) enabled quantitation of multiple virus harvests; the ^{35}S-radioactivity was efficiently incorporated into MMTV gp60, gp52, p44, gp37.7-33, and p24, although p17, p13, and p8 were not highly labeled due to low methionine content (Yagi, et al., 1978c).

Fig. 28. Effects of cytodisruptive drugs on MMTV. MMTV was prepared from spent media of MJY-alpha cells labeled for 24 h with ^{3}H-uridine (A), or labeled and treated with 2.1 μM cytochalasin B (B), or 0.14 μM colcemid (C). Final isopycnic gradients were fractionated and radioactivity determined as described.


1.16 g/cc
1.18 g/cc
A
B
C
1500
1000
500
^{3}H dpm
3
9
Fraction Number

Exposure to CB resulted in 1.7-2.6 fold increases in virion-associated radioactivity throughout the 24 h treatment period (Table 23). Relative levels of MMTV continued to be elevated 2-3 fold 24 h following CB removal. CD had the opposite effect, and reduced levels of immunoprecipitable MMTV by 40-80% during the 24 h treatment period. In addition, extracellular MMTV continued to be depressed by 60-70% during the 24 h period following CD removal. Reduction in MMTV production was also detected during and following treatment with 0.28 μM colcemid, although prolonged treatment (36-48 h) was required to observe this effect (Table 23).

2. EFFECT OF MICROFILAMENT DISRUPTION ON MMTV COMPOSITION.

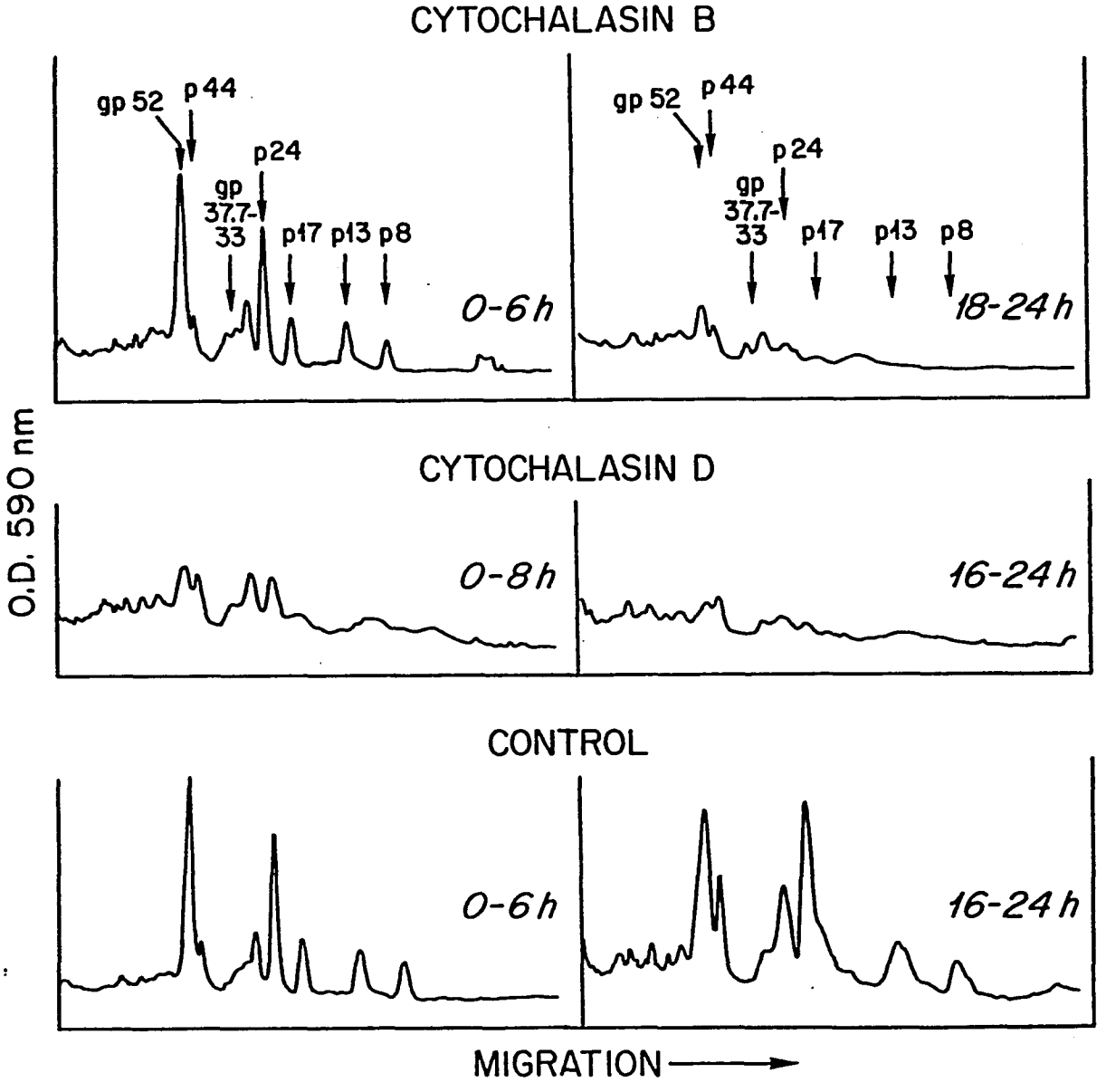
To determine whether cytochalasin-induced effects on MMTV production were accompanied by alterations in virion polypeptide composition, isopycnically purified MMTV from cultures treated with CB or CD was subjected to SDS-PAGE. Treatment with equimolar doses of either cytochalasin resulted in modifications in typical MMTV profiles, although the duration of treatment period required to observe these changes differed for CB and CD. As shown in Fig. 29, changes in MMTV polypeptide profile were detected within 8 h of CD exposure, and were observed throughout the treatment period. In contrast, exposure of MJY-alpha cells to CB did not result in detectable

Table 23. Effects of cytodisruptive drugs on MMTV production.

Treatment period[1]	Relative levels of MMTV-associated radioactivity			
		Treatment		
	Control	2.1 μM CB	2.1 μM CD	0.28 μM Colcemid
16 h	1.00	1.75	0.20	0.72
24 h	1.00	2.62	0.57	1.16
36 h	1.00	***[3]	***	0.48
48 h	1.00	***	***	0.38
Recovery period[2]				
24 h	1.00	2.24	0.32	0.44

[1] Levels of MMTV-associated radioactivity were quantitated by immune precipitation of ^{35}S-labeled MMTV isopycnically purified from drug treated or control MJY-alpha cells as described in Materials and Methods.
[2] Levels of MMTV-associated radioactivity were quantitated by immune precipitation of ^{35}S-labeled MMTV isopycnically purified from media harvested 24 h after drug removal.
[3] CB and CD treatment was carried out for 24 h only.

Fig. 29. SDS-PAGE analysis of isopynically purified MMTV from MJY-alpha cells treated with 2.1 μM CB, or 2.1 μm CD, or no drug. Representative profiles of MMTV harvested from 2 treatment periods during 24 h exposure to the drug are presented. Proteins in gels were stained with coomassie blue, and gels were scanned at 590 nm.


CYTOCHALASIN B
gp 52
p44
p24
gp 37.7-33
p17
p13
p8
0-6h
18-24h
O.D. 590 nm
CYTOCHALASIN D
0-8h
16-24h
CONTROL
0-6h
16-24h
MIGRATION

alterations in MMTV polypeptide profile until 16-24 h of exposure. Radiolabeling of MJY-alpha cells with ^{35}S-methionine and ^{3}H-glucosamine prior to exposure to CB and CD also revealed alterations in the relative levels of three MMTV proteins, gp60, p44, and p24 (Table 24). The major internal core protein p24, which comprises 22-24% of the total virion protein, was decreased by 25-44% within 16 h and by 50-61% after 16-24 h exposure to either cytochalasin compound. The other nonglycosylated protein, p44, was transiently increased 2-fold during the first 16 h of CD, but not CB, treatment. Exposure to either cytochalasin resulted in a 1.5-2 fold increase throughout treatment in gp60, the external, envelope-associated glycoprotein (Table 24).

Comparison of the levels of glycoprotein-associated ^{3}H-glucosamine and ^{35}S-methionine radioactivity revealed that the ratios of ^{3}H to ^{35}S in gp60, gp52, and gp37.7-33 were depressed (Table 25). This alteration was most apparent in gp52, the major external virion glycoprotein and suggested the occurrence of modifications in the carbohydrate moieties of the proteins.

In addition to alterations in relative levels of MMTV polypeptides, 4-5 high molecular weight proteins were detected in MMTV from CB- or CD-treated cells (Fig. 30). These proteins were not intensely stained with commassie blue (Fig. 29), although they were labeled with

Table 24. Effects of cytodisruption on MMTV polypeptides.

MMTV polypeptide		Relative % of polypeptide-associated radioactivity[1]			
		Control	CB	CD	Colcemic
0-16 h					
gp60:	^{3}H	20.3	25.1	35.7[2]	26.7
	^{35}S	12.8	21.0[2]	20.4[2]	16.8
gp52:	^{3}H	50.1	44.7	42.9	43.9
	^{35}S	21.8	17.6	14.1	17.8
p44:	^{35}S	14.8	19.2	27.8[2]	26.0[2]
gp37.7-33:	^{3}H	28.3	29.3	21.4	28.8
	^{35}S	24.9	21.6	23.5	22.6
p24:	^{35}S	23.6	13.5[2]	14.0[2]	22.3
16-24 h					
gp60:	^{3}H	19.3	40.9[2]	41.0[2]	32.7
	^{35}S	12.9	24.3[2]	23.8[2]	21.4
gp52:	^{3}H	51.2	42.8	41.7	49.2
	^{35}S	17.9	17.6	12.2	12.3
p44:	^{35}S	32.3	23.9	30.4	36.0
gp37.7-33:	^{3}H	28.6	21.3	17.9	28.2
	^{35}S	17.2	18.8	25.6	18.4
p24:	^{35}S	21.5	10.7[2]	8.0[2]	11.7[2]

[1] MJY-alpha cells prelabeled with ^{35}S-methionine and ^{3}H-glucosamine were treated with 2.1 μM CB or CD, or 0.28 μM colcemid. Proportions of ^{35}S- and ^{3}H-associated with MMTV polypeptides were determined for each 6-8 h harvest of MMTV during the drug treatment. Values represent averages of polypeptide- associated radioactivity for MMTV harvested between 0-16 h or 16-24 h.

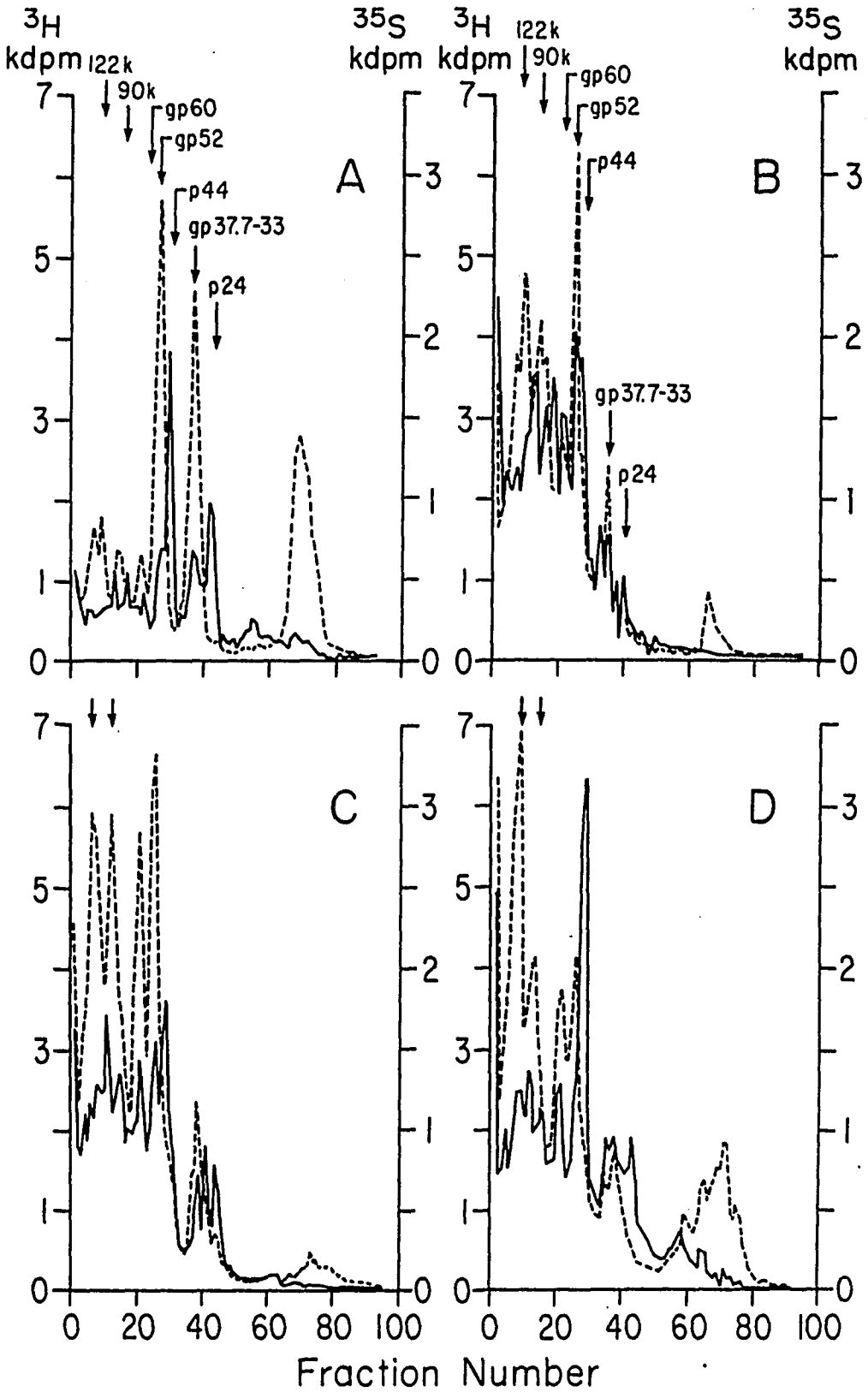
[2] Significant in one-way Dunnett's test alpha=0.05.

Table 25. Glycosylation Ratio.

	^{3}H radioactivity/^{35}S radioactivity in MMTV glycoproteins[1]					
	24 h exposure			48 h exposure		
Treatment	gp60	gp52	gp37.7-33	gp60	gp52	gp37.7-33
Control	4.78	9.25	4.95	3.21	7.25	4.33
CB 2.1 μM	3.28	5.53	3.32	-	-	-
CD 2.1 μM	3.39	6.46	2.02	-	-	-
Colcemid 0.28 μM	3.24	3.50	2.83	2.80	5.65	3.04

[1]Cultures were doubly labeled with ^{35}S-methionine and ^{3}H-glucosamine, treated with cytodisruptive drugs, and virions were purified and subjected to SDS-PAGE. Relative levels of radioactivity associated with virion glycoproteins were determined as described in Materials and Methods.

Fig. 30. SDS-PAGE of MMTV from MJY-alpha cells prelabeled with ^{35}S-methionine (——) and ^{3}H-glucosamine (----) for 24 h before drug treatment. A. MMTV from untreated control cultures 36-42 h after isotopic labeling. B. MMTV harvested at 18-24 h from cells continuously treated with 2.1 μM CB.
C. MMTV harvested 18-24 h from cells treated with 2.1 μM CD. D. MMTV harvested 26-36 h from cells treated with 0.28 μM colcemid.


3H kdpm
35S kdpm
122k
90k
gp60
gp52
p44
gp37.7-33
p24
A
B
C
D
Fraction Number

^{35}S-methionine Two species, with molecular weights of 90,000 and 120,000 also incorporated ^{3}H-radioactivity indicating that they were glycoproteins. These high molecular weight proteins accounted for approximately 50% of the total virion-associated radioactivity after 24 h treatment, and were still present in MMTV harvested 24 h after drug removal. To determine whether these polypeptides were related to MMTV virion proteins, purified, ^{35}S-labeled MMTV from cultures treated with CB or CD were disrupted in non-ionic detergent and subjected to immune precipitation with antiserum to MMTV. The high molecular weight proteins were identified in the immune precipitates by SDS-PAGE, suggesting that they shared antigenic specificities with virion proteins. Because of limited quantities of virus generated in these experiments, further tests of nonspecific binding were not carried out. It is possible these proteins were nonspecifically bound to S. aureus, although proteins with similar electrophoretic mobility have not been previously detected in immune precipitations from lysates of untreated cells or virions.

3. EFFECTS OF MICROTUBULE DISRUPTION ON MMTV COMPOSITION.

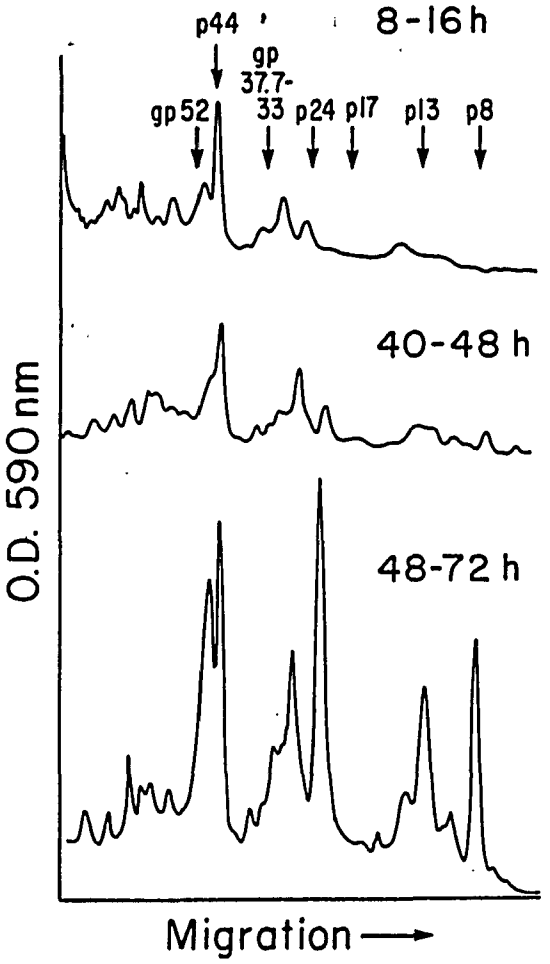
Exposure to 0.28 μM colcemid resulted in reversible alterations in MMTV polypeptide composition, as demonstrated by comparison of SDS-PAGE profiles of MMTV harvested during (40-48 h) and following (48-72 h) colcemid

treatment (Fig. 31). Using radiolabeled precursors as before, alterations in levels of MMTV polypeptides were detectable within 16-24 h of drug exposure (Fig. 30). As quantitated in Table 24, the nonglycosylated core protein, p24, was decreased by 40-60%, whereas, gp60 increased by 50% (Table 24). The level of p44 was significantly increased within the first 16 h of colcemid exposure, although this difference was not maintained between 16-24 h. As observed with cytochalasin treatment, the $^{3}H/^{35}S$ ratio for all MMTV glycoproteins, including gp60, was depressed following exposure to colcemid (Table 25). In addition, 4-5 high molecular weight glycosylated and nonglycosylated proteins were present (Fig. 30). These polypeptides had similar electrophoretic mobilities as components observed in MMTV from cytochalasin-treated cells. Colcemid-induced alterations in MMTV polypeptides were dependent upon the presence of the drug. Within 24 h after colcemid removal, levels of MMTV polypeptides were similar to controls although the high molecular weight proteins persisted.

4. EFFECTS OF CYTODISRUPTION ON MMTV POLYPEPTIDE PRODUCTION.

To determine whether alterations in cell-associated MMTV polypeptides accompanied changes in MMTV virion polypeptide profile, synthesis of MMTV proteins was inves-

Fig. 31. SDS-PAGE of isopycnically purified MMTV from MJY-alpha cells during and following treatment with 0.28 μM colcemid. MMTV was harvested at 8 h intervals throughout the 48 h treatment period; two harvests, obtained during 8-16 and 40-48 h of treatment are presented. Following colcemid removal, a single additional 24 h harvest (48-72 h) was obtained. Profiles of MMTV from untreated cultures are similar to those in Fig. 29.


p44
gp 37.7-33
gp52
p24
p17
p13
p8
8-16h
40-48 h
48-72 h
O.D. 590 nm
Migration

tigated. MJY-alpha cells treated with CB, CD, or colcemid for 24 h were labeled with ^{3}H-leucine throughout the 24 h treatment period, and with ^{35}S-methionine during the final 3 h of drug exposure. Although several trials were complicated by high molecular weight protein contamination, immunoprecipitation with anti-MMTV antisera did detect ^{35}S and ^{3}H-labeled MMTV polypeptide precursors, intermediates, and products in drug-treated cells. It was not possible to identify whether the 95,000 and 110,000 radiolabeled species corresponded to known MMTV gag precursors or to the cross-reacting polypeptides detected in MMTV virions from cytochalasin-treated cells. Quantitation of polypeptide-associated radioactivity (Table 26) revealed levels of MMTV polypeptide synthesis and accumulation were similar in control and cytochalasin-treated cells. Minor changes in relative levels of several MMTV polypeptides were detected, and further study is required to determine whether consistent deviations result from CB- or CD-induced alterations of MMTV polypeptide processing.

Exposure to 0.28 μM colcemid resulted in 30-40% decreases in all ^{35}S-labeled cell-associated MMTV polypeptides (Table 26). Similar decreases in radiolabel incorporation into total MJY-alpha cell-associated protein were also detected (see below).

Table 26. Effects of cytodisruption on cell-associated MMTV polypeptides.

	Incorporation into cell-associated MMTV polypeptide (dpm/mg protein)[1]															
	$Pr110^{gag}$		$Pr95^{gag}$		$Pr76^{gag}$ - $Pr76^{env}$		gp52		$Pr38^{gag}$		gp37.7-33		p24		Total	
Sample	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S
Control	169.5	272.8	126.0	217.5	211.0	485.5	109.7	210.4	117.5	207.6	160.2	191.5	48.2	119.2	942.1	1704.5
CB 2.1 M	116.0	192.8	93.9	204.4	332.3	743.0	95.6	286.5	163.9	284.5	143.8	244.4	65.5	123.7	1011.0	2079.3
CD 2.1 M	80.1	116.3	96.1	173.3	161.6	502.8	106.6	224.2	123.3	177.8	135.0	170.7	68.5	119.1	771.2	1484.2
Colcemid																
0.28 M	141.9	193.7	47.2	146.9	138.1	287.6	93.4	204.7	107.8	193.0	123.5	165.1	39.6	68.3	691.5	1259.3

[1]MJY-alpha cells were treated with 2.1 M CB, 2.1 M CD, or 0.28 M colcemid for 24 h, and lebeled with ^3H-leucine throughout the treatment period, and with ^{35}S-methionine during the final 3 h of treatment. Labeled cells were immune precipitated with anti-MMTV andtisera and analyzed by SDS-PAGE as described. Protein was determined by Lowry assay, and data expressed as:

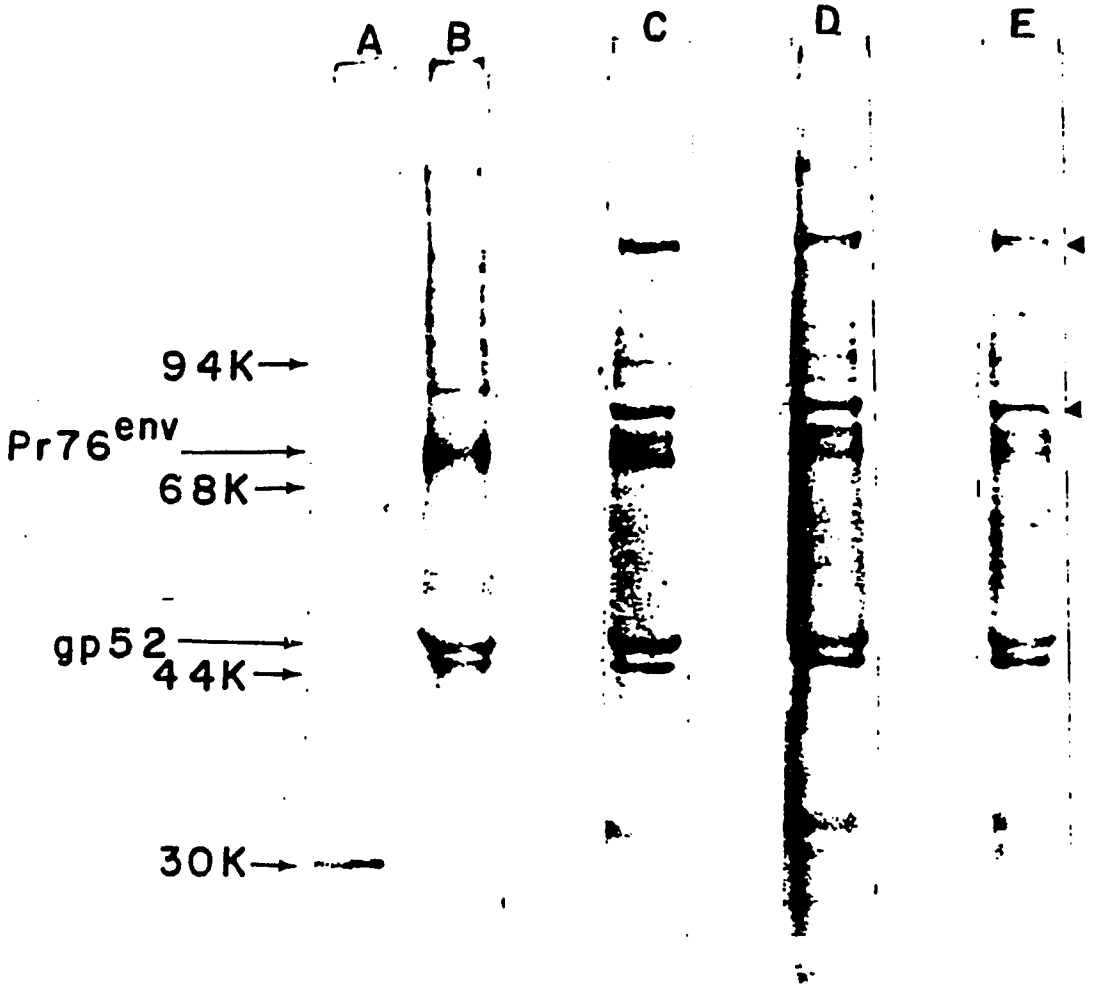
$$\frac{\text{dpm radioactivity incorporated}}{\text{total cell protein}}$$

Cell-associated MMTV *env* proteins were identified using anti-gp52 antisera in the immune precipitations of labeled cell lysates. SDS-PAGE analysis revealed CB, CD, or colcemid treatment resulted in alterations in electrophoretic mobility of principal *env* precursor $Pr76^{env}$, while the presence of other MMTV *env* proteins were unaffected (Fig. 32). These findings suggested initial alterations in MMTV glycoprotein processing occurred which did not prevent subsequent precursor cleavage. High molecular weight polypeptides (90,000 and 120,000 mw) were also identified in these immune precipitates from cells exposed to CB, CD, or colcemid. It is not known whether these polypeptides are similar to virion-associated high molecular weight polypeptides.

5. EFFECTS OF CYTODISUPTIVE AGENTS ON MMTV SURFACE ANTIGENS.

The effects of CB, CD, or colcemid on cell surface expression of MMTV proteins were assessed using rabbit anti-MMTV antisera and FITC-conjugated goat anti-rabbit IgG. A FACS analyzer was also employed to quantitate the degree of immunofluorescence following staining of viable cells with anti-MMTV. A uniform bright green punctate fluorescence on drug-treated and control cell surfaces was observed by fluorescence microscopy, suggesting cyto-

Fig. 32. Effects of cytodisruption on cell-associated MMTV proteins. Confluent, HC-stimulated MJY-alpha cells were treated with 2.1 μM CB (C), 2.1 M CD (D), 0.28 μM colcemid (E), or no drug (B) for 24 h. Cells were labeled with ^{3}H-leucine throughout the treatment period and with ^{35}S methionine during the final 3 h. Lysates were immune precipitated with anti-gp52, and analyzed by SDS-PAGE as described. Molecular weight markers (A) are included for comparison.


A
B
C
D
E
94K
Pr76env
68K
gp52
44K
30K

disruption did not alter distribution of MMTV antigens on MJY-alpha cell surfaces. Relative levels of MMTV surface expression were then compared by FACS; 2.1 μM CB or 0.28 μM colcemid treatment resulted in 3-5% decreases in the proportion of released MJY-alpha cells staining above background, and in the relative intensities of cell-associated fluorescence, (Table 27). Although these differences appeared to be small, they were statistically significant with $p<<0.0001$, due to the large numbers of observations (1×10^4 cells analyzed) per sample.

Determinations of MMTV expression on surfaces of CD-treated cells were carried out only twice due to the low numbers of cells released from these cultures. However, 2.1 μM CD treatment decreased the proportion of cells fluorescing above background, and reduced the mean intensity of cell-associated fluorescence (Table 28). These data suggest that CD, like CB and colcemid did cause a reduction in MMTV surface expression.

6. EFFECT OF CYTODISRUPTIVE DRUGS ON CELLULAR MORPHOLOGY AND METABOLISM.

The morphology and metabolism of MJY-alpha cells were monitored to ascertain any toxic effects of CB, CD,

Table 27. Effects of CB and colcemid on MMTV surface expression.

	Dilution of MMTV antisera[1]					
	1:50		1:100		1:250	
Sample	Mean[2]	%[3]	Mean	%	Mean	%
Control	231.7	98.9	225.4	98.7	209.7	97.6
CB	205.7	93.4	221.1	97.9	198.0	88.4
Colcemid	226.6	94.3	220.3	94.3	206.7	91.9

[1] MJY-alpha cells were treated with 2.1 μM CB or 0.28 μM colcemid as described and cell surface MMTV antigens or released cells stained using rabbit anti-MMTV antisera and FITC-conjugated goat anti-rabbit IgG. Staining with pre-immune sera resulted in 16% of cells fluorescing above background, with a mean fluorescence intensity of 172. Studies were done twice with antisera dilutions of 1:50-1:500 and a representative sample is shown.
[2] Average log fluorescence intensity, as described in Materials and Methods.
[3] Proportion of cells fluorescing above background. Differences in means for CB- or colcemid-treated samples were statistically significant in two-way Student t tests with $p \ll 0.0001$ at all antisera dilutions employed.

Table 28. Effects of CD on MMTV surface antigen expression[1]

	Dilution of anti-MMTV antisera			
	1:10		1:50	
	Mean	%	Mean	%
Control	214.7	99.1	226.7	99.2
CD 2.1 μM	205.7	87.5	220.0	87.2

[1] MJY-alpha cells were treated in the presence or absence of 2.1 μM CD for 24 h, and MMTV cell surface antigens were fluorescently labeled as described. Data are presented from two independent experiments. Differences in means for CD-treated samples are statistically significant in two-way Student t tests with $p << 0.0001$.

and colcemid. Viability of CB- or CD-treated cells was equivalent to untreated controls even following prolonged exposure to elevated doses of drug (21 μM for 2-10 days). Treatment of MJY-alpha cells with 2.1 μM CB did result in morphologic changes characteristic of this drug, as shown by comparison of figures 33 and 34. These alterations included zieotic-type blebbing at the cell surface (Fig. 34A), enucleation in 0.4% of treated cells, and inhibition of cytokinesis, with generation of binucleate cells in 3.6% of the population. There were no detectable alterations in the ultrastructure of intracytoplasmic A-type particles or extracellular B-type particles (Fig. 34B), but the numbers of microvilli were greatly reduced. In contrast, exposure of MJY-alpha cells to CD did not induce enucleation or an abundance of zieotic-type blebbing, although binucleated cells were present at a frequency of 5.3% (Fig. 35A, 35B). Electron microscopic examination revealed a significant reduction in numbers of microvilli and in budding virion particles (Fig. 35B), but no alterations in morphology of A- or B-type particles. The cytologic appearances of CB- and CD-treated cells were similar to untreated controls within 24 h of drug removal (Fig.

Fig. 33. Untreated control MJY-alpha cells: A. Confluent monolayer releasing viable cells in situ. Bar= 50 μ. B. Parallel cell layers stained with May-Grünwald-Giemsa (MGG).
Bar = 25 μ.
C. Electron micrograph depicting B-type particles ( ) and microvilli
( ) on cell surfaces. Bar=1 μ.
D. Intracellular A-type particles. Bar = 0.2 μ. E. Extracellular B-type particles. Bar = 0.2 μ.

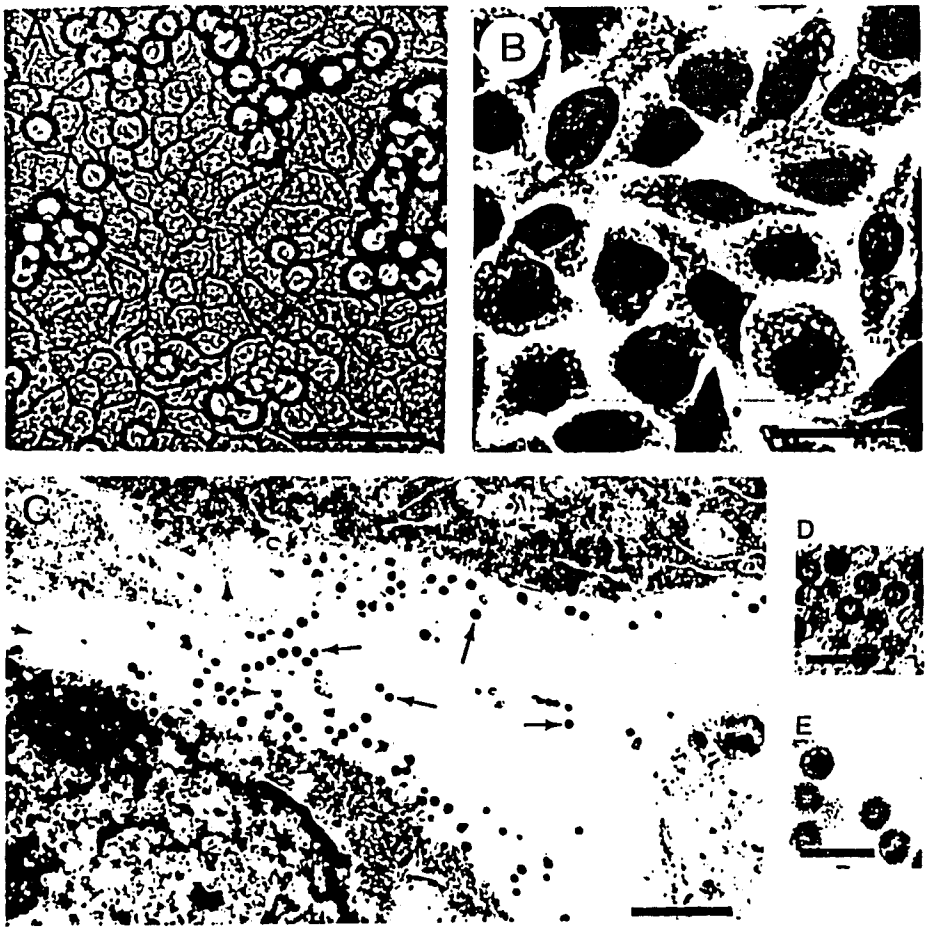

A
B
C
D
E

Fig. 34. MJY-alpha cells treated with CB: A. MJY-alpha cells exposed to 2.1 μM CB for 24 h. Note zeiotic blebbing ( ), and binucleate cells ( ). MGG, Bar = 25 μ.
B. Electron micrograph of cells treated as in A, with B-type ( ) and A-type ( ) particles. Bar = 1 μ.
C. CB-treated cells following 48 h in drug-free medium contained multinucleated cells in the process of nuclear division ( ). MGG, Bar = 25 μ.
D. Electron micrograph of CB-treated cells 48 h after treatment. Bar = 2 μ.

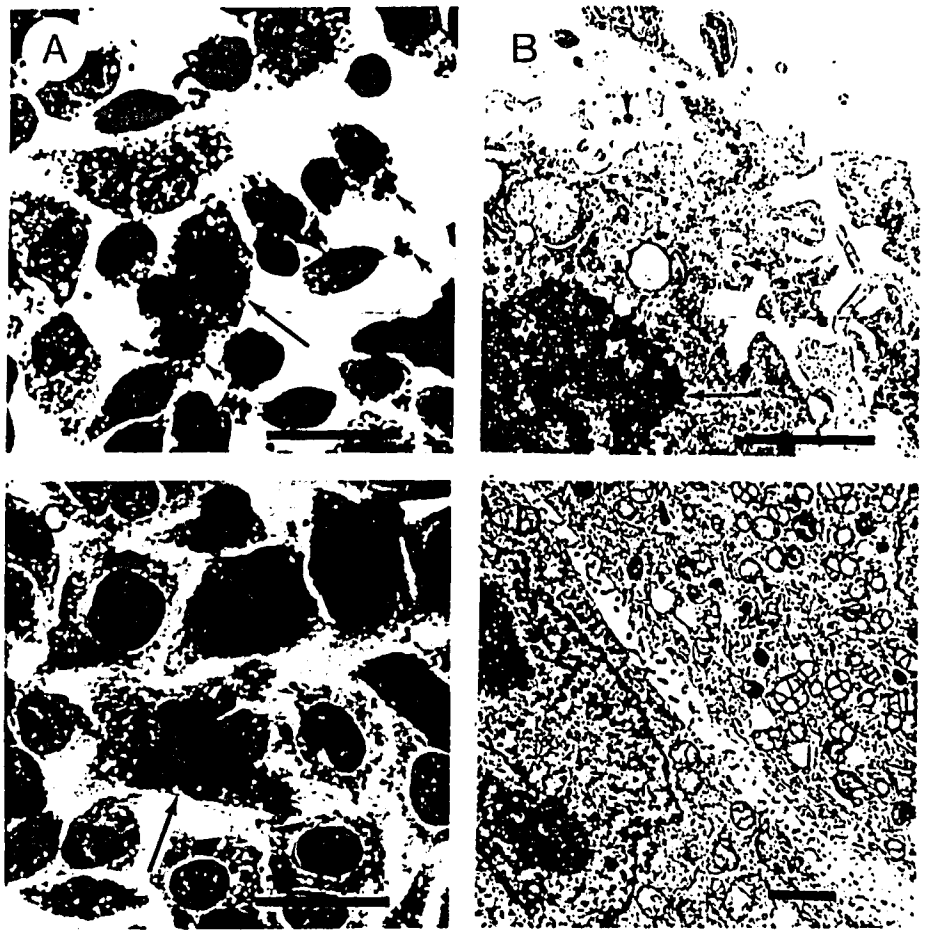
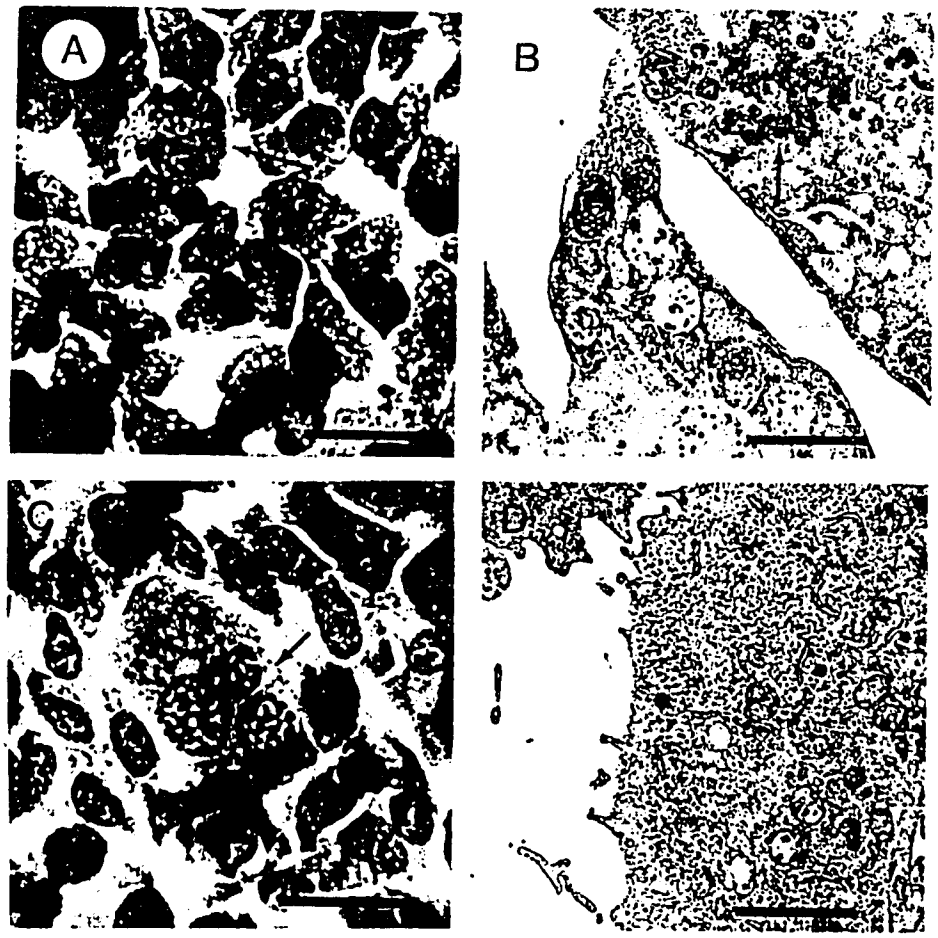

A
B

Fig. 35. MJY-alpha cells treated with CD: A. MJY-alpha cells treated with 2.1 μM CD for 24 h. Note the presence of binucleate cells ( ). Bar = 25 μ.
B. Electron micrograph of cells treated as in A. Clusters of A-type are still present ( ). Bar = 2 μ.
C. CD-treated cells after 48 h in drug-free medium containing multinucleated cells ( ). Bar = 25 μ.
D. Electron micrograph of CD-treated cells 48 h after treatment. Bar = 2 μ.


A
B

34C, 34D, 35C, 35D), although CD-treated cells continued to exhibit decreases in numbers of microvilli and budding virions (Fig. 35D).

Confluent monolayers of MJY-alpha cells treated with 0.28 μM colcemid lacked pseudopodia, and appeared spherical after 48 h exposure, but exhibited no reductions in cellular viability. Monolayers were in stationary growth phase when exposed to colcemid, and only 5.3% of the cells were arrested in mitosis by the end of the 48 h treatment period. Morphologic alterations were reversed within 24 h after drug removal (Fig. 36B). Colcemid had no effect on the ultrastructure of MJY-alpha cells or A- or B-type MMTV particles.

The effects of cytodisruptive agents on cellular metabolism were assessed by measuring glucose utilization, the redox state of treated cells, and their uptake and incorporation of radiolabeled precursors. Both lactate production and the cellular NAD+/NADH ratio in CB-treated cells decreased by 13%, suggesting a reduced rate of glycolysis (Table 29). The congener, CD, did not affect these parameters. Addition of radiolabels during the 24 h CB treatment period revealed a 50-70% reduced uptake and incorporation of ^{3}H-glucosamine, whereas, the levels of ^{14}C-amino acids and ^{3}H-uridine were not affected (data not shown). More sensitive analyses of precursor

Fig. 36. MJY-alpha cells treated with 0.28 μM colcemid for 48 h: A. in situ, Bar = 50 μ. B. Colcemid-treated cells 24 h after return to druq-free medium
Bar = 50 μ.

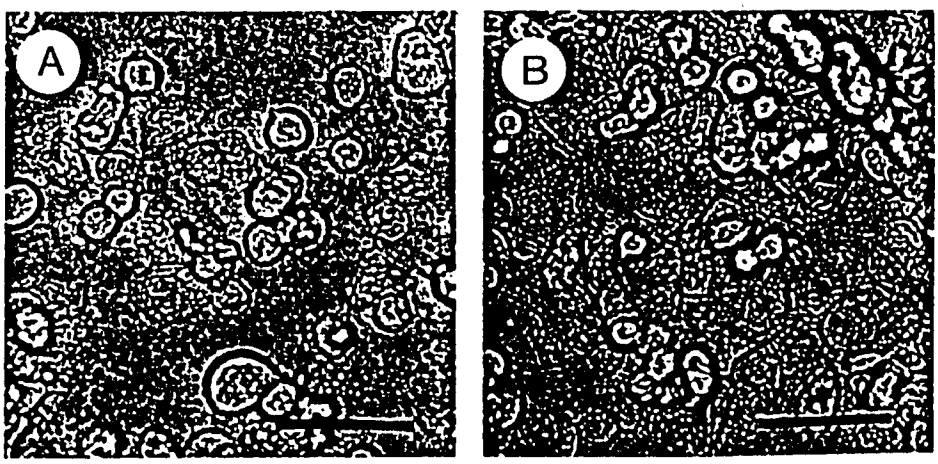

A
B

Table 29. Effect of cytodisruption on the cellular redox state and lactate production.

Treatment[1]	NAD+ / NADH[2]	Lactate produced (μM/petri dish/24 h)[3]
None	420	7.81 +/- 0.37
CB 2.1 μM	355	5.93 +/- 0.40
CD 2.1 μM	410	7.58 +/- 0.62
Colcemid 0.28 μM	-	7.65 +/- 0.19

[1] HC-treated MJY-alpha cells were exposed to cytochalasins for 24 h, and to colcemid for 48 h, and parallel controls were maintained without additions.
[2] Cultures were washed, scraped, precipitated with perchloric acid; lactate and pyruvate were measured enzymatically, and free NAD+ and NADH were calculated according to the procedure of Krebs (1968).
[3] Lactate was chemically determined as described from spent media harvested from treated and control cultures.

uptake and utilization, using 1 h pulse-labeling following 23 h of CB treatment consistently detected reductions of ^{14}C-radioactivity in the TCA-soluble, but not TCA-precipitable cellular fractions (Table 30). Under identical pulse-labeling procedures, no depression was observed when the non-glycogenic amino acid ^{3}H-leucine was substituted for the ^{14}C-amino acid tracer. Unlike CB, CD had no inhibitory effect on uptake and incorporation of any metabolic precursors when examined using 1 h or 24 h labeling procedures (Table 30). In a similar manner, 24 h labeling studies did not reveal any effects of colcemid exposure on uptake or utilization of ^{3}H-glucosamine, ^{14}C-amino acids, or ^{3}H uridine. However, 1 h pulse-labeling studies did reveal that colcemid, like CB, reduced intracellular levels of ^{14}C-amino acids but not ^{3}H-leucine (Table 30).

Studies described in Results section C. 6. demonstrated that little if any, of the cell-associated MMTV is released from MJY-alpha cells as soluble antigen. To determine whether exposure to cytodisruptive agents resulted in detectable increases in shedding of MMTV polypeptides, cleared media supernatants were prepared from drug-treated and control ^{35}S-labeled MJY-alpha cells. SDS-PAGE analysis of anti-MMTV immune precipitates did not detect MMTV precursors, intermediates, or products, demonstrat-

Table 30. Effect of cytodisruptive drugs on uptake and incorporation of amino acids and amino sugars

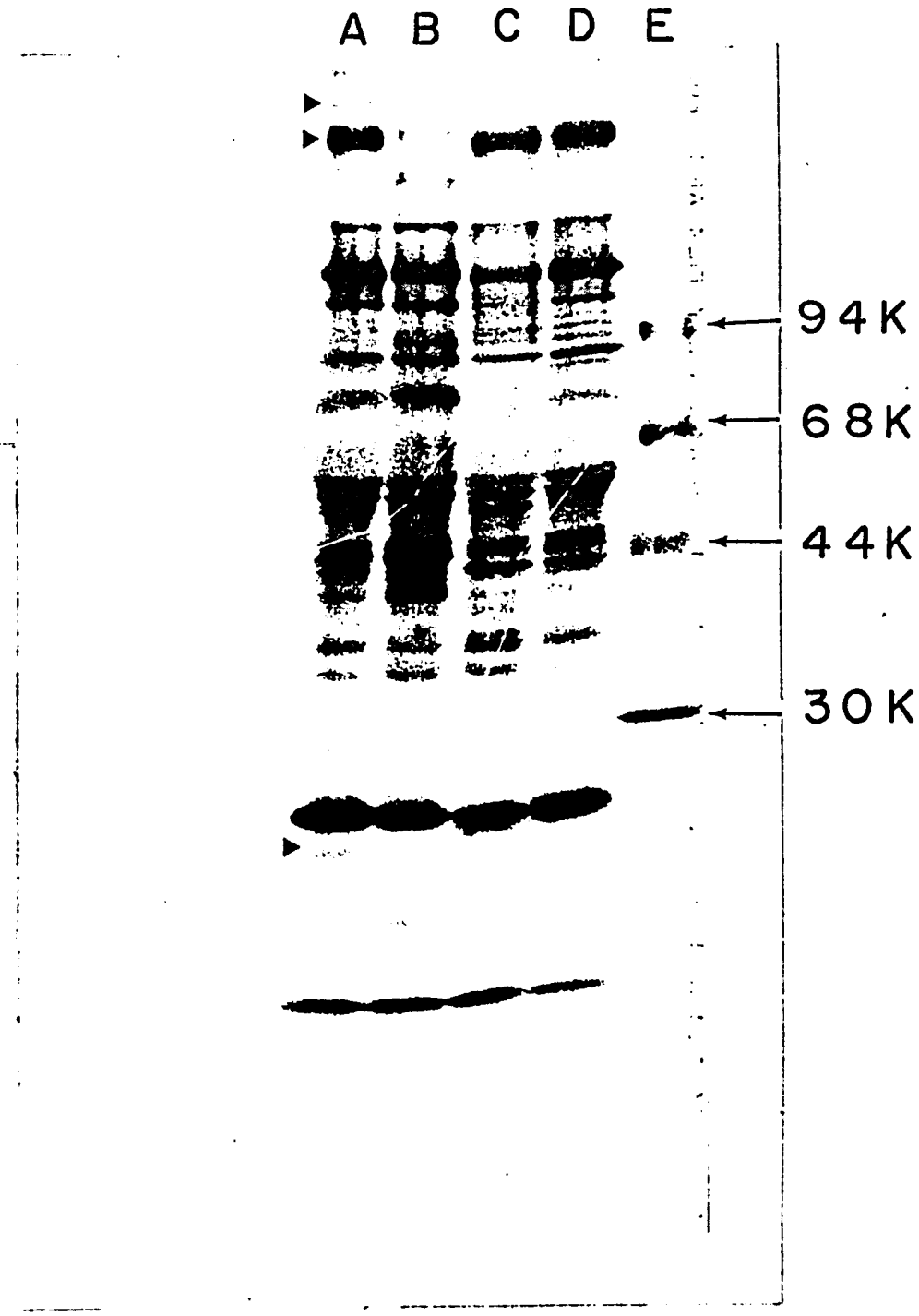
	Relative uptake and incorporation of radioactivity[1]				
	TCA-soluble			TCA-precipitable[2]	
Treatment	^{3}H glucosamine	^{14}C amino acids	^{3}H leucine	^{14}C amino acids	^{3}H leucine
None	1.00	1.00	1.00	1.00	1.00
CB 2.1 μM	0.12	0.68	1.00	0.95	1.00
CD 2.1 μM	1.00	1.00	1.00	1.00	1.00
Colcemid 0.28 μM	0.71	0.78	1.00	0.85	1.00

[1] MJY-alpha cells were treated with cytochalasins or colcemid for 24 h, and radiolabels were added during the final h of treatment. TCA-soluble and precipitable radioactivity were determined as described.

[2] Incorporation of ^{3}H-glucosamine into TCA precipitable material after 1 h labeling of these cultures with 5-10 μCi ^{3}H-glucosamine/ml media was negligible.

inq large amounts of MMTV-associated protein were not shed into culture medium after cytodisruptive drug treatment. Aliquots of cleared media supernatants were also subjected to SDS-PAGE prior to immune precipitation to determine whether druq exposure altered release of non-MMTV, MJY-alpha cell-associated polypeptides. As shown in Fiq. 37, 20-25 ^{35}S-labeled polypeptides were synthesized, and accumulated durinq the 6 h labeling period. Levels of the majority of ^{35}S-labeled polypeptides released into the medium were unaffected by cytodisruption (Fig. 37). However, exposure to CB, CD, or colcemid did result in complete loss of a 200,000 and a 20,000 mw protein, and levels of several additional polypeptides were altered. The identity of these proteins is unknown, and further studies are required to determine the significance of these changes.

Fig. 37. Effects of cytodisruption on release of proteins from MJY-alpha cells. Cleared media supernatants were prepared as described from untreated MJY-alpha cells labeled with ^{35}S-methionine for 4 h (A) or from cells treated with 2.1 μM CB (B), 2.1 μM CD (C), or 0.28 μM colcemid (D) for 24 h, and labeled during the final 4 h with ^{35}S-methionine; mw markers (E) are included for comparison. Experiment was performed 2-3 x and a representative profile is presented.


A
B
C
D
E
94K
68K
44K
30K

Chapter IV

DISCUSSION.

A. MMTV POLYPEPTIDE COMPOSITION.

The protein composition of MMTV includes 6 polypeptide products of the MMTV *env* (gp52, gp37.7-33) and *gag* (p24, p17, p14, p8) genes. Incorporation studies using ^{35}S-labeled polypeptides revealed that gp52, gp37.7-33, p24, p14, and p6 were principal virion constituents. ^{35}S-labeled *gag* protein p24 and *env* proteins gp52 and gp37.7-33 were present in approximately equimolar levels, indicating principal MMTV polypeptides were incorporated into MMTV particles at the same rate during a 24 h labeling period. These ratios remained relatively constant throughout a 48 h chase period, suggesting that intracellular pools of these ^{35}S-labeled proteins were equivalent. Mechanisms regulating intracellular levels and processing of MMTV proteins are poorly understood, and it is not known how pool sizes of *gag* and *env* proteins with different subcellular distributions are maintained. MMTV *gag* proteins have been reported synthesized on free ribosomes (Schochetman and Schlom, 1977), and may be

incorporated into intracytoplasmic type A particles; env proteins were immune precipitated from smooth and rough endoplasmic reticulum, and from plasma membranes. Immune precipitation of radiolabeled lysates of MJY-alpha cells using anti-MMTV antisera yielded only low levels of mature MMTV proteins, making it impossible to directly assess pool sizes of gag and env products.

B-type particles are produced by assembly of MMTV proteins and RNA, with budding from plasma membranes of infected cells. Assuming the population of B-type particles obtained from MMTV-producing cultures is uniform, it is possible to determine the number of MMTV polypeptides incorporated per virion particle by quantitative analysis of ^{14}C-incorporation into each MMTV polypeptide using the method of Adolph and Haselkorn (1972). As illustrated in Appendix A, the number of molecules of each protein can be calculated from relative proportion of total virion-associated protein occupied by that protein, and from the molecular weight of the species. By this method, 1500-2400 molecules of each principal MMTV polypeptide were estimated to be present in each MMTV virion.

Previous studies (Sarkar and Moore, 1974) demonstrated MMTV glycoproteins gp52 and gp37.7-33 were arranged in characteristic, spike-like projections uniformly distributed on virion surfaces. Using published values for the spike dimensions and interprojection distances, the number of surface

spikes on the MMTV virion membrane was determined to be 199-304 (see Appendix B). Calculations based on this value and on the number of glycoprotein molecules per virion suggest that each spike is composed of 6-11 molecules each of gp52 and gp37.7-33. The subunit structure of the MMTV spike has not been extensively investigated, although Racevskis and Sarkar (1980) used the reversible cross-linking reagent dithiobis-succinimidyl proprionate (DTBSP) to identify a multimer with minimum molecular weight of 230,000, consisting of 3 molecules each of gp52 and gp37.7-33. The authors suggested that this polypeptide aggregate comprised the intact MMTV spike. The results presented here indicate that a larger complex of gp52 and gp37.7-33 represents the MMTV spike. A dimer of the structure postulated by Racevskis and Sarkar, including 6 molecules each per spike, would satisfy the minimum calculated stochiometry reported here.

Investigation of MMTV polypeptide pattern under reducing or nonreducing conditions did not detect inter-protein disulfide bonds in MMTV-S. These data are contrary to the findings of Dion, et al., (1979b), who reported gp52-gp37.7-33 and p24-gp37.7-33 disulfide-linked complexes, but are consistent with the data of Racevskis and Sarkar (1980) who did not detect native inter-protein disulfide complexes prior to DTBSP cross-linking under non-reducing conditions. Our findings suggested generation of the eccen-

tric nucleoid of B-type particles does not require inter-protein disulfide bond formation. Other post-budding events, such as cleavage of $Pr38^{gag}$ to p24 and p8, may be responsible for type B particle apearance (Dickson and Atterwill, 1979).

B. CYTOSKELETAL INVOLVEMENT IN MMTV.

Studies reported here establish that the B-type retrovirus MMTV contains actin-like polymerization and DNase I-binding activities, and attribute these activities to the nonglycosylated 44,000 mw polypeptide of MMTV. Similarities in published amino acid compositions of MMTV p44 and rabbit muscle skeletal actin support this conclusion, although sequence analysis of p44 is not yet available. Although present in relatively few copies (350 molecules p44/virion), the presence of p44 is apparently due to its incorporation into budding virions, and not to adsorption to extracellular particles or contamination of actin-containing vesicles with MMTV. Copurification of cellular organelles containing actin was not a source of contamination, as demonstrated by the absence of such structures when preparations of purified MMTV are examined by electron microscopy and the lack of other cellular proteins in MMTV polypeptide profiles gener-

ated by SDS-PAGE (Yaqi, 1973; Yagi and Compans, 1977). In addition, there was no evidence for adhesion of actin to the exterior of MMTV; p44 was not accessible to trypsin in intact virions, and could not be polymerized without prior disruption of the viral envelope, indicating that p44 was located within the virion particle. Assignment of an interior location to p44 is also supported by the inability to label this protein in intact virions by lactoperoxidase-catalyzed iodination (Witte, et al., 1973).

The function of cellular actin in MMTV replication is unknown, as is its role in extracellular virions. Enveloped RNA and DNA viruses representing the retroviruses, ortho- and paramyxoviruses, rhabdoviruses, and poxviruses classes all contain a 44,000 mw protein, suggesting a common mechanism of incorporation or functional role of this protein in the replication schemes of budding virions (Damsky, et al., Hiller, et al., 1979; Naito and Matsumoto, 1978; Tyrrell and Ehrnst, 1979; Wang, et al., 1976). The involvement of actin and other cytoskeletal elements in virus replication has been investigated using the cytodisruptive cytochalasins, vinca alkaloids, and local anaesthetics. Several of these studies indicated the cytoskeleton participates in replication of budding virions. Colcemid- or cytochalasin-induced disruption of cellular microtubules and microfilaments altered production and composition of MMTV virions. These findings were specific and not attribu-

table to the other activities of these agents. The data suggested that disruption of retrovirus replication is another consequence of cellular cytoskeletal interruption.

The precise manner by which cellular structures contribute to MMTV production is unknown. Probable mechanisms include participation in the intracellular movement of protein and viral forms, and in the budding of virion particles from the plasma membrane. Altered processing of MMTV glycoproteins was suggested by immune precipitation of $Pr76^{env}$ with decreased electrophoretic mobility from drug-treated cells. Immunoelectron microscopic studies were not carried out to determine whether cytodisruptive drugs altered local surface distribution of MMTV antigens. Cytochalasins could directly inhibit MMTV maturation at cell membranes by eliminating interactions between microfilaments and budding virions, or indirectly by nonspecifically reducing microvilli formation. Analysis of cell surfaces of drug-treated cells by FACS detected only minor alterations in cell surface reactivity to anti-MMTV antisera, despite the great reduction of budding particles as revealed by electron microscopic studies.

Concomitant with alterations in MMTV production was the presence of novel virion-associated proteins, as well as, changes in relative levels of MMTV polypeptides, and decreases in glycoprotein $^{3}H/^{35}S$ ratios. The origin of new virion-associated proteins, which were detected in drug-

treated cells by immune precipitation with anti-gp52 antisera, is unknown; they may represent products of altered viral or cellular glycoprotein processing which cross react with anti-MMTV and anti-gp52 antisera, or they may be contaminants adhering to MMTV polypeptides. The contribution of these proteins to ^{35}S-radioactivity in immunoprecipitates used for virus quantitation may artifactually overestimate amounts of virions produced in drug-treated cultures. Consequently, if MMTV production is quantitated by determining levels of known MMTV proteins in purified MMTV from control and drug-treated cultures, a 50% increase in the first 18 h of CB treatment, followed by a 3-4 fold decrease between 18-24 h is observed, compared to the 1.7-2.5 fold increases in MMTV for these time points obtained by immunoprecipitation. Determination of MMTV production from cultures treated with CD or colcemid by quantitation of known virion polypeptides yields values 10-15% lower than levels detected by immune precipitation. Reductions in MMTV yield were consistently observed, and may be attributed to inclusion of high molecular weight proteins in the immunoprecipitates.

In addition to changes in MMTV production, relative levels of virion polypeptides gp60, p44, and p24 were also affected by cytodisruption. Gp60 has been shown to be extremely sensitive to proteolytic cleavage, and it has been suggested that this glycoprotein may be an uncleaved form of major virion glycoprotein gp52 (Yagi, et al., 1978c).

The level of gp60 present in MMTV particles is dependent on the environment of the MJY-alpha cells, and is increased by exposure to anti-MMTV antibody, (Yagi, et al., 1978a) or to exogenous mouse interferon (Yagi, et al., 1980). The mechanism for these increases in gp60, including those observed after CB, CD, or colcemid exposure is unknown. Changes in the levels of p44 and the major core protein p24 may be the result of independent activities of the drugs on MMTV synthesis and maturation. Alterations in these polypeptides have never been reported. P24 is located in the core of the virion particle, and decreases in its level following drug exposure suggests these virion particles may be biologically altered. Further studies are required to determine the significance of these alterations, and to clarify the mechanism of the virion alterations resulting from disruption of cellular cytoskeleton.

C. INTRACELLULAR PROCESSING OF MMTV PROTEINS.

Four MMTV glycoprotein precursors and products were identified in MJY-alpha cells: precursors $Pr79^{env}$, and $Pr76^{env}$, which are exclusively cell-associated, and the glycoproteins gp52 and gp37.7-33, which are located in MMTV virions and infected cells. In addition, a presumed viral glycoprotein,

gp60, was also detected in both virions and cells; whether this glycoprotein is a product of the MMTV env gene has yet to be firmly established. Previous in vitro translation studies indicated $Pr76^{env}$ was the initial polyprotein product of the env gene. The other precursor, $Pr79^{env}$, was detected by immune precipitation with anti-gp52 antisera in MMTV-S, but not in MMTV-P-producing mouse mammary cells (Racevskis, and Sarkar, 1978, Dickson and Atterwill, 1980). Anderson and coworkers (1979) demonstrated $Pr79^{env}$ contained fucose, a marker for terminal glycosylation, and suggested the relatively fucose-deficient $Pr76^{env}$ was further glycosylated to $Pr79^{env}$, and subsequently cleaved to gp52 and gp37.7-33. However, Sarkar and Racevskis (1983) recently reported relatively large amounts of fucose-containing $Pr76^{env}$ shed into the medium of MMTV-S-infected MuMT73 cells. They suggested $Pr79^{env}$ was an altered processing product of uncleaved $Pr76^{env}$ which was released intact from infected cells. Firestone (1982) detected $Pr79^{env}$ only in HC-treated HTC-rat cells transfected with the MMTV genome, and suggested it was an altered glycosylation product of $Pr76^{env}$ found exclusively in HC-treated cells. However, in these studies, $Pr76^{env}$ and $Pr79^{env}$ were present in control, HC-stimulated, and HC-attenuated MMTV-S-infected MJY-alpha cells. HC treatment was not required for $Pr76^{env}$ synthesis. In this cell line, steroid treatment appeared to affect env precursor processing in MJY-alpha cells. HC exposure did result

in 5-fold decreases in the half life of $Pr76^{env}$, and in altered relative incorporation of ^{3}H-mannose and glucosamine into Pr76 and $Pr7^{env}$. ^{35}S-methionine pulse precursor processing in MJY-alpha cells. ^{35}S-methionine pulse labeling studies simultaneously detected $Pr76^{env}$ and $Pr79^{env}$; Further incubation of these cells in isotope-free medium for 12 h did not result in any cellular accumulation of $Pr79^{env}$. Sensitive immune precipitation studies also did not identify this polypeptide in spent culture medium from MJY-alpha cells. These data suggest $Pr76^{env}$ is processed to a highly glycosylated, short-lived intermediate, $Pr79^{env}$. $Pr79^{env}$ is then probably processed by proteolytic cleavage to gp52 and gp37.7-33, as suggested by Anderson (1978). Neither Dickson (1980) nor Racevskis and Sarkar (1983) identified a cell-associated gp37.7-33 containing mannose. However, immune precipitation studies presented here identified a band of ^{3}H radioactivity in the entire gp37.7-33 region after ^{3}H-mannose labeling, suggesting mannose is incorporated into all sizes of this heterogeneous polypeptide. This conflict with previous reports probably represents altered patterns of glycosylation among the different mammary tumor cell lines.

D. EFFECTS OF HC ON MMTV.

MMTV production in *in vitro* mammary tumor cell culture is stimulated by exposure to glucocorticoids. Interaction of hormone-receptor complexes with specific sequences of DNA stimulated transcription of MMTV RNA resulting in increased levels of MMTV protein and virions. Results of immune precipitations revealed short-term exposure of MJY-mammary tumor cells to 14 μM HC resulted in 2-5 fold increases in synthesis of MMTV proteins; however, the quantity of total cell-associated MMTV protein in HC-stimulated and control MJY-alpha cells were roughly equivalent (1.1-1.2 fold greater than control). More rapid processing of MMTV precursors in HC-stimulated cells, detected by pulse chase studies of $Pr76^{env}$, may prevent increases in cell-associated MMTV protein.

Prolonged exposure of MJY-alpha cells to 14 μM HC resulted in reductions in MMTV yield and in MMTV protein synthesis to levels obtained in untreated cells. The mechanism of this reduction is not known although glucocorticoid responsiveness was detected by treatment with high HC concentrations or by intermediate culture in HC-free medium. Attenuation of glucocorticoid-induced alterations in other steroid-responsive cell types has not been reported. However, similar results have been obtained in polypeptide hormone-receptor systems; prolonged exposure of insulin-responsive cells in vitro to insulin resulted in diminished glucose transport due to decreased levels of cell surface insulin

receptors. Analoqous *in vivo* or *in vitro* examples of steroid-induced "down requlation" of qlucocorticoid receptors have not been reported, although lack of response after prolonqed, hiqh-dose steroid treatment of certain immune diseases has been described. It is not known whether these therapeutic failures are related to steroid-induced attenuation of responsive cells.

Alternatively, it is possible that glucocorticoid treatment selected steroid-resistant MJY-mammary tumor cells, or induced increases in levels of steroid-metabolizinq enzymes. However, siqnificant effects of HC exposure on growth rate or viability of MJY-alpha or beta cells were not detected, and HC responsiveness was restored within one passage in druq-free medium. In a preliminary study, equivalent stimulation of MMTV in HC-attenuated cells was detected with HC or dexamethasone (a non-metabolizable glucocorticoid), sugqestinq steroid metabolism was not a significant factor in preventinq MMTV stimulation.

Exposure of MJY-alpha or beta cells to HC resulted in relatively low (2-5 fold) increases in MMTV production compared to stimulations reported in other cell lines. Levels of MMTV produced in MJY mammary cultures maintained in the absence of exoqenous steroid are relatively high (5-10 μg/1 x 10^8 MJY-alpha cells) and MJY mammary cells may be sensitive to low level of endoqenous cortisol present in fetal bovine serum (1 x 10^{-8} M) which may stimulate MMTV produc-

tion in the absence of exogenous steroid. Previous reports by Dickson (1974) demonstrated that similar low steroid concentrations increased MMTV production in mMtc-5 cells. Alternatively, MJY-mammary tumor cells may be relatively resistant to glucocorticoid stimulation. As a result, it is not known whether these findings of prolonged HC treatment may be applied to other MMTV-producing tumor and non-neoplastic mammary epithelial cells lines, or generalized to other steroid-responsive systems.

E. SUSPENSION CULTURES.

In vitro cultivation of mouse mammary tumor cells has employed solid substratum for cell proliferation and MMTV production, but the requirement of adhesion for cell growth or virus production has never been evaluated. As described, the epithelial-like MJY-alpha mammary tumor cell line was successfully adapted to growth in suspension. Examination of some of the growth and metabolic characteristics of the suspension (MJY-beta) cells revealed differences compared to the parental monolayer cells. Saturation density of MJY-beta cells was not static, and could be elevated two-fold by increasing the frequency of culture media replenishment, suggesting cell growth was inhibited by exhaustion of nut-

rients, or by accumulation of waste products, or both. It is not known whether similar increases of cell number could be effected in monolayer cultures of MJY-alpha cells, in which growth rate slows as cells become confluent. Differences in the tumorigenicity of MJY-alpha and beta cells have not been investigated. Heppner and coworkers (1972) have obtained subsets of cells varying in tumorigenicity, growth rate, morphology, and MMTV expression from a single mammary tumor, and further study is required to determine whether MJY-alpha and -beta cells also exhibit differential in vivo growth characteristics.

Significant increases in MMTV production were detected after cultivation of MJY-mammary tumor cells in suspension, demonstrating MMTV production in vitro did not require polarized cellular organization resulting from adherence to a solid substratum. Increases in MMTV production were detected without concomitant alterations in cell growth, suggesting the rate of MMTV production per cell was independent of cell replication. The reason for MMTV stimulation in suspension culture is unknown. Although increases in lactate production and more stringent serum requirements suggested a more rapid metabolic rate in MJY-beta cells compared to MJY-alpha cells, it is not known whether these alterations increased MMTV production directly, or were independent effects of suspension culture. Determinants of MMTV production in mammary tumor cells are not known. Prior investiga-

tions (McGrath, 1971; Nagle and Fine, 1978; Fine, et al., 1976) revealed certain medium supplements enhanced MMTV production in mammary tumor cells grown in chemically defined medium in vitro, but effects of altered cultivation techniques on MMTV production have not been previously described. It is possible that cultivation on inert substrata inhibits optimal MMTV production. MJY-alpha cells release virions only from anterolateral cell surfaces, while cultivation in suspension permits MMTV production from the entire plasma membrane. However, estimation of MMTV production per unit of available cell surface area revealed differences in surface area did not entirely account for the large increases in virus production (Appendix C). Polarized organization of organelles in monolayer cells may have inhibited optimal MMTV production. It is possible released cells (1-5% total cell number per monolayer culture) may represent the principal source of MMTV, producing MMTV at a rate similar to that of suspension cells. Alternatively, cultivation in suspension may result in greater synthesis or more efficient processing of MMTV polypeptides into virions.

Chapter V

CONCLUSIONS

These studies addressed several aspects of MMTV composition and replication:

Analysis of MMTV polypeptide composition revealed principal gag (p24, p14, p8) and *env* (gp52, gp37.7-33) polypeptides were present in approximately equivalent amounts. This stochiometric relation was not due to inter-protein disulfide bonds. An additional virion polypeptide, p44, was detected in 5-6 fold lower levels than other MMTV proteins; p44 exhibited DNase I-binding and polymerization-like activities similar to the cellular protein, actin, suggesting a cytoskeletal polypeptide incorporated into extracellular MMTV particles.

Involvement of cytoskeletal elements in MMTV production was demonstrated in MJY-alpha cells. Disruption of microfilaments by cytochalasins or of microtubules by colcemid resulted in altered production and composition of extracellular MMTV virions. The lack of significant effects on MMTV polypeptide synthesis suggested that an intact cytoskeleton was required for proper MMTV maturation and release from infected cells.

Studies of cell-associated MMTV polypeptides were facilitated by development of specific immune precipitation, ELISA, and FACS techniques. Immune precipitation identified 15 ^{35}S-labeled polypeptide precursors and products of MMTV env and gag genes after labeling MJY-alpha cells with ^{35}S-methionine. Similarly, glycosylated forms of MMTV env precursors $Pr76^{env}$ and $Pr79^{env}$, and products gp52 and gp37.7-33 were identified using carbohydrate labels ^{3}H-glucosamine and ^{3}H-mannose In addition, intracellular sulfated forms of gp52, gp37.7-33, and $Pr76^{env}$ precursor were identified, although the only sulfated env protein in extracellular virions was gp52.

Addition of HC to the culture medium increased numbers of MMTV particles chronically shed from MJY-alpha cells within 24 h. Short-term glucocorticoid exposure also affected env protein processing; the half-life of Pr76 was reduced 5-fold after HC treatment and relative incorporation of ^{3}H into $Pr76^{env}$ and $Pr79^{env}$ after ^{3}H-mannose or ^{3}H-glucosamine labeling was altered. Prolonged HC exposure abrogated the stimulatory effects, and MMTV production returned to levels equivalent to untreated cultures. However, HC responsiveness could be restored in these cultures by passage in HC-free medium. These studies demonstrated a more complex interrelationship among MMTV production, mammary tumor cells, and HC exposure than previously suggested.

MJY-alpha tumor cells were adapted to grow in suspension. Cellular morphology and growth rate of these MJY-beta cells were not altered, although changes in metabolic parameters were identified. Cultivation in suspension resulted in increases in MMTV production which were independent of steroid-induced stimulation of MMTV. These findings demonstrated that *in vitro* growth of epithelial mammary tumor cells, and production of MMTV do not require a particular morphologic orientation or a solid substratum.

APPENDIX A

Determination of numbers of protein molecules per virion particle using the method of Adolph and Haselkorn (1972):

Number of protein molecules, Ni, per MMTV particle is determined by:

$$Ni = \frac{mpi}{wi}$$

where m=mass of protein per particle=2.66 x 10^{-8}

(Sarkar et al., 1974)

pi = fraction of total protein in polypeptide i

wi = molecular weight of polypeptide i

^{14}C incorporation into MMTV polypeptides after 24 h ^{14}C-amino acid labeling was used to detemine the fraction of total protein in polypeptide i, as reccommended by Adolph and Haselkorn (1972).

APPENDIX B

Determination of number of MMTV spikes per virion particle.

For closest packing of spikes on MMTV surface:

$$\text{no. of MMTV spikes/particle} = \frac{\text{surface area of MMTV particle}}{\text{effective area of each spike}} \times 0.74$$

1. Surface area of MMTV particle:
   a. Assume a spherical virion particle with diameter = 105 nm
   b. Surface area of sphere =4 pi r2

      Surface area of MMTV particle = 4 pi (52.5 nm)2

      $= 3.464 \times 10^{-14}\ m^2$

2. Effective area of MMTV spike.

   MMTV spikes, arranged in a hexagonal array, have a diameter of 54.4 Å, and interprojection distance of 73.7 Å (Sarkar and Moore 1974

   Effective area of each spike

$$= \text{pi} \left(\frac{\text{spike diameter}}{2} \right)^2 + \left(\frac{\text{interprojection dist.}}{2} \right)^2$$

$$= pi \frac{54.4\,Å}{2} + \frac{73.7\,Å}{2}$$

$$= 1.2886 \times 10^{-16}\ m^2$$

$$\text{No. of spikes per particle} = \frac{3.464 \times 10^{-14}\ m^2}{1.2888 \times 10^{-16}\ m^2} \times 0.74$$

$$= 199 \text{ spikes}$$

N. B. : MMTV diameter estimate may be artifactually low due to drying. If larger diameters reported by Banq (130 nm, 1956a) for calculations, number of spikes per virion becomes 304.85.

No. of qlycoprotein molecules per spike.

Assuminq all virion-associated qlycoprotein molecules are incorporated into a spike structure:

$$\text{no. of molecules/spike} = \frac{\text{no. of qlycoprotein molecules per virion}}{\text{no. of spikes per virion}}$$

qlycoprotein	no. of molecules per spike if 199 spikes/virion	no. of molecules per spike if 304.8 spikes/virion
qp52	9.1	5.9
qp37.7-33	10.9	7.1

APPENDIX C

Comparison of available surface area for virus budding on MJY-alpha and beta cells.

1. MJY-alpha cells.

a. MJY-alpha cells produce MMTV virion only at anterolateral plasma membranes.

b. MJY-alpha cells have a polygonal shape with cell diameter=14.7 X 10^{-6} and a height above petri dish of 5 X 10^{-6} m

c. Assuming a cuboidal shape (giving greatest surface area):
Area = 4 length X height + length
available surface area = 4.96 x 10^{-10} m^2

d. Assuming a cylindrical shape with diameter 14.7 X 10^{-6} m and a height of 5 x 10^{-6} m (giving minimum surface area)
available surface area = 4 X 10^{-10} m^2

2. MJY-beta cells.

1. Assume a spherical cell with measured diameter = 14.7 X 10^{-6} m

2. Surface area = 4 pi r2
= 6.79 X 10^{-10} m^2

Surface area of MJY-beta cell is only 1.7 x greater than the smallest typical MJY-alpha cell; increases in surface area cannot completely account for increases in MMTV production in MJY-beta cells.

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