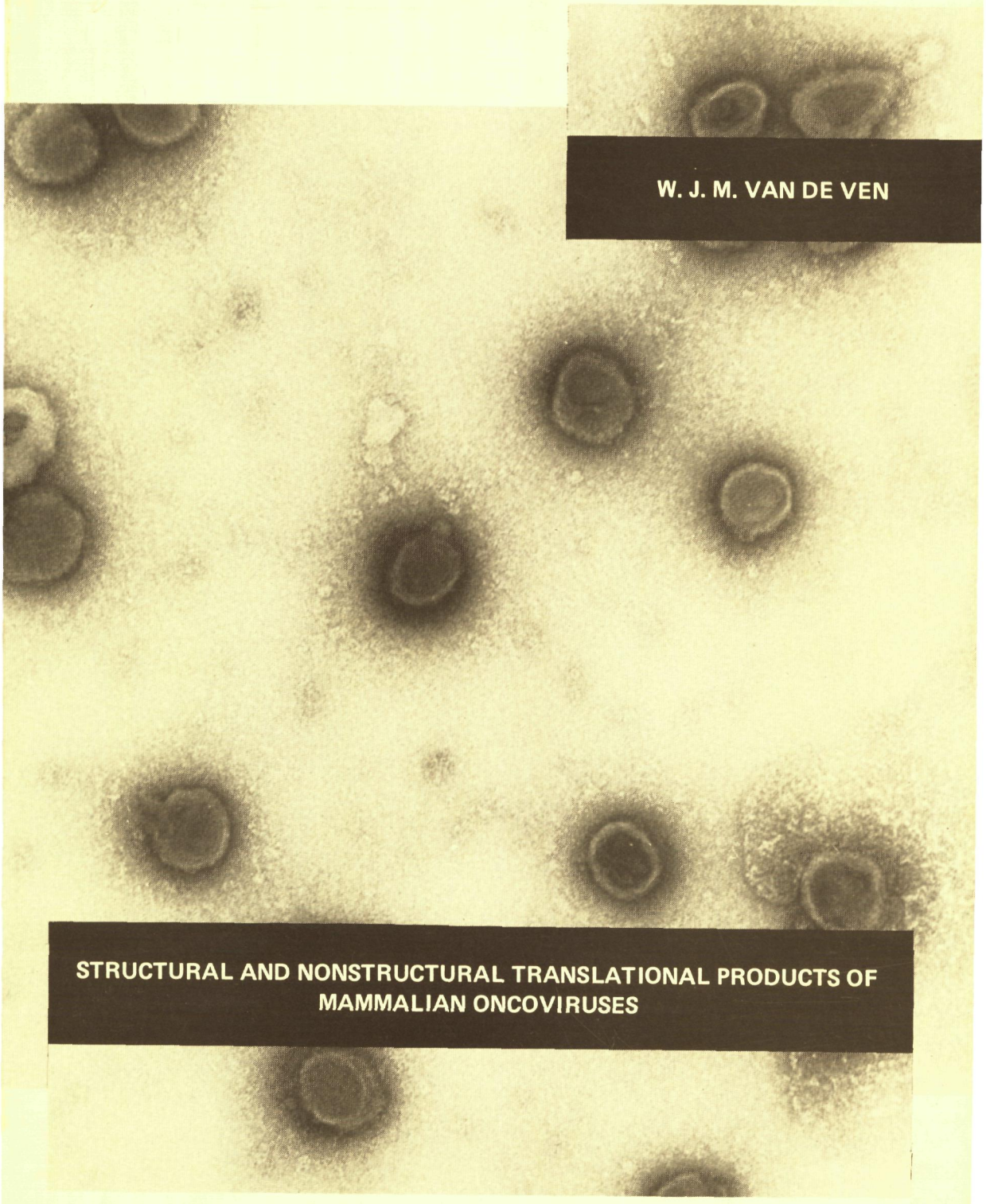

W. J. M. VAN DE VEN

STRUCTURAL AND NONSTRUCTURAL TRANSLATIONAL PRODUCTS OF MAMMALIAN ONCOVIRUSES

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Cover photograph: Electron micrograph of Rauscher murine leukemia virus particles.

Voor Elly

Aan mijn vader en moeder

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CONTENTS

CHAPTER I

INTRODUCTION

There is abundant evidence that along with other carcinogens of physical or chemical origin, viruses can induce tumors and are associated with neoplasia in a broad spectrum of vertebrate species (1-4). Since the first succesful demonstration of tumor induction by a virus in the early 1910s (5-7), numerous tumor inducing or oncogenic viruses have been isolated, both DNA viruses and RNA viruses. The oncogenic DNA viruses include papova viruses, adenoviruses, and herpesviruses while oncoviruses are the only group of RNA viruses able to cause tumors (4). Upon in vivo inoculation into appropriate hosts, oncogenic viruses induce neoplasms of a large variety of histological types. Details about the oncogenic effects of tumor inducing viruses on animals are described by Gross (1).

The oncoviruses, which are studied in the present thesis, induce mainly neoplasms of hemopoietic origin (leukemias) or neoplasms of connective tissue (sarcomas) but they can also induce neoplasms of epithelial origin (carcinomas) (1,4). The mechanism of neoplastic transformation has still to be settled. However, there are theories postulating that the transition of a cell from a normal to a transformed phenotype may be either the consequence of a modified expression of the cellular genome (oncogene theory postulated by Huebner and Todaro (8)), or of a modification of the genetic content of the cell (protovirus theory postulated by Temin (9)). Thus, the geretic information for neoplastic transformation may already be present in the cell but is generally under repression. Alternatively, geretic information is introduced into the cell genome by genetic transformation (i.e the integration of new genes) or mutation. In the last decade, these two not completely exclusive alternatives, have strongly influenced the development of theories on cancer and attempts to solve the problem of its etiology.

In early studies on the pathogenesis of the oncoviruses, basic aspects of neoplastic transformation were examinec ir animals (5-7). At the present time, however, most of the research on these viruses can be performed in tissue culture although neoplasia is a disease which is defined only in whole organisms. An analog of neoplastic transformation, called cellular transformation, has provided an in vitro model for quantitative

studies on neoplastic transformation (10-12). Thus, cells altered in vitro by oncoviruses or by other oncogenic agents are essentially the same in many newly acquired biological and biochemical properties as cells cultured from neoplasms induced in animals by those agents. Moreover, they often develop into tumors when transferred to healthy animals under proper conditions (13). The introduction of in vitro models for cell-virus systems was a major improvement of the research on the virus-induced transformation. The role of viruses in human cancer is still obscure. Yet, the study of virus-induced transformation is relevant to a better understanding of cancer in general because many oncogenic viruses are vehicles of transforming genes with functional or structural homology to cellular genetic information. The viral genome is less complex than the cellular genome by several orders of magnitude. Thus, through the isolation of viruses, transforming genes are directly accessible to experimentation. "It is indeed this aspect of viruses that makes them invaluable to the biologist, whom they present with the unique opportunity to observe in relative isolation the active determinants of biological specificity, which are truly the stuff of which all life is made".*

The aim of the study described in this thesis was to investigate the structure and assemblage of oncoviruses, and to characterize the expression of the viral genomes in order to provide further insight in the molecular biology of oncoviruses. Therefore, we analyzed viral and subviral structures by electronmicroscopic and biochemical techniques and we studied the synthesis of virus-specific structural and nonstructural polypeptides in virus-infected tissue culture cells and in a protein synthesizing system (oocytes of Xenopus laevis). Moreover, we studied the viral genome in transfection experiments.

We described a method for the preparation and purification of oncoviral substructures, which is in fact the first method for the isolation of pure viral envelopes. It enabled us to further characterize the structural virus-specific polypeptides in these viral substructures. In studies on gene expression of Rauscher murine leukemia virus either under normal physiological conditions or under conditions of impaired glycosylation we contributed to the elucidation of the pattern of viral

* Quoted from last sentence of reference 14.

structural polypeptide synthesis. Subsequently, using a similar experimental approach, we could further characterize temperature-sensitive mutants of Rauscher murine leukemia virus of different classes. Moreover, in an extension of studies on gene expression, we could identify transforming virus-coded polyproteins containing leukemia virus-specific structural components covalently bound to nonstructural components, possibly representing transformation-specific sequences. Finally, in another approach to study viral genes, we showed that by transfecting mouse cells with purified DNA isolated from cells releasing high titers of oncoviruses, we could introduce new viral genes into the cellular genome. In virus rescue experiments we showed the rescue of oncoviruses from cells infected with a temperature-sensitive oncovirus and transfected with low molecular weight proviral DNA fragments of a wild-type oncovirus.

As an introduction to the experimental work described in this thesis we will present some relevant aspects of the molecular biology of oncoviruses, followed by a preface to the papers.

CLASSIFICATION AND STRUCTURE OF ONCOVIRUSES

Taxonomically, oncoviruses or oncovirinae constitute one of the three subfamilies of the retroviridae; the two other subfamilies are represented by the spumavirinae (foamy virus group) and the lentivirinae (Maedi/visna group) (15). Oncoviruses, are enveloped, spherical viruses with a diameter of approximately 100 nm; they contain a virus-coded RNA-dependent DNA polymerase and a diploid RNA genome encapsidated within a protein core (16,17). They consist of proteins (about 65%), lipids (about 30%), carbonydrates (about 3%) and RNA (about 2%) (2). The majority of the virion polypeptides are virus-specific and are coded for by the high molecular weight genomic RNA of the virus; in contrast most of the other components such as RNA species with lower molecular weights than the genomic RNA, carbohydrates and lipids are mostly cellular components derived from the infected cell.

Since their initial discovery by Ellerman and Bang (5) and Rous (6), oncoviruses have been isolated from a large number of species; they are apparently ubiquitous among vertebrates. Representative isolates

have been obtained from primates (baboons), domestic animals including avian species (chickens, sheep, pigs, cattle), small rodents (mice, hamsters, rats), reptiles (snakes), and piscine species.

The oncoviruses have been classified primarily on the basis of morphological criteria and, according to this classification, they have been designated as type-A, type-B, type-C or type-D (18,19,20). The designation "type-A" is used for virus-like particles that are present intracellularly in a variety of mouse tumors (21). These particles are often found associated with the endoplasmic reticulum (22). Type-B oncoviruses, characterized by their eccentrally located nucleoids and the presence of characteristic spikes on their envelopes (23), have been isolated from tumors of the mouse (24), the quinea pig (25,26) and cattle (27,28). Most extensively studied is the type-B virus of the mouse, the

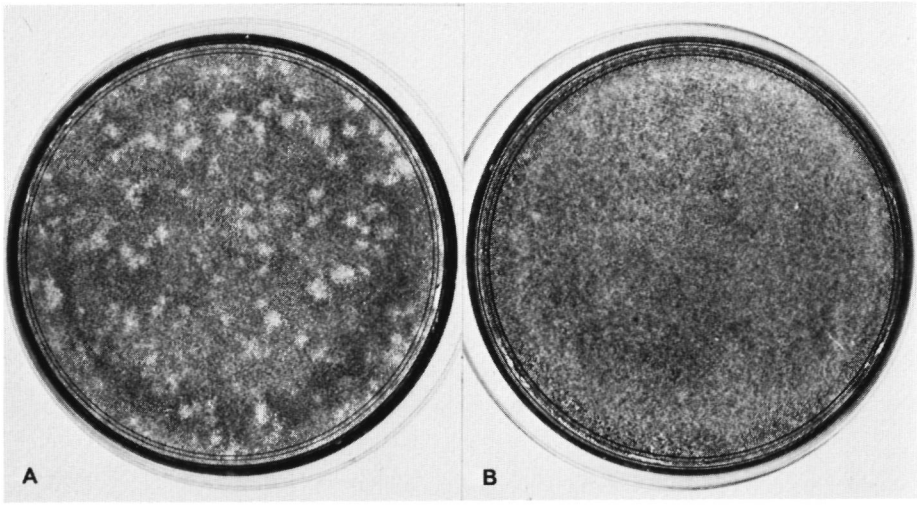

Fig. 1) XC-plaque assay on mouse embryo fibroblasts infected with Rauscher murine leukemia virus. Mouse embryo cells were infected with Rauscher murine leukemia virus (plate A) or mock infected (plate B). After incubation for 6 days, the mouse cells were UV irradiated and XC cells were added. XC cells are derived from a rat tumor induced by the avian Rous sarcoma virus. These cells form syncitia when cocultivated with murine leukemia virus producing mouse embryo cells. Three days later cells were fixed with methanol and stained with hematoxylin. The development of visible syncitial plaques in the infected preparation is evident. No plaques appear in the control, uninfected preparation.

mouse mammary tumor virus (29,30,37). Based upon similar serological characteristics of their major structural proteins, type-B particles probably represent maturational products of intracellular type-A particles (31,32). The main morphological characteristic of type-C oncoviruses are their centrally located nucleoids and the virion assembly pattern during the budding process at the plasma membrane of the infected cell (23). Type-C viruses are widely spread among mammals. Moreover, all known non-mammalian oncoviruses belong to this morphological group. Finally, type-D oncoviruses are larger in diameter than type-B or type-C viruses and have pleomorphic shaped nucleoids (20). Representative isolates of this type are the Mason-Pfizer monkey virus (33,34), an oncovirus endogenous to the langur monkey (35) and possibly a number of isolates of squirrel monkey origin (35,36).

Another classification criterion is pathogenicity. The mouse mammary tumor virus mentioned above is the only oncovirus associated with a carcinoma (37). Other oncoviruses cause leukemias of various types or sarcomas. Most leukemia viruses and avian sarcoma viruses are independent viral entities. Some avian oncoviruses, the mammalian sarcoma viruses and several other mammalian viral isolates require leukemia viruses as helper viruses for their replication (38,39,40). They occur as so-called pseudotypes, that is their genomes are encapsidated with the aid of their helper viruses. In the absence of helper virus they can be latently present in so-called nonproducer cells from which they can be rescued again upon infection by a helper virus (2). The replication-defective oncoviruses appear to have arisen as a result of genetic recombination between type-C oncoviral and host cell genetic sequences, that may code for malignant transformation (41-44). Representative isolates have been obtained from four different mammalian species including two rodent species, the mouse (45-48) and rat (49,50), one carnivore species, the cat (51,52), and one primate species, the woolly monkey (53). The leukemia and sarcoma viruses include viruses that do not occur in nature but apparently originated from recombinational events that were selected for during decades of cancer research in experimental animals. Finally, it should be mentioned that a large number of type-B, type-C or type-D viruses are endogenous, i.e. their proviruses are stably integrated in the germ line of many if not all avian and mammalian species. Sometimes they can be detected in hybridization experiments using cDNA

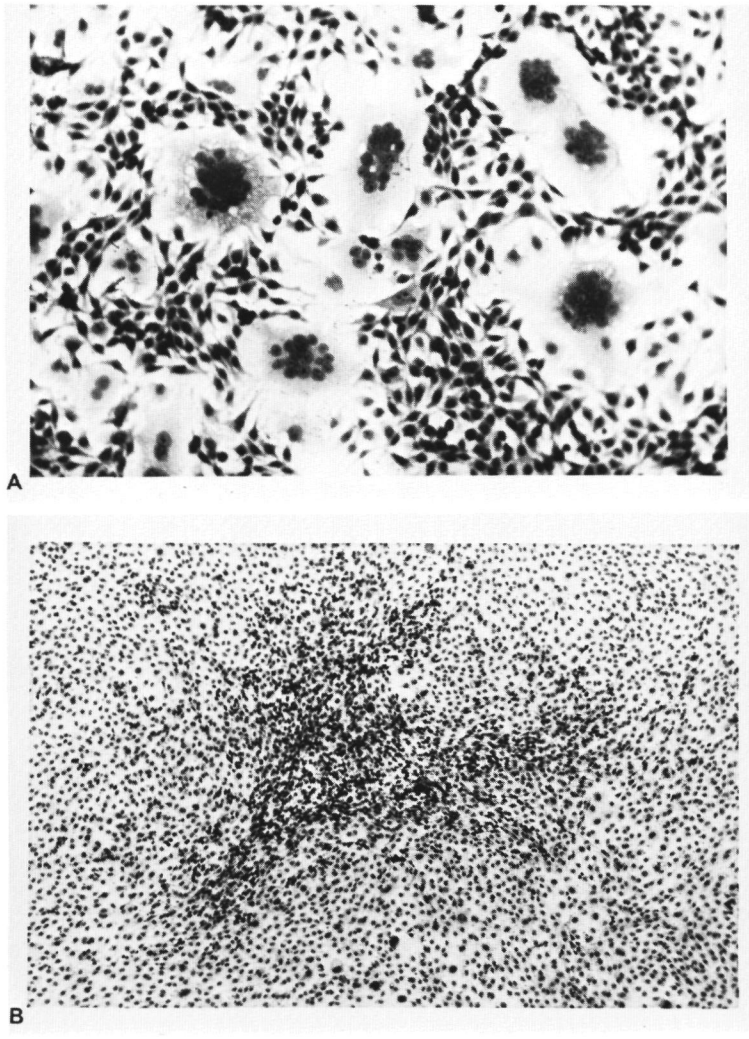

Fig. 2)
A) Syncitia in an XC-plaque induced by Rauscher murine leukemia virus.
B) Focus of transformed mouse embryo fibroblasts after exposure to Kirsten sarcoma virus.

of related viruses as a probe; sometimes virus expression is induced in various ways. In other cases endogenous viruses are released "spontaneously". Although for historical reasons, endogenous type-C viruses are sometimes referred to as leukemia viruses, their implication in the development of tumors is only well documented for endogenous mouse viruses. The biological significance of endogenous viruses is not understood. In view of the fact that only mammalian type-C oncoviruses and type-C pseudotypes of replication-defective transforming oncoviruses were used in our studies, we will restrict this introduction mainly to these categories. Oncoviruses of both categories can be assayed in tissue culture by various techniques (2). A generally applied assay for autonomously replicating ecotropic murine leukemia viruses is the XC-plaque assay. This assay is based on the observation that mouse cells releasing these viruses form syncitia when cocultivated with XC cells (Figure 1A+B and Figure 2A). In general, autonomously replicating murine leukemia viruses do not transform mouse embryo fibroblasts in tissue culture. However, some selected cell lines are susceptible to transformation by some of these viruses and can thus be used in a focus assay (2). In contrast to autonomously replicating murine leukemia viruses, murine sarcoma viruses transform mouse embryo fibroblasts in tissue culture and therefore, they are generally assayed by focus assay (Figure 2B). It should be noted here that Abelson leukemia virus is a replication-defective virus that causes leukemia and transforms fibroblasts in tissue culture. Therefore, it resembles the murine sarcoma viruses. Furthermore, we want to mention a focus assay in which bone marrow cells are used as indicator cells. In this assay foci are induced upon infection with the Friend leukemia virus complex (54).

RELATEDNESS OF DIFFERENT TYPE-C ONCOVIRUS ISOLATES

Based upon the observation that complete, infectious type-C oncoviruses are released spontaneously by animals of certain mammalian species and by cultured cells of these animals, the hypothesis has been postulated that the information for the production of these viruses might be transmitted genetically from parent to offspring along with other cellular genes (8,9,55,56). Apparently, type-C oncoviral sequences are integrated

within the genome of a great number of different species their expression being subjectec to the same regulatory processes that affect cellular genes. Although normally repressed, these endogenous viral genes can be activated by a variety of factors (genetic factors, hormones, radiation, chemical carcinogens, infecting viruses) (57). Following activation, the viral genes can sometimes escape from host control and the released infectious type-C oncoviruses can be transmitted horizontally to animals of the same species (58-64). Moreover, during the course of evolution type-C viruses have been transmitted to and become stably associated with the germ line of species that are only remotely related phylogenetically (65-70). An implication of these findings is that oncovirus isolates of taxonomically distant species may be closely related. Based upon nucleic acid hybridization studies, it appears that there are two lineages of ancestral type-C viruses, one represented by the type-C viruses found in rodents, and one represented by those found in certain primates. Within the first lineage, oncoviral isolates related to one group of endogenous rodent viruses include endogenous viruses within the pig genome (68), a group of isolates of gibbon apes (61) and a woolly monkey isolate (71). All these isolates are supposed to be evolutionary related to ancestral mouse viruses (72). Another highly related group seems to be endogenous rodent viruses of the rat (73) and the hamster (69,74,75). In addition, a horizontally-transmitted type-C virus of cats seems to be derived originally from another endogenous rodent virus (69). Representative viruses of the second lineage include isolates from old world primates (76,77), a class of endogenous feline oncoviruses (78) and probably a group of oncoviruses endogenous to ungulates (79,80). The data mentioned above are consistent with numerous serological data and with recent amino acid sequence studies of structural proteins (Dr. S. Oroszlan, personal communication) and, in the case of the avian viruses, nucleotide sequence studies, all providing evidence for the evolutionary relatedness of the different isolates of type-C oncoviruses. The whole family of retroviruses has so many basic properties in common as far as their morphology and replication cycle is concerned, that we are convinced that they have evolved from a common ancestor. The same may be true for a number of other families of viruses but in no other family one encounters so many viruses that are so closely related.

The mechanism of infection of a susceptible host cell by mammalian type-C oncoviruses is not yet completely understood. Entry may occur by viropexis, by fusion of the viral envelope with the plasma membrane or by localized dissolution of the viral and cellular membrane (81,82,83). Furthermore, a specific interaction between the cell surface and the viral envelope seems to be involved in the entry of the virus into the host cell (84,85).

An infecting oncovirus introduces the RNA-dependent DNA polymerase (reverse transcriptase) together with the viral genome into the cytoplasm of the cell. This enzyme is capable of DNA synthesis on an RNA or DNA template and contains RNAse H activity (degradation of the RNA in a RNA-DNA hybrid) (86,87,88). After entry of the virus into the cell, the high molecular weight genomic RNA is transcribed with the help of the reverse transcriptase and this synthesis occurs in the cytoplasm during the first hours after infection (89,90).

Based upon data from *in vitro* and *in vivo* experiments, it appears that the reverse transcription begins near the 5'-end of one of the two genomic RNA subunits (91,92,93), progresses a short distance to the 5'-end of the RNA molecule (94,95) and, subsequently, continues at the 3'-end of the same or another RNA subunit (88,93,96,97,98). The initial intermediate in the reverse transcription reaction is probably an RNA-DNA hybrid consisting of viral RNA hydrogen-bonded to full length (-)strand DNA (96). Next, a double-stranded linear DNA intermediate is synthesized, consisting of the long (-)strand DNA hydrogen-bonded to relatively short (+)strand DNA fragments (88). Thereafter, a complete linear double-stranded DNA molecule is formed (99,100). This full length linear DNA apparently contains the entire genetic information of the virus, since upon transfection of appropriate recipient cells, progeny virus was released (101,102). Only full geromic length molecules are infectious and can give rise to progeny virus production (103). Subsequent to the formation of the linear dsDNA provirus, a closed circular dsDNA form of the viral genome is found. This form was first identified in cells infected with the avian oncovirus Rous sarcoma virus (100,104) but later also in mammalian cells infected with mammalian oncoviruses (105,106).

The circular form has been visualized by electron microscopy (106,107, 108). Circularization is probably necessary for integration into the cellular genome (88). In contrast to the linear Rous sarcoma virus DNA molecules, whose accumulation has been demonstrated in enucleated cells (89), closed-circular DNA is found only in the nuclei of infected cells (107). From transfection experiments, it appears that the closed-circular DNA molecule is of full genome size (99,102,107). However, the closed-circular DNA has a 5 fold lower specific infectivity than the linear form (102). In the last step of the infection process, the newly synthesized oncoviral DNA is integrated into the cellular genome. There is some suggestive evidence that integration of the viral genome into cellular DNA is necessary for transformation and for virus production (104). However, the possibility that some provirus remains unintegrated and can function in that state has not been ruled out definitively.
Upon transcription of the integrated proviral DNA, viral RNA is synthesized. Cellular RNA polymerase II is probably involved in this process, since synthesis of viral RNA is sensitive to α-amanitin (109,110,111). Furthermore, mitosis is required for the initiation of the viral RNA synthesis (112,113). The synthesized viral RNA can be found as messenger RNA associated with polyribosomes and becomes incorporated in progeny virus. Both messenger RNA and virion RNA have the same plus polarity. However, there is substantial evidence that they constitute separate pools (114). Basic properties of the viral genome, the characteristics of the viral messenger RNA and the synthesis of the virus-specific polypeptides will be described separately.
Assemblage of the virus particles and virus release takes place by budding from the plasma membrane. In a process of extracellular maturation which can be followed in an electron microscope, newly released virus particles undergo a transition from particles with an electron lucent to an electron dense core ((22) see also chapter II). Concomitantly, the viral RNA, present in newly released virions in the form of 30S-40S components which are not or only weakly linked to each other, become more extensively linked resulting in a 60-70S RNA complex (115,116,117).

The RNA present in type-C oncoviruses is composed of different RNA species. The RNA species with the highest molecular weight sediments in a neutral sucrose gradient of about 50-70S (118,119) and appears to carry all viral genetic material. RNA species of lower molecular weight include ribosomal RNAs and RNA species sedimenting in the range of 4-7S (119-122). With the exception of part of the 4S RNAs no virus-specific function can be attributed to the lower molecular weight species present in the virions. The 4S RNA class consists of a selected population of cellular tRNA species which are free inside the virus-particle or hydrogen bonded to the 50-70S complex (121,123,124). One specific tRNA molecule per haploid genome can serve as a primer in the RNA-dependent DNA synthesis *in vitro* and presumably has the same function *in vivo* (125,126). In Rous sarcoma virus and Moloney murine leukemia virus the initiator tRNAs have been identified as $tRNA^{Trp}$ and $tRNA^{Pro}$ respectively (127,128), they are attached by basepairing with their 3'-end closely to the 5'-end of the genomic RNA (128-131).

Based upon particle weight determination (132), equilibrium sedimentation (133), electron microscopic measurements (134,135) and gel electrophoresis (136), the estimated molecular weight of the 50-70S RNA is between 4.5×10^6 and 7.0×10^6 daltons. By dimethylsulfoxide treatment or heating the high molecular weight 50-70S RNA complex can be dissociated into 30-40S subunits (137). This indicates that the 50-70S RNA is a complex whose components are held together by hydrogen bonds. Later evidence indicated that there are two 30-40S subunits per 50-70S complex (138-142). In electron microscopic studies on the structure of partially denatured 50-70S virion RNAs, linear complexes were observed and, as could be visualized by tagging with SV40 DNA both free ends contained poly(A) (143). These complexes called dimer linkage structures are Y-shaped and possess a loop in each subunit (143-145). The molecular weight of the 30-40S RNA subunits estimated by gel electrophoresis varies in the range of 2.2×10^6-3.5×10^6 daltons (146-147). The higher values represent RNA species from nondefective transforming viruses. These type of orcoviruses, with Rous sarcoma virus as the prototype, have the largest genomes. The lower values are characteristic for defective oncoviruses from which one or more

genes are deleted.

The 30-40S virion RNAs have a poly(A) track of about 200 residues at the 3'-ends (148-150) and their 5'-ends possess the typical cap structure m7G5'ppp5'NmpNp (151-153). Thus, they have termini typical for eukaryotic mRNAs. Furthermore, the sequence of the 5'-end has additional interesting features which are probably important characteristics of the genome. As an example we discuss here the 5'-end of the avian sarcoma virus genome because this genome is studied in most detail at the moment. Mammalian oncoviruses genomes, however, are not basically different. Hybridization studies and sequence analysis have revealed a terminal redundancy in the viral genome (98,154-156). About 20 nucleotides at the 5'-end are repeated at the 3'-end immediately adjacent to the terminal poly(A) track. In view of the observation that DNA synthesis starts at the tRNA site and proceeds to the 5'-end of the genome, terminal redundancy provides possible mechanisms for reverse transcription of the remaining genomic RNA (93,98). The 5'-end also contains a region complementary to the 3'-end of eukaryotic 18S ribosomal RNA (157-160). Moreover, an initiation codon AUG is located in the direct neighborhood of this site (159, 160). These sequences probably represent a ribosome binding site and an initiation site for the translation of the first gene at the 5'-end of the genome. Finally, inverted repeat sequences are observed at the 5'-end. These structures may be involved in the formation of dimer linkage structures (160).

TYPE-C ONCOVIRAL GENES AND THEIR EXPRESSION

Oncoviral genes

All autonomously replicating type-C oncoviruses contain three genes which code for the virus-specific virion polypeptides (161,162). The gene coding for the internal structural polypeptides is called the gag-gene; the pol-gene directs the synthesis of the virion associated reverse transcriptase and the env-gene codes for the envelope polypeptides. Furthermore, according to the current conception about their genomes, autonomously replicating and transforming type-C oncoviruses, which until now have only been found in avian species, contain an additional gene.

This gene is termed src-gene and is involved in cellular transformation. Based upon oligonucleotide mapping and frequencies of genetic recombination, the gene order within the genome of these oncoviruses is 5'-gag-pol-env-src-3' (161,163,164,165). The transformation-defective avian leukosis viruses lack the src-gene but apart from that their gene order is the same. The gene order of the nontransforming mammalian type-C oncoviruses is probably identical to the one for avian viruses (166,167). The genes of transforming mammalian oncoviruses are discussed below. Typically, infection by oncoviruses hardly influences the cellular synthetic apparatus quantitatively and does not result in cell lysis. Relative to the total cellular RNA and protein synthesis, the oncovirus-specific synthesis of macromolecules is low accounting for only about 0.5%-2% of the total (168). Therefore, very sensitives techniques such as nucleic acid hybridization and competition or precipitation radioimmunoassays are required to detect the oncovirus-specific gene-products in a mixture with host cellular components.

Oncoviral mRNA species

Analysis of the transcriptional products of the viral genomes in nucleic acid hybridization studies revealed the presence of several virus-specific mRNA species in type-C oncovirus infected cells (169-175). In cells infected with autonomously replicating and transforming avian type-C oncoviruses, three different virus-specific mRNA species have been identified. The mRNA species are different in size and have sedimentation values of about 38S, 28S and 21S. With selected cDNA probes the genetic content of these mRNA species and of mRNA species in cells infected with various deletion mutants of the same oncoviruses has been characterized (172). In studies on mammalian oncovirus-specific mRNA species similar results were obtained (167,174,175). In Figure 3 a schematic representation of characterized mRNA species from mammalian oncovirus infected cells is depicted.

Furthermore, it appeared from hybridization studies that oncoviral mRNA species present in type-C oncovirus infected cells contain noncontiguously coded sequences (167,172,176). This phenomenon due to splicing of RNA molecules appear to be a more general feature in eukaryotes. For instance the juxtaposition of RNA sequences encoded by noncontiguous sequences

of DNA has also been observed in adenovirus mRNAs and the mRNAs of SV40 as well as in a number of cellular mRNAs (177-181). The molecular mechanism responsible for this apparently very delicate and specific process is still completely unknown. In a recent hybridization and heterocuplex study by Rothenberg et al. (167), the 21S mRNA species in a Moloney murine leukemia virus infected cell was compared with the genomic RNA. It appeared that the 21S mRNA contained a 500 nucleotide sequence which maps at the 5'-end of the genomic RNA and a 2.8 kilobase sequence which maps at the 3'-end of the genome. It is not yet clear whether or not this 5'-terminal sequence has a specific function in protein synthesis.

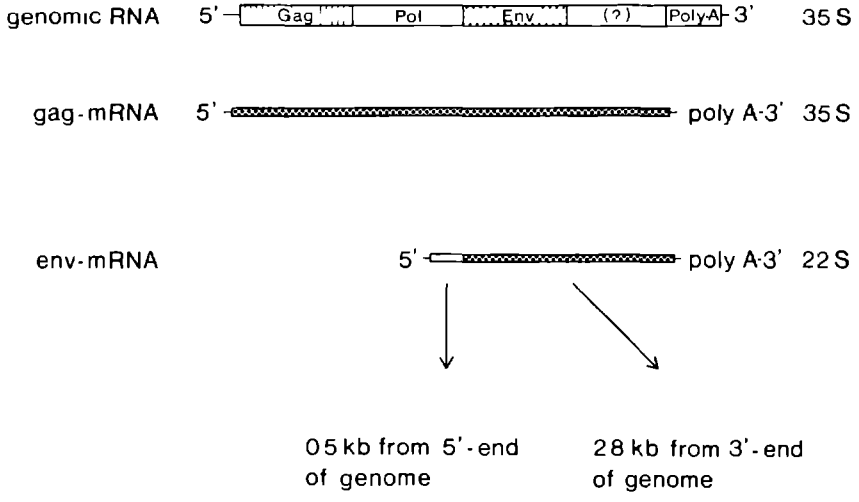

Fig. 3) Schematic representation of intracellular oncovirus-specific mRNA species as identified in mammalian oncovirus infected cells.

Oncoviral structural polypeptides

Analysis of the biosynthesis of virus-specific polypeptides in cells infected with type-C oncoviruses has led to the elucidation of synthetic processes involved (168-182). In almost all studies specific antisera prepared against disrupted virus particles of purified virion polypeptides have been used. Therefore, prior to their synthesis, the properties and the serological characteristics of oncovirus-specific polypeptides are discussed. Although much is known about the polypeptides of the avian oncoviruses, we will restrict our discussion to the mammalian type-C oncoviruses.

In prototype mammalian type-C virions, Rauscher murine leukemia virions, four different internal structural polypeptides and four envelope structural polypeptides have been identified (168). According to the nomenclature agreed upon at a Tumor Viral Immunology Workshop ('83,184) these polypeptides are named p30, p15, p12, p10, gp70, gp45, p15(E) and p12(E) ("p" standing for "protein", "gp" for glycoprotein, "(E)" for location in the viral envelope; the figures refer to estimated molecular weights in kilo-daltons). A similar nomenclature is used for other classes of oncoviruses.

Serologically, each virus-specific virion polypeptide of type-C oncoviruses is characterized by multiple antigenic determinants (for a review see reference 182). One polypeptide may contain group-specific antigenic determinants (shared by certain viruses within a given host species), interspecies-specific antigenic determinants (shared by certain viruses of different animal species), and type-specific antigenic determinants (unique to an individual virus strain). Aviar and mammalian type-C oncoviral polypeptides are serologically completely different.

The largest internal structural polypeptide of mammalian type-C virions is the major group-specific antiger p30. This most extensively studied internal polypeptide possibly forms the core shell of the virion (185). In addition to group-specific antigenic determinants, it contains interspecies-specific and, although less pronounced, type-specific antigenic determinants. The isoelectric points of p30 isolated from diverse mammalian type-C viruses vary between 6.2 and 8.0.

The internal structural polypeptide p15 is a strongly hydrophobic polypeptide and possesses type-specific, group-specific as well as broadly

reactive interspecies-specific antigenic determinants. The isoelectric points of the hydrophobic polypeptides from diverse type-C mammalian oncovirus isolates vary between 6.1 and 8.3. Its presence in the viral core as well as in the viral envelope is discussed in chapter II of this thesis.

The internal structural polypeptide p12 is a phosphoprotein (186). It is acidic; the isoelectric points of isolates from diverse type-C mammalian oncoviruses vary between 4.1 and 5.9. The phosphoproteins bind specifically to homologous viral genomic RNA but not to RNAs of heterologous type-C oncoviruses (187,188). Polypeptide p12 contains some group-specific antigenic determinants, but as appeared from competition immunoassays, it is highly type-specific and thus provides a valuable tool to discriminate between mammalian type-C oncovirus isolates.

The internal structural polypeptide p10 is an arginine-rich strongly basic protein which has been reported to be associated with the viral RNA in the form of a ribonucleoprotein complex within the mature viral core (185,189). The isoelectric point of isolates from diverse, type-C mammalian oncoviruses, vary between 9.1 and 11.6. Group-specific and interspecies-specific reactivity has been demonstrated in serological studies with these polypeptides.

In addition to the internal structural polypeptides, the type-C oncoviral reverse transcriptase is also located in the viral core. In contrast to the avian type-C oncoviral reverse transcriptase which contains two subunits, the enzyme from murine type-C oncoviruses consists of a single polypeptide with a molecular weight of about 70,000 daltons (87,182). This polypeptide possesses type-specific, group-specific and interspecies-specific antigenic determinants.

The largest envelope structural polypeptide is gp70, a glycoprotein with an apparent molecular weight of about 70,000 dalton based on sodium dodecyl sulfate polyacrylamide gel electrophoresis. This polypeptide is located on the cell surface (190,191,192) and possesses group-, interspecies-, and type-specific antigenic determinants which apparently reside in the protein moiety (193). The isoelectric points of isolates from diverse mammalian type-C oncoviruses vary between 4.1 and 5.3.

In addition to gp70 in some isolates one finds a 45,000 molecular weight glycoprotein, gp45. Based upon amino acid composition, serological properties and peptide mapping, this glycoprotein seems to be related to

gp70 (168,194).
The envelope polypeptide p15(E), which possesses group- and interspecies-specific antigenic determinants, is a nonglycosylated acidic polypeptide and has a tendency to aggregate in the absence of detergents (195). gp70 is probably linked by disulfide bonds to p15(E) (196,197).
The envelope polypeptide p12(E) is related to p15(E) as appeared from chymotryptic peptide analysis (192,198).

Gag-gene translational products

In early studies on the synthesis of virus specific structural polypeptides in avian type-C oncovirus infected cells, it was shown that the primary gag-gene product was a high molecular weight precursor polypeptide (199,200). Post-translational cleavage of this precursor polypeptide resulted in the production of the nonglycosylated internal structural polypeptides. Later, in similar experiments, an analogous precursor-product relationship was found in mammalian type-C oncovirus infected cells (168,182). In pulse-chase experiments with Rauscher murine leukemia infected cells it appeared that the internal structural polypeptides p30, p15, p12 and p10 are cleavage products of a common precursor polypeptide of about 75,000 dalton termed $Pr75^{gag}$ according to the current nomenclature (182,201-208). The same results were obtained in in vitro translation studies using 35S genomic RNA (209-216) or the 35S mRNA species from infected cells (217,218). From a number of intracistronic mapping studies the sequence of the Rauscher murine leukemia subgene products was deduced as NH_2-p15-p12-p30-p10-COOH (182,219). At the moment the primary gag-gene precursor polypeptides, their cleavage products and the order of these subgene products of a large number of different mammalian and avian oncoviruses have been described (182). The results of some of these studies are depicted in Figure 4 which was obtained from Dr. J.R. Stephenson. It is obvious from this figure that the identification of the subgene products according to the current nomenclature is very confusing when related oncoviral isolates are concerned. In order to overcome this difficulty a nomenclature system taking into account immunologic, biochemical and genetic properties has been proposed by Stephenson et al. (182). According to this proposal the gag-subgene products are called gag-a, gag-b, gag-c and gag-d going from left to right on the map.

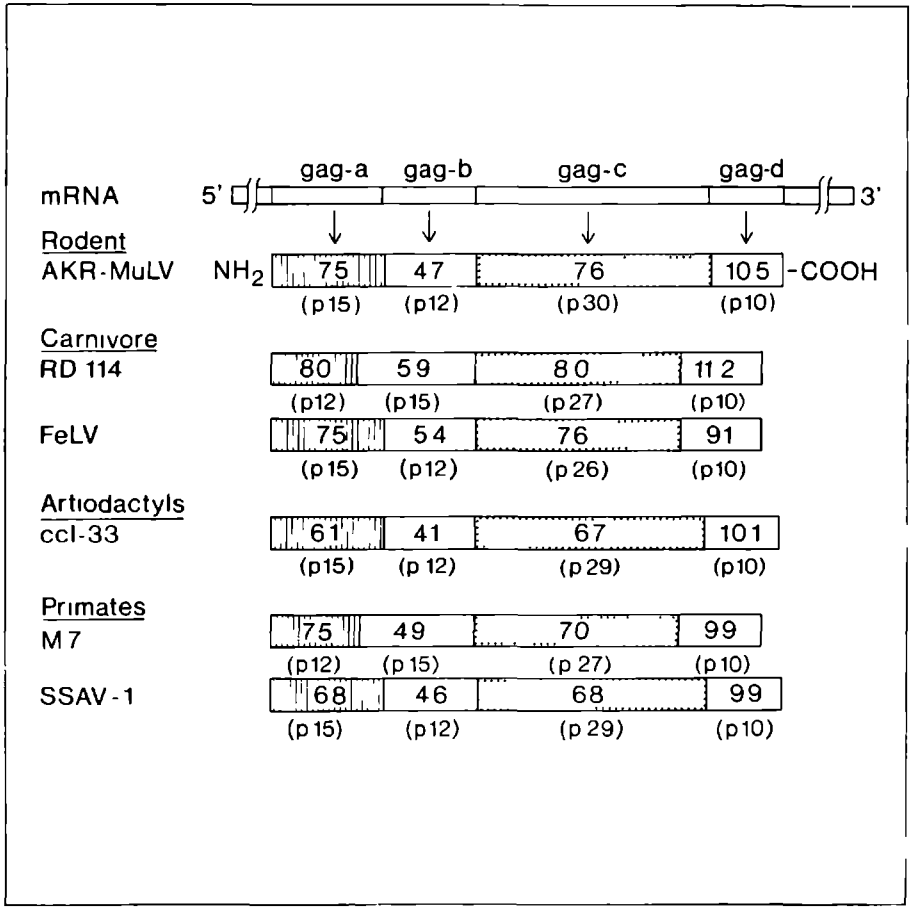

Fig. 4) Proposed arrangement of viral proteins within representative mammalian type-C viral gag-gene-coded precursor polypeptides. The figures indicated in the bar graphs represent the isoelectric points of the individual proteins, while the values between brackets represent the molecular weight as determined by agarose gel filtration in the presence of 6 M GuHl. (Figure obtained from Dr. J.P. Stephenson; see reference 182).

Sofar, uncomplete knowledge about functional analogy between virus-specific products of more distantly related oncoviruses has hampered the general introduction of this useful nomenclature.

Pol-gene translational products

The enzyme reverse transcriptase seems to be synthesized according to a peculiar mechanism (207,213,220). In mammalian type-C oncovirus infected cells a high molecular weight precursor polypeptide was observed which could be precipitated with antisera raised against the internal structural polypeptides as well as against the reverse transcriptase (207). Apparently, the primary translational product in the synthesis of reverse transcriptase is a gag-pol-precursor polypeptide. In vitro translation studies using 35S genomic RNA, the synthesis of a simi ar high molecular weight precursor polypeptide was observed (213-216) and appeared to be more pronourced wher certain suppressor tRNA species were added (213). These results suggest that the synthesis may be due to occasional readthrough at the gag-gene termination site which is sensitive to certain suppressor tRNA species. These observations also suggest, that the 35S mRNA species is the messenger for the internal structural polypeptides as well as for the reverse transcriptase. Furthermore, a readthrough mechanism at low frequency could also explain the difference in the production of the relatively large amounts of internal structural polypeptides and small amounts of the enzyme.

Env-gene translational products

Like the internal structural polypeptides, the envelope polypeptides gp70, gp45, p15(E) and p12(F) are derived from a common precursor polypeptide (201,202,204-206,221). In Rauscher murine leukemia virus infected cells this primary translational product of the env-gene is a high molecular weight glycosylated polypeptide of about 82,000 daltons ($gpPr82^{env}$). The same precursor polypeptide is observed in translation studies using the 21S mRNA species (218). The arrangement of the subgene products within the primary env-gene translational product is probably NH_2-gp70-p15(E)-COOH (221).
In Figure 5 a scheme of the expression of the mammalian murine leukemia viral genes is depicted.

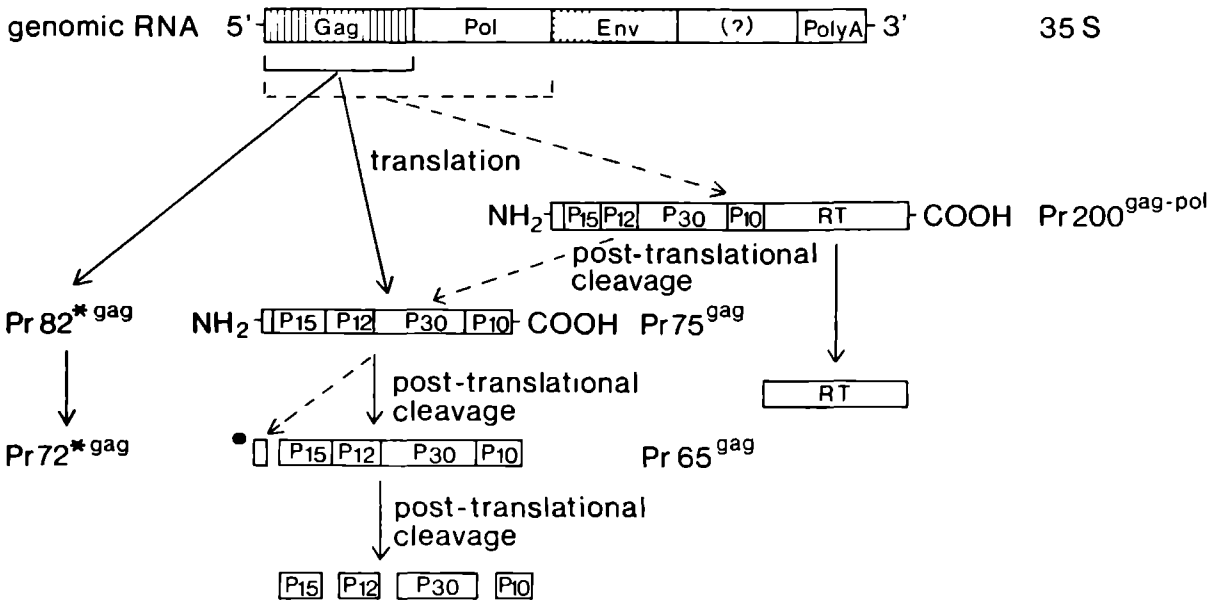

Fig. 5A) Model for the synthesis and post-translational processing of the gag- and pol-gene coded precursor polypeptides.

* *Precursor polypeptides synthesized under artificial conditions (i.e. in the presence of canavanine).*

• *The primary translational product of the gag-gene contains in addition to the four subgene products a 13,000-15,000 molecular weight polypeptide. This polypeptide is located at the amino terminal site of the precursor polypeptide (S. Oroszlan, personal communication).*

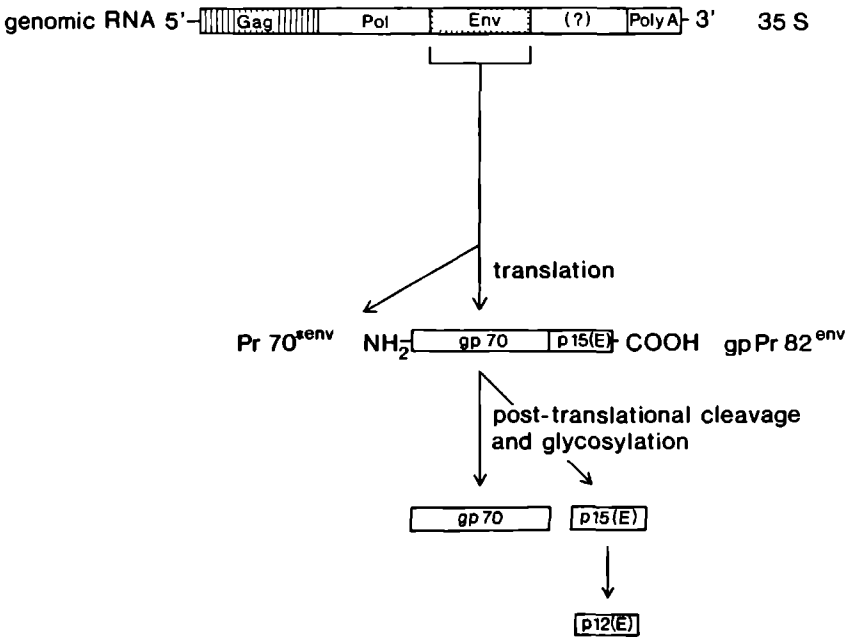

Fig. 5B) Model for the synthesis and post-translational processing of the env-gene coded precursor polypeptide.

**Precursor polypeptide synthesized under artificial conditions (i.e. in the presence of inhibitors of glycosylation).*

All mammalian oncoviruses capable to transform fibroblasts *in vitro* are replication-defective (2) and are believed to have originated as a consequence of genetic recombination between type-C helper-viruses and host cell genetic sequences coding for malignant transformation (41-44). Representative isolates of mammalian transforming oncoviruses have been obtained from a number of mammalian species including the mouse, rat, cat and woolly monkey. Some isolates are from naturally occurring sarcomas but most murine transforming viruses were isolated from mice or rats after inoculation of murine leukemia viruses.

Initially hybridization studies have demonstrated partial homology between the transforming replication-defective oncoviruses and the type-C oncoviruses which have been used for their isolation (222-227). Further characterization of the genomes of the transforming oncoviruses has been performed using heteroduplex mapping, oligonucleotide mapping, restriction enzym analysis, and transfection and hybridization techniques (228-235).

Until now, a characterization of polypeptides coded for by transforming genes has been limited. Recently, a 60,000 molecular weight protein has been detected in avian sarcoma virus transformed cells as well as in *in vitro* translation studies using the 3'-terminal third of nondefective avian sarcoma virus RNA (236,237,238). Peptide mapping revealed that in both cases the same protein was detected (237). This protein appears to be the product of the avian *src*-gene and probably possesses protein kinase activity (237).

Polypeptides coded for by mammalian transforming genes have not yet been described but identification of such polypeptides is the main goal of a number of laboratories. Using cell-free translation systems, Parks and Scolnick showed, that partially purified viral RNA of Harvey sarcoma virus codes for a protein of about 21,000 molecular weight (239). This protein did not correspond with translational products obtained with viral RNA of the helper type-C oncovirus but there is no evidence that this product is related to the transformation-specific gene product.

Since mammalian transforming oncoviruses seem to be evolved as the result of a genetic recombination between type-C oncoviral sequences and cellu-

TABLE I

TYPE-C VIRAL ANTIGEN EXPRESSION IN MAMMALIAN ONCOVIRUS TRANSFORMED NONPRODUCER CELL LINES

Virus Origin	Virus Isolates	Transformed Nonproducer Cell Line	Expression of Type-C Oncoviral Gene Products						
			gag-coded				pol-coded	env-coded	
			gag-a (p15)	gag-b (p12)	gag-c (p30)	gag-d (p10)	rt	(gp70)	(M_r) ‡
Mouse	NP-Mo-MSV	M-NRK	-	-	-	-	-	-	-
	Friend SFFV	NP9	+	-	-	-	-	-	NT
	Balb-MSV	MAI-3206	+	+	-	-	NT	-	NT
	Abelson-MuLV	Ab2-CCL64	+	+	-	-	-	-	120,000
	T8-MCF	T8-CCL64	+	+	-	-	-	-	130,000
	S*L*Mo-MSV	$MiC1_1$	+	+	+	-	-	+	58,000
Rat	KiMSV	K-NRK	-	-	-	NT	NT	-	NT
	HaMSV	H-NRK	-	-	-	NT	NT	-	NT
Cat	FeSV	FeSV/CCL64	+	+	-	-	-	-	135,000
Woolly Monkey	WSV	WSV-NRK	+	+	+	-	NT	-	55,000

This table is adopted from Table 1 in reference 240 and other data in references 240-243.

‡ Molecular Weights of Polyproteins Containing Gag-gene Coded Antigenic Determinants.

lar genetic sequences, studies on the expression of type-C oncoviral translational products in cells nonproductively transformed by mammalian oncoviruses were performed. From these studies it appeared that this frequently observed expression is generally restricted to a part of the gag-gene (182,240). The part which is expressed is always contiguous and includes the 5'-terminal gag-subgene for p15. In Table I the expression of gag-coded polypeptices in cells nonproductively transformed by diverse mammalian transforming viruses is depicted. Further molecular size analysis of these viral antigens revealed that in some cases high molecular weight polyproteins (50,000-130,000 dalton) containing gag-gene coded antigenic reactivity were present (see Table I) (182,240-243). These observations suggest a recombinational event within the gag-region of the type-C oncovirus genome. Furthermore, the nonstructural components of the polyproteins may represent transformation-specific cellular sequences. Evidence for this possibility may be obtained from the feline system, where the 60,000 molecular weight cleavage product of the precursor polyprotein has been shown to possess immunologic cross-reactivity with certain antisera directed against the tumor-specific feline oncornavirus-associated cell membrane antigen (FOCMA) (241,242). From heteroduplex mapping it is known that some sarcoma viruses are the result of more than one recombinational event; presumably secundary recombinations occur during the cloning procedures used to purify these viruses (230).

STRUCTURAL AND NONSTRUCTURAL TRANSLATIONAL PRODUCTS OF MAMMALIAN ONCOVIRUSES: INTRODUCTION TO THE PAPERS

Upon expression in the host cell of integrated mammalian oncoviral genes, infectious type-C oncovirus particles can be produced. Such particles are in fact vehicles of oncoviral genes. The virus-specific structural components of these particles are characteristic for the oncovirus and they are encoded by the encapsidated oncoviral genome. However, in addition to oncoviral genes coding for oncovirus-specific structural polypeptides also other genes (some of them being probably involved in cellular transformation) can be encapsidated and, upon infection, integrated in host cell genome. Thus, oncovirus particles provide mechanisms to

the cell to transfer or to acquire new genetic sequences. In the experimental part of this thesis we describe some typical features of those mechanisms.
We further characterized the structure of the type-C oncoviral particles in an electronmicroscopic and biochemical study using Rauscher murine leukemia virus as a prototype (Chapter II). We studied the synthesis of the oncovirus-specific structural polypeptides under natural as well as artificial conditions in pulse-chase experiments with mouse cells infected with Rauscher murine leukemia virus (Chapter III). Using a similar experimental approach, we characterized temperature-sensitive mutants of Rauscher murine leukemia virus (Chapter IV); In translation experiments, we studied the genomic RNA of some mammalian transforming oncoviruses (Chapter V) and, finally, we examined in transfection experiments the biological activity of integrated oncoviral genetic sequences and *in vitro* synthesized proviral DNA (Chapter VI).

Chapter II: Structural studies on Rauscher murine leukemia virus: Isolation and characterization of viral envelopes.
W.J.M. van de Ven, A.J.M. Vermorken, C. Onnekink, H.P.J. Bloemers and H. Bloemendal.
J. Virol. In Press (1978).

Structural analysis of mammalian type-C oncoviral particles has been limited mainly to studies on the outer surface of these particles or to studies on the centrally located spherical nucleoids or cores. In the latter cases, the viral envelopes were removed from the virions by disruption with detergents and ether. In this study we present a procedure for the isolation of oncoviral envelope structures from virions of Rauscher murine leukemia virus. Using a detergent in combination with ether, we carefully stripped the viral envelopes from the virions and fractionated the oncoviral structures through flotation in a discontinuous sucrose gradient in one centrifugation run. Flotation rather than sedimentation was a crucial step in obtaining pure viral envelope preparations. The purified oncoviral envelope fraction contained in addition to the *env*-gene coded structural polypeptides, a *gag*-gene coded structural polypeptide, namely p15. According to Bolognesi *et al.* (244),

this polypeptide is associated with the outer surface of
Its presence in the purified envelope fraction can be due t
hydrophobic character of p15. However, it might be possible t
linkage between p15 and the viral envelope is a functional ste
assembly. In the perspective of the recent identification of mam
transforming oncovirus-specific translational products (see Chapte
Chapter V), encapsidation of these products into the pseudotype viric
can be expected. Therefore, the pseudotype virions may be used as star-
ting material for preparative isolation of the polyproteins, which are
probably involved in cellular transformation. Preliminary results indi-
cate that indeed these polyproteins can be isolated from pseudotype vi-
rions (J.R. Stephenson and A.S. Kahn, personal communication).

Chapter III: Effect of impaired glycosylation on the synthesis of envelope proteins of Rauscher murine leukemia virus.
W.J.M. van de Ven, C. Onnekink, A.J.M. Vermorken, and H.P.J. Bloemers.
Virology 82, 334-344 (1977).

Oncoviral structural polypeptides are formed through post-translational processing of precursor polypeptides (Chapter I). In the study described in chapter III, using JLS-V9 cells productively infected with Rauscher murine leukemia virus, we examined in pulse-chase experiments the synthesis and processing of virus-specific envelope precursor polypeptides under natural and artificial conditions. To detect the newly synthesized oncovirus-specific polypeptides, we applied a sensitive immunoprecipitation procedure in this study since oncovirus-specific polypeptides represent about 0.5-2% of the total cellular polypeptide content.
In the presence of 2-deoxy-D-glucose or cytochalasin B glycosylation was impaired and the synthesis of the env-gene coded precursor polypeptide $gpPr82^{env}$ was inhibited. Instead, the synthesis of a new glucosamine-deficient polypeptide with a molecular weight of about 70,000 was observed. This polypeptide, $Pr70^{*env}$, appeared to carry antigenic determinants of the subgene products of $gpPr82^{env}$ and, thus, may represent the unglycosylated primary translational product of the env-gene. Glycosylation of the envelope precursor polypeptide is apparently a necessary condition for its proper processing, since in the presence of the inhibitors of

glycosylation production of the envelope structural polypeptides was inhibited, while proteolytic cleavage of the gag-gene precursor polypeptide was not affected. Furthermore, after removal of cytochalasin B, which in contrast to 2-deoxy-D-glucose reversibly inhibits glycosylation, glycosylated $Pr70^{*env}$-related polypeptides as well as the normal envelope structural polypeptides were formed.

Chapter IV: Impaired processing of precursor polypeptides of temperature-sensitive mutants of Rauscher murine leukemia virus.
W.J.M. van de Ven, D. van Zaane, C. Onnekink, and H.P.J. Bloemers.
J. Virol. 25, 553-561 (1978).

A similar experimental approach as applied in chapter III was followed in this study on the characterization of temperature-sensitive mutants of Rauscher murine leukemia virus. These mutants of the Rauscher strain were obtained through the courtesy of Dr. J.R. Stephenson and they can be divided in the following classes: class I mutants, defective at 39°C in replication function before the synthesis of viral group-specific antigens, class II mutants, defective at 39°C in functions between the synthesis of viral group-specific antigens and production of virions, and class III mutants, defective at 39°C in the production of infectious virions, although virus-like particles are formed. In our experiments, we have infected the mutants at the permissive temperature (31°C) and thereafter, we have studied the synthesis and processing of the virus-specific precursor polypeptides at the permissive and nonpermissive temperature. It appeared that in cells infected with a class I mutant (ts17 or ts29) or a class II mutant (ts25 or ts26), the processing of the primary gag-gene translational product was impaired. The proteolytic cleavage was blocked beyond $Pr65^{gag}$. Concomitantly, the formation of the env-gene related polypeptide p12(E) of all four mutants was blocked at the nonpermissive temperature. In contrast, cells infected with the class III mutant ts28 showed a normal turnover of the gag- and env-gene coded precursor polypeptides.

Chapter V: Translation of type-C viral RNAs in Xenopus laevis oocytes. Evidence that the 120,000 molecular weight polyprotein expressed in Abelson leukemia virus transformed cells is viral coded.
Roberta K. Reynolds, Wim J.M. van de Ven, and John R. Stephenson.
Submitted for publication.

Cells nonproductively transformed by mammalian type-C transforming viruses can express polypeptides related to the gag-gene of a leukemia helper virus; these polypeptides are apparently derived progressively from the 5'-end of the leukemia helper virus genome (see Chapter I, Table I). In view of the fact that the replication-defective transforming oncoviruses appear to represent recombinants between a portion of a leukemia helper virus genome and cellular genes presumably coding for malignant transformation, expression of gag-gene polypeptides in nonproductively transformed cells was further analyzed. From a number of studies it appeared that cells nonproductively-transformed by each of several such viruses express polyproteins of 55,000-130,000 molecular weight containing amino terminal gag-gene polypeptides (Chapter I). These polyproteins sometimes contain nonstructural components covalently linked to the gag-gene related structural components. To resolve whether or not these polyproteins are encoded by the transforming oncovirus genome, we encapsidated the genomes of Abelson murine leukemia virus and woolly monkey sarcoma virus with the aid of an appropriate leukemia helper virus in pseudotype virions, isolated the genomic RNAs of both isolates and translated them in Xenopus laevis oocytes. The primary translational product of Abelson murine leukemia virus genomic RNA is a polyprotein corresponding in molecular weight and serological properties to a polyprotein expressed in Abelson murine leukemia virus transformed cells (243). This polyprotein contains in addition to the gag-gene coded structural components p15 and p12 a nonstructural component. Translation of woolly monkey sarcoma virus genomic RNA resulted in the synthesis of a 55,000 molecular weight polyprotein containing the translational products of gag-a, gag-b, and gag-c of the woolly helper virus. A similar protein was expressed in woolly sarcoma virus transformed nonproducer cells. From this study, we conclude that the nonstructural component of the polyprotein expressed in Abelson murine leukemia virus-transformed cells is viral-coded and that such a polyprotein is either not present in woolly sarcoma virus-

transformed cells or is subject to rapid post-translational cleavage. Translation in Xenopus laevis oocytes is a useful experimental approach for testing sera and for characterizing the serological properties of similar nonstructural components of various other transforming oncovirus isolates. In view of the fact that the nonstructural component in the Abelson murine leukemia virus-specific polyprotein could represent the product of cellular-acquired transforming sequences which are etiologically involved in spontaneous tumors of their natural hosts, development of immunological assays for this and other nonstructural components may lead to a better understanding of cancer.

Chapter VI: Cells infected with a temperature-sensitive murine leukemia virus and transfected with fragments of wild-type murine leukemia virus proviral DNA release progeny virus infectious at the nonpermissive temperature.
W.J.M. van de Ven, F.A.V.J. van der Hoorn, H.J. Dodemont, and H.P.J. Bloemers.
Submitted for publication.

To study proviral DNA of oncoviruses, transfection experiments can be performed in which appropriate recipient cells are treated with purified DNA (245,246). In such experiments, we have studied DNA isolated from cells releasing high titers of either wild-type or temperature-sensitive oncovirus. By generating specific oncovirus producing cells, we showed that by transfection oncovirus-specific genetic sequences can be introduced into the recipient cells.
Apparently, these studies are limited to experiments with DNA that contains intact viral genomes of autonomously replicating oncoviruses (245, 246). In an attempt to study non-infectious proviral DNA fragments (i.e. proviral DNA fragments that upon transfection do not give rise to progeny virus release) we have coinfected recipient cultures with a temperature-sensitive oncovirus. At the nonpermissive temperature, we could isolate progeny viruses. They contained antigenic determinants of the oncovirus that was used for the synthesis of the proviral DNA fragments and, furthermore, these viruses were infectious at the restrictive temperature. We conclude from these virus-rescue assays that some factors

necessary for the observed oncovirus release were provided by the thermosensitive mutant.

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CHAPTER II

STRUCTURAL STUDIES ON RAUSCHER MURINE LEUKEMIA VIRUS: ISOLATION AND CHARACTERIZATION OF VIRAL ENVELOPES

the cell to transfer or to acquire new genetic sequences. In the experimental part of this thesis we describe some typical features of those mechanisms.

We further characterized the structure of the type-C oncoviral particles in an electronmicroscopic and biochemical study using Rauscher murine leukemia virus as a prototype (Chapter II). We studied the synthesis of the oncovirus-specific structural polypeptides under natural as well as artificial conditions in pulse-chase experiments with mouse cells infected with Rauscher murine leukemia virus (Chapter III). Using a similar experimental approach, we characterized temperature-sensitive mutants of Rauscher murine leukemia virus (Chapter IV); In translation experiments, we studied the genomic RNA of some mammalian transforming oncoviruses (Chapter V) and, finally, we examined in transfection experiments the biological activity of integrated oncoviral genetic sequences and *in vitro* synthesized proviral DNA (Chapter VI).

Chapter II: Structural studies on Rauscher murine leukemia virus: Isolation and characterization of viral envelopes.

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J. Virol. In Press (1978).

Structural analysis of mammalian type-C oncoviral particles has been limited mainly to studies on the outer surface of these particles or to studies on the centrally located spherical nucleoids or cores. In the latter cases, the viral envelopes were removed from the virions by disruption with detergents and ether. In this study we present a procedure for the isolation of oncoviral envelope structures from virions of Rauscher murine leukemia virus. Using a detergent in combination with ether, we carefully stripped the viral envelopes from the virions and fractionated the oncoviral structures through flotation in a discontinuous sucrose gradient in one centrifugation run. Flotation rather than sedimentation was a crucial step in obtaining pure viral envelope preparations. The purified oncoviral envelope fraction contained in addition to the *env*-gene coded structural polypeptides, a *gag*-gene coded structural polypeptide, namely p15. According to Bolognesi *et al.* (244),

this polypeptide is associated with the outer surface of the viral core. Its presence in the purified envelope fraction can be due to the very hydrophobic character of p15. However, it might be possible that the linkage between p15 and the viral envelope is a functional step in virion assembly. In the perspective of the recent identification of mammalian transforming oncovirus-specific translational products (see Chapter I and Chapter V), encapsidation of these products into the pseudotype virions can be expected. Therefore, the pseudotype virions may be used as starting material for preparative isolation of the polyproteins, which are probably involved in cellular transformation. Preliminary results indicate that indeed these polyproteins can be isolated from pseudotype virions (J.R. Stephenson and A.S. Kahn, persoral communication).

Chapter III: Effect of impaired glycosylation on the synthesis of envelope proteins of Rauscher murine leukemia virus.
W.J.M. van de Ven, C. Onnekink, A.J.M. Vermorken, and H.P.J. Bloemers. Virology 82, 334-344 (1977).

Oncoviral structural polypeptides are formed through post-translational processing of precursor polypeptides (Chapter I). In the study described in chapter III, using JLS-V9 cells productively infected with Rauscher murine leukemia virus, we examined in pulse-chase experiments the synthesis and processing of virus-specific envelope precursor polypeptides under natural and artificial conditions. To detect the newly synthesized oncovirus-specific polypeptides, we applied a sensitive immunoprecipitation procedure in this study since oncovirus-specific polypeptides represent about 0.5-2% of the total cellular polypeptide content.
In the presence of 2-deoxy-D-glucose or cytochalasin B glycosylation was impaired and the synthesis of the env-gene coded precursor polypeptide $gpPr82^{env}$ was inhibited. Instead, the synthesis of a new glucosamine-deficient polypeptide with a molecular weight of about 70,000 was observed. This polypeptide, $Pr70^{*env}$, appeared to carry antigenic determinants of the subgere products of $gpPr82^{env}$ and, thus, may represent the unglycosylated primary translational product of the env-gene. Glycosylation of the envelope precursor polypeptide is apparently a necessary condition for its proper processing, since in the presence of the inhibitors of

glycosylation production of the envelope structural polypeptides was inhibited, while proteolytic cleavage of the gag-gene precursor polypeptide was not affected. Furthermore, after removal of cytochalasin B, which in contrast to 2-deoxy-D-glucose reversibly inhibits glycosylation, glycosylated $Pr70^{*env}$-related polypeptides as well as the normal envelope structural polypeptides were formed.

Chapter IV: Impaired processing of precursor polypeptides of temperature-sensitive mutants of Rauscher murine leukemia virus.
W.J.M. van de Ven, D. van Zaane, C. Onnekink, and H.P.J. Bloemers.
J. Virol. 25, 553-561 (1978).

A similar experimental approach as applied in chapter III was followed in this study on the characterization of temperature-sensitive mutants of Rauscher murine leukemia virus. These mutants of the Rauscher strain were obtained through the courtesy of Dr. J.R. Stephenson and they can be divided in the following classes: class I mutants, defective at 39°C in replication function before the synthesis of viral group-specific antigens, class II mutants, defective at 39°C in functions between the synthesis of viral group-specific antigens and production of virions, and class III mutants, defective at 39°C in the production of infectious virions, although virus-like particles are formed. In our experiments, we have infected the mutants at the permissive temperature (31°C) and thereafter, we have studied the synthesis and processing of the virus-specific precursor polypeptides at the permissive and nonpermissive temperature. It appeared that in cells infected with a class I mutant (ts17 or ts29) or a class II mutant (ts25 or ts26), the processing of the primary gag-gene translational product was impaired. The proteolytic cleavage was blocked beyond $Pr65^{gag}$. Concomitantly, the formation of the env-gene related polypeptide p12(E) of all four mutants was blocked at the nonpermissive temperature. In contrast, cells infected with the class III mutant ts28 showed a normal turnover of the gag- and env-gene coded precursor polypeptides.

Chapter V: Translation of type-C viral RNAs in Xenopus laevis oocytes: Evidence that the 120,000 molecular weight polyprotein expressed in Abelson leukemia virus transformed cells is viral coded.
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Submitted for publication.

Cells nonproductively transformed by mammalian type-C transforming oncoviruses can express polypeptides related to the gag-gene of a leukemia helper virus; these polypeptides are apparently derived progressively from the 5'-end of the leukemia helper virus genome (see Chapter I, Table I). In view of the fact that the replication-defective transforming oncoviruses appear to represent recombinants between a portion of a leukemia helper virus genome and cellular genes presumably coding for malignant transformation, expression of gag-gene polypeptides in nonproductively transformed cells was further analyzed. From a number of studies it appeared that cells nonproductively-transformed by each of several such viruses express polyproteins of 55,000-130,000 molecular weight containing amino terminal gag-gene polypeptides (Chapter I). These polyproteins sometimes contain nonstructural components covalently linked to the gag-gene related structural components. To resolve whether or not these polyproteins are encoded by the transforming oncovirus genome, we encapsidated the genomes of Abelson murine leukemia virus and woolly monkey sarcoma virus with the aid of an appropriate leukemia helper virus in pseudotype virions, isolated the genomic RNAs of both isolates and translated them in Xenopus laevis oocytes. The primary translational product of Abelson murine leukemia virus genomic RNA is a polyprotein corresponding in molecular weight and serological properties to a polyprotein expressed in Abelson murine leukemia virus transformed cells (243). This polyprotein contains in addition to the gag-gene coded structural components p15 and p12 a nonstructural component. Translation of woolly monkey sarcoma virus genomic RNA resulted in the synthesis of a 55,000 molecular weight polyprotein containing the translational products of gag-a, gag-b, and gag-c of the woolly helper virus. A similar protein was expressed in woolly sarcoma virus transformed nonproducer cells. From this study, we conclude that the nonstructural component of the polyprotein expressed in Abelson murine leukemia virus-transformed cells is viral-coded and that such a polyprotein is either not present in woolly sarcoma virus-

transformed cells or is subject to rapid post-translational cleavage. Translation in Xenopus laevis oocytes is a useful experimental approach for testing sera and for characterizing the serological properties of similar nonstructural components of various other transforming oncovirus isolates. In view of the fact that the nonstructural component in the Abelson murine leukemia virus-specific polyprotein could represent the product of cellular-acquired transforming sequences which are etiologically involved in spontaneous tumors of their natural hosts, development of immunological assays for this and other nonstructural components may lead to a better understanding of cancer.

Chapter VI: Cells infected with a temperature-sensitive murine leukemia virus and transfected with fragments of wild-type murine leukemia virus proviral DNA release progeny virus infectious at the nonpermissive temperature.
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Submitted for publication.

To study proviral DNA of orcoviruses, transfection experiments can be performed in which appropriate recipient cells are treated with purified DNA (245,246). In such experiments, we have studied DNA isolated from cells releasing high titers of either wild-type or temperature-sensitive oncovirus. By generating specific oncovirus producing cells, we showed that by transfection oncovirus-specific genetic sequences can be introduced into the recipient cells.
Apparently, these studies are limited to experiments with DNA that contains intact viral genomes of autonomously replicating orcoviruses (245, 246). In an attempt to study non-infectious proviral DNA fragments (i.e. proviral DNA fragments that upon transfection do not give rise to progeny virus release) we have coinfected recipient cultures with a temperature-sensitive oncovirus. At the nonpermissive temperature, we could isolate progeny viruses. They contained antigenic determinants of the oncovirus that was used for the synthesis of the proviral DNA fragments and, furthermore, these viruses were infectious at the restrictive temperature. We conclude from these virus-rescue assays that some factors

necessary for the observed oncovirus release were provided by the thermosensitive mutant.

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CHAPTER II

STRUCTURAL STUDIES ON RAUSCHER MURINE LEUKEMIA VIRUS: ISOLATION AND CHARACTERIZATION OF VIRAL ENVELOPES

STRUCTURAL STUDIES ON RAUSCHER MURINE LEUKEMIA VIRUS: ISOLATION AND CHARACTERIZATION OF VIRAL ENVELOPES

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SUMMARY

A preparative method for isolating pure viral envelopes from a type-C RNA tumor virus, Rauscher murine leukemia virus, is described. Fractionation of virions of Rauscher murine leukemia virus was studied after disruption of the virions with the detergents sodium dodecyl sulfate or Nonidet P-40 in combination with ether. Fractionation was performed through flotation in a discontinuous sucrose gradient and, as appeared from electron microscopic examination, a pure viral envelope fraction was obtained, in this way. Using sensitive competition radioimmunoassays or sodium dodecyl sulfate polyacrylamide gel electrophoresis after immunoprecipitation with polyvalent and monospecific antisera directed against Rauscher murine leukemia virus proteins, the amount of the gag- and env-gene encoded structural polypeptides in the virions

and the isolated envelope fraction was compared. The predominant viral structural polypeptides in the purified envelope fraction were the env-gene encoded polypeptides, gp70, p15(E) and p12(E), while, except for p15, there was only a relatively small amount of the gag-gene encoded structural polypeptides in this fraction.

INTRODUCTION

Methods for the fractionation of virions into an envelope-enriched fraction and a core-enriched fraction have been described for a number of oncornaviruses (19). The preparation of pure cores of certain type-C virions has been achieved by centrifugation of disrupted virions through continuous or discontinuous sucrose gradients (5,28). Based upon the data from a number of such studies, the localization of the gag-gene encoded structural polypeptides of type-C viruses relative to the viral core was obtained (see ref. 3).

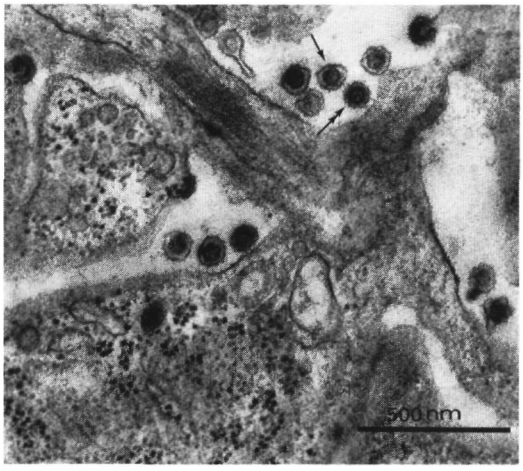
Other viral proteins are apparently constituents of the viral envelope. They are derived from one precursor polypeptide encoded for by the env-gene (13). From neutralizing (25), surface labeling (14), cytotoxic (10) and immunoelectron microscopic studies (24), there is now considerable evidence that a glycoprotein of about 70,000 daltons is localized on the viral envelope. Furthermore, a polypeptide of about 15,000 daltons described by Ikeda et al. (12) as p15(E) has also been localized in or on the viral envelope (3,11) as well as probably p12(E) (3), a polypeptide serologically related to p15(E) (13). However, the isolation of purified viral envelopes of type-C virions has not yet been described. In this paper we want to report the isolation and characterization of viral envelope structures from disrupted virions of Rauscher murine leukemia virus (R-MuLV) through flotation in a discontinuous sucrose gradient in one centrifugation run.

Cell lines and virus

NIH-Swiss-3T3 mouse embryo cells (29) infected with and producing R-MuLV, were grown as monolayers in Dulbecco's modified Eagle's medium completed with 10% fetal calf serum.
R-MuLV was isolated as described by Duesberg and Robinson (6).
R-MuLV labeled with L-(^{35}S)methionine (Radiochemical Centre, Amersham, England) was isolated from culture medium of monolayers labeled in Hank's basic salt solution supplemented with 10% dialyzed calf serum and amino acids.

Preparation of subviral components

Subviral components were prepared through treatment of R-MuLV with Nonidet P-40 (Shell Nederland Chemie, Rotterdam) or sodium dodecyl sulfate (Merck, Darmstadt) in combination with ether. Based on pilot experiments the following procedure was used for the preparation of subviral components through Nonidet P-40 and ether treatment. Five µg of Nonidet P-40 were added to 200 µl of a virus suspension containing about 0.2 mg of purified R-MuLV in TNE buffer. After an incubation of 5 minutes at 4°C, 2 volumes of cold ether were added and the suspension was shaken gently during 10 minutes. The aqueous phase separated by low speed centrifugation was centriguged in a Beckman Ti-50 rotor for 3 hours at 50,000 rpm and the pellet was further processed for electron microscopic examination. In order to purify subviral components, the disrupted virions were layered onto a discontinuous gradient of sucrose in TNE buffer of the following composition: 2 ml 1.78 M sucrose (density 1.30 g/cm^3), 3 ml 1.70 M sucrose (density 1.28 g/cm^3), 2 ml 0.94 M sucrose (density 1.14 g/cm^3), 2 ml 0.82 M sucrose (density 1.12 g/cm^3), and 1 ml 0.68 M sucrose (density 1.10 g/cm^3). Centrifugation was performed in a Beckman SW-40 rotor during 17 hours at 30,000 rpm. Subviral fragments banded on the sucrose layers with densities of 1.28 g/cm^3, and 1.12 g/cm^3 were centrifuged after five-fold dilution with TNE buffer in a Beckman Ti-50 rotor for 3 hours at 50,000 rpm. Thereafter, the pellets were processed for electron microscopic or gel electrophoresis



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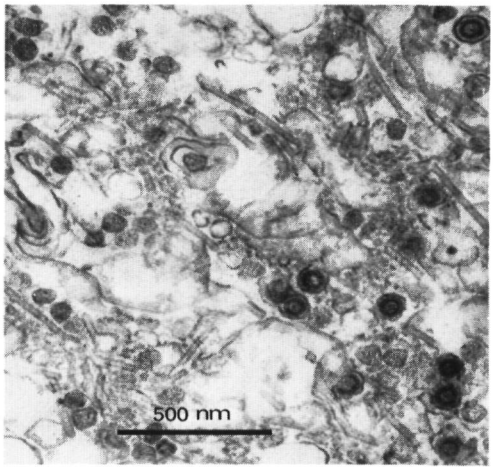

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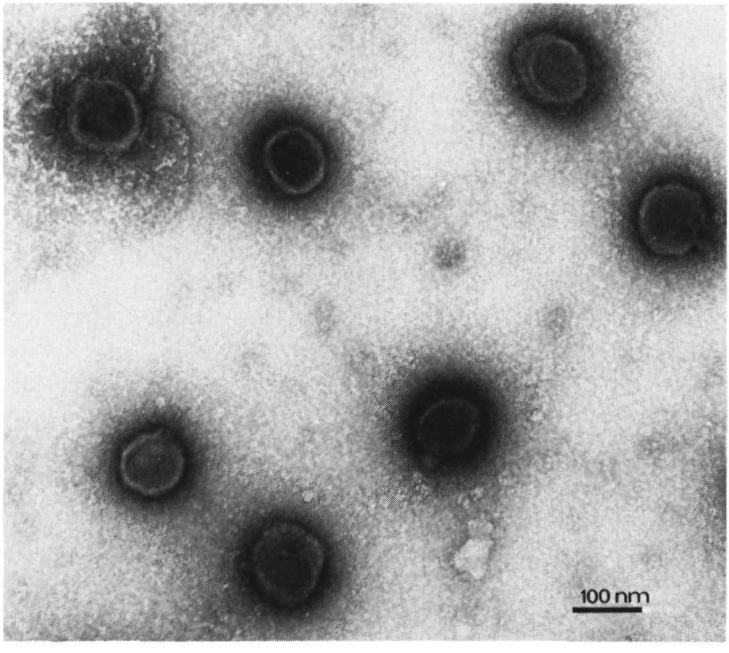

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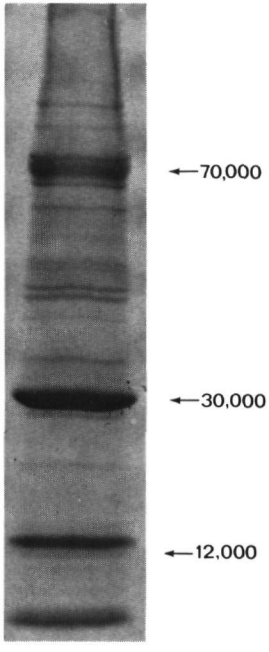

C

analysis.

In order to obtain a pure preparation of viral envelopes of R-MuLV, flotation rather than sedimentation in the discontinuous sucrose gradient was preferred. In flotation experiments, the same discontinuous sucrose gradients as described above were used; in those experiments, however, the disrupted virions were suspended in the bottom layer with a final sucrose concentration of 1.8 M (density 1.30 g/cm^3). Centrifugation was performed as described above. Treatment with sodium dodecyl sulfate and ether was performed similarly using 2 ug of sodium dodecyl sulfate instead of Nonidet P-40.

Immunoprecipitation and polyacrylamide gel electrophoresis

R-MuLV-specific polypeptides were detected by the immunoprecipitation procedure described by Van Zaane et al. (31). Characterization of the antisera was performed by means of immunoprecipitation of virus-specific polypeptides, labeled with a ^{14}C-amino acid mixture (Radiochemical Centre, Amersham, England). Anti-gp70 and anti-p15 serum were a generous gift of Drs. M. Strand and J.T. August. Characteristics of these sera were described by Strand and August (27). The other antisera used were prepared and described by Van Zaane et al. (31). Analysis of the immunoprecipitates were performed by polyacrylamide gel electrophoresis according to Laemmli (15) on sodium dodecyl sulfate containing (7-18%) polyacrylamide gradient slab gels (SDS-PAGE). Visualization of radioactivity was performed by scintillation autoradiography according to Bonner and Laskey (4).

Fig. 1. A) Thin section of NIH-Swiss-3T3 cells infected with Rauscher leukemia virus. Several stages of the morphogenesis of the virus can be identified. The preparation shows two types of virions: a mature type with an enveloped dense nucleoid (arrow →), and an immature type with two concentric shells surrounded by the viral envelope (arrow ↠).
B) Intact Rauscher murine leukemia virus particles, isolated as described in Materials and Methods, prefixed with glutaraldehyde and stained with uranyl acetate. The particles are penetrated by the stain, so that an internal structure is revealed.
C) SDS-PAGE analysis of the polypeptides of isolated virions; Coomassie Brilliant Blue staining.
D) Thin section of a preparation of virions disrupted with sodium dodecyl sulfate and ether as described in Materials and Methods. Nondisrupted virus particles present in the preparation, have the morphology of immature virions. It should be noted, that because of differences in sedimentation, the distribution of virions and substructures as shown in the figure is not representative for the whole pellet.

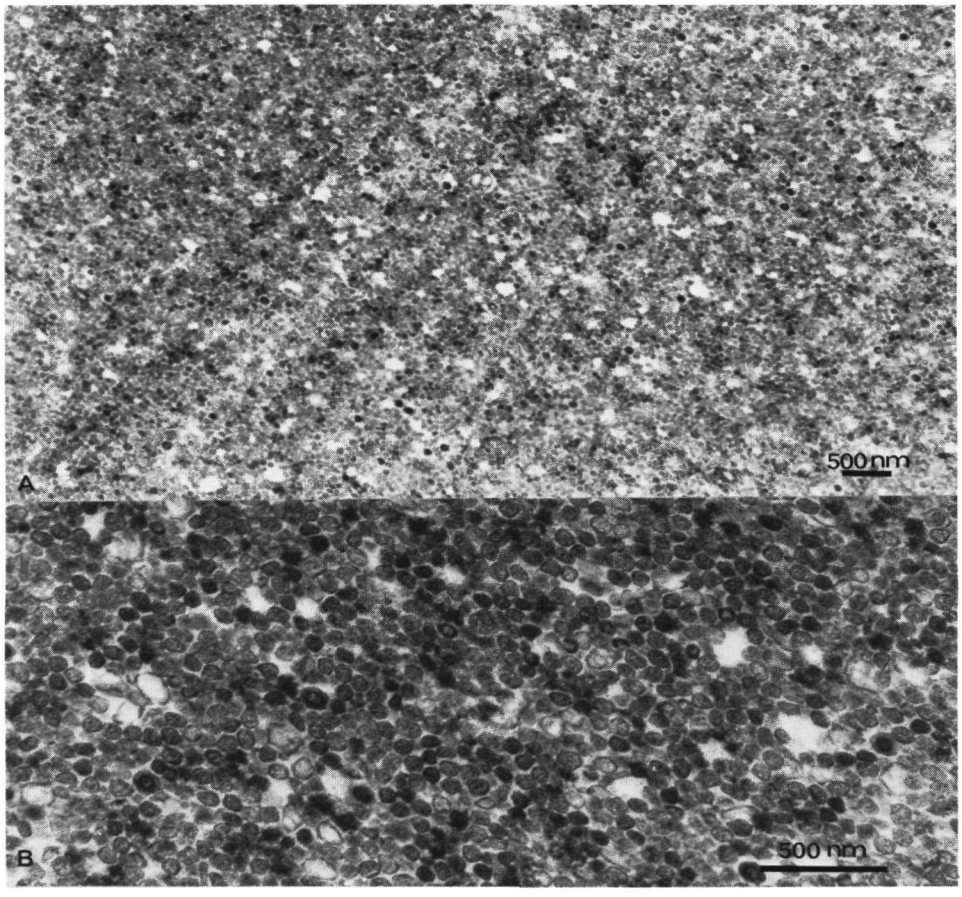

Fig. 2.A) Thin section of R-MuLV cores isolated after treatment of the virions with sodium dodecyl sulfate and ether. The envelope of the virions has been removed.
B) Same preparation as A) at higher magnification.

Radioimmunoassay procedures

The structural polypeptides of R-MuLV, p30, p15, p12 and p10 (gift from Dr. J.R. Stephenson) were each labeled with ^{125}I at high specific activity by the chloramine T method of Greenwood et al. (9); (^{125}I from Radiochemical Centre, Amersham, England). Competition radioimmunoassays were performed as described by Barbacid et al. (1).
Briefly a limiting amount of antiserum against the appropriate viral antigen was incubated with serial dilutions of unlabeled antigen for 1 hour at 37°C. ^{125}I-labeled antigen (about 10,000 cpm) was then added, and incubation continued for 3 hours at 37°C and a further 18 hours at 4°C. Thereafter, 0.025 ml of undiluted pig anti-goat immunoglobulin G was added to each reaction mixture and samples were incubated 1 hour at 37°C followed by 3 hours at 4°C. After centrifugation at 2500 rpm for 15 minutes and aspiration of the supernatant, ^{125}I-radioactivity in the precipitate was measured in a Searle 1285 gamma counter.

Electron microscopy

For thin section electron microscopy, purified virus and subviral fractions were fixed in 2.5% (w/v) glutaraldehyde in sodium cacodylate buffer, pH 7.4, for 1 hour (21), washed twice in sodium cacodylate buffer, and postfixed with OsO_4. Before dehydration the preparations were treated with 0.5% (w/v) uranyl acetate (8). After dehydration in a graded series of ethanol and propylene oxide, the preparations were embedded in Epon 812. Ultrathin sections cut with a LKB microtome (glass knives) were stained with uranyl acetate and lead citrate (20). Alternatively, for negative staining a drop of virus suspension, prefixed with glutaraldehyde, was placed on a Formvarcarbon-coated grid. After 1 minute the excess fluid was removed by touching the grid with a piece of filter paper and the preparation was stained immediately with uranyl acetate, a negative staining method which had previously been applied for examination of the morphology of the virions (18). The preparations were examined in a Philips EM 200 electron microscope.

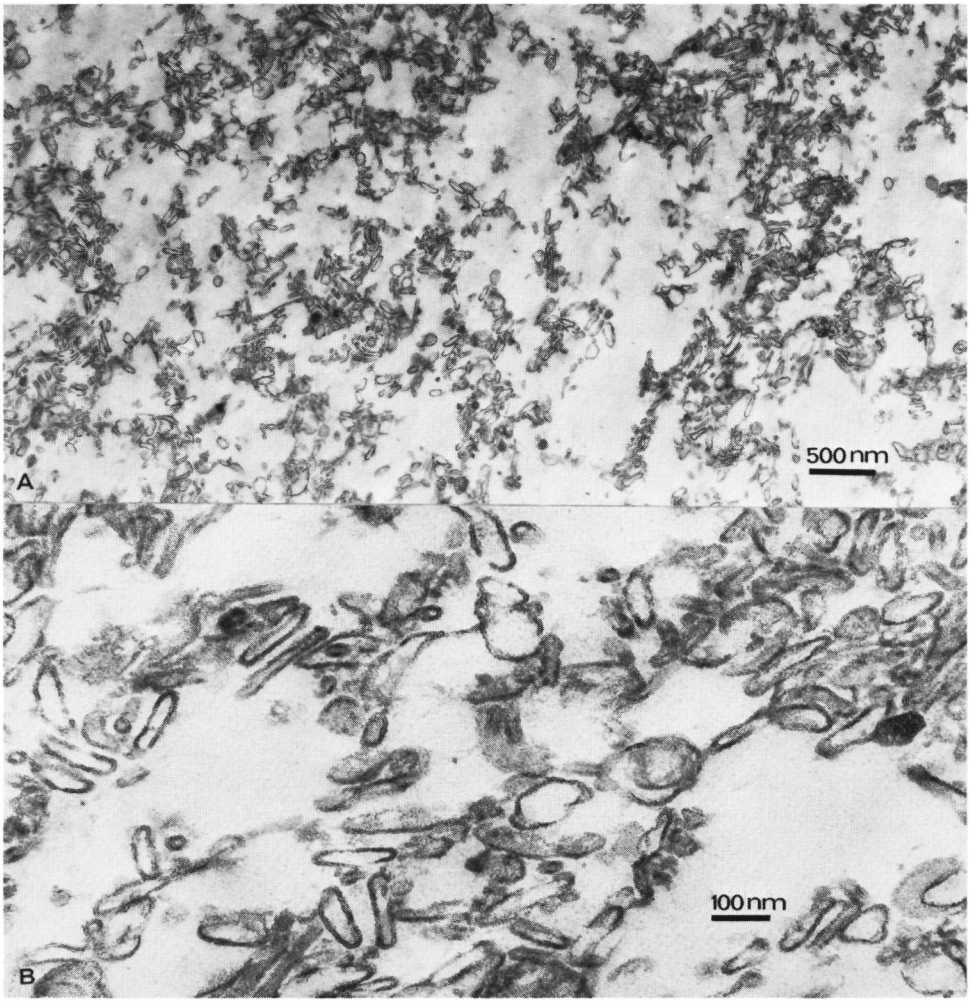

Fig. 3. A) Thin section of R-MuLV envelopes isolated through flotation after treatment with Nonidet P-40 and ether. Note the homogeneity of the preparation.
B) Same preparations as A) at higher magnification.

R-MuLV virions used in this study were isolated from tissue culture medium of chronically infected NIH-Swiss-3T3 cells (Fig. 1A). Samples of virions purified by sucrose gradient centrifugation were examined by thin section and negative staining (Fig. 1B) electron microscopy. From these studies, it appears that in the virion preparations, two types of virions are present (not shown); a mature type with an enveloped dense nucleoid, and an immature type with two concentric shells surrounded by the viral envelope (22) (see also Fig. 1A). The purified virions are morphologically intact since no viral substructures can be observed and the morphology of the isolated virions resembles that of free intercellular virus particles. The virion preparations are very pure, as can be seen from SDS-PAGE analysis of the samples (Fig. 1C).

Treatment with detergent and ether

In order to strip the viral envelope from the virion two different surface active agents were used, namely Nonidet P-40 and sodium dodecyl sulfate. Additional treatment with ether was performed in order to prevent trapping of the viral cores in reconstituted viral membrane fragments (2).

Treatment of R-MuLV virions with sodium dodecyl sulfate and ether resulted in the disruption of the virions. Electron microscopic examination (Fig. 1D) revealed that the viral envelope was stripped from the virions, leaving core particles (diameter of 70-80 nm) and membrane structures, representing the viral envelopes or fragments thereof. Furthermore, non-disrupted virions could be observed. The morphology of these virions always resembled the structure of immature particles. Disruption of R-MuLV virions with Nonidet P-40 and ether gave the same results as treatment with sodium dodecyl sulfate and ether. By subsequent density gradient centrifugation viral core particles could be isolated (16, and Fig. 2). Purified viral envelopes could be obtained by purification through flotation in a discontinuous sucrose gradient. The envelope fraction (Fig. 3) was banded on the sucrose layer with a density of 1.12 g/cm^3. Even after ether treatment, viral cores were found in the

viral envelope fraction when the preparation was sedimented instead of floated. As can be seen in Fig. 3, the viral envelope preparation from a flotation experiment is pure and the morphology (triple layer) of the membrane structures is preserved.

Analysis of the viral envelope fraction

In order to compare the amount of the gag-gene encoded structural polypeptides in the virion and the isolated envelope fraction, sensitive competition radioimmunoassays for p30, p15, p12 and p10 were used. The results are summarized in Table 1.

Table 1. Amounts of gag-gene encoded structural polypeptides in purified viral envelopes of R-MuLV relative to R-MuLV virions [a].

Structural polypeptide	*Relative associated antigenic reactivity in purified viral envelopes (%)* [b]
p30	*2*
p15	*40*
p12	*2*
p10	*2*

a) *Associated levels of antigenic reactivity of gag-gene encoded structural polypeptides in R-MuLV virions and purified viral envelopes were assayed as described in Materials and Methods.*

b) *The associated antigenic reactivity in purified viral envelopes is expressed as % of the reactivity present in the same amount (mg protein) of R-MuLV virions. Protein determinination was performed according to the Lowry method (17).*

Only relatively small amounts of the gag-gene encoded structural polypeptides p30, p12 and p10 were present in the envelope fraction. However, a larger amount of the gag-gene product p15 was detected in this fraction.

The envelope preparations were further analyzed by SDS-PAGE after immunoprecipitation with polyvalent and monospecific antisera directed against R-MuLV proteins. The serological activity of these antisera was tested as previously described (31) in a radioimmunoprecipitation assay with cell lysates of R-MuLV infected NIH-Swiss-3T3 cells labeled with a ^{14}C-amino acid mixture. The results are shown in Fig. 4.

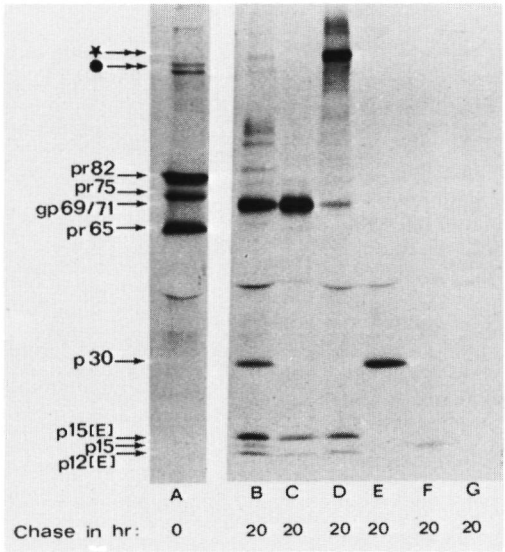

Fig. 4) Characterization of the antisera.
Subconfluent monolayers of NIH-Swiss-3T3 cells (10^6 cells/25 cm^2) infected with R-MuLV were pulse-labeled for 30 minutes with 100 µCi (25µCi/ml) of a ^{14}C-amino acid mixture and radioactivity was chased for 0 or 20 hours as indicated in the figure. After lysis of the cells as described in Materials and Methods, virus-specific polypeptides were analyzed by SDS-PAGE and scintillation autoradiography after immunoprecipitation with: anti-R-MuLV serum (A,B), with anti-gp70 serum (C), with anti-p15(E), p12(E) serum (D), with anti-p30 serum (E), with anti-p15 serum (F), or with anti-BSA serum (G).

In Fig. 4A the env-precursor polypeptide pr82 and the gag-precursor polypeptides pr75 and pr65, immunoprecipitated with anti-R-MuLV serum from pulse labeled cells, are shown. In addition two high molecular weight polypeptides (Marked • in Fig. 4A) were precipitated. Immunoprecipitation analysis with anti-p30 serum and with anti-p15 serum revealed, that in addition to pr75 and pr65, also these high molecular weight polypeptides were precipitated. Furthermore, the envelope precursor was precipitated only with anti-gp70 serum and anti-p15(E),p12(E) serum (results not shown). In pulse-chase experiments after a chase period of 20 hours, all major virion polypeptides, with the exception of p12 and p10, were precipitated by the anti-R-MuLV serum (Fig. 4B). Anti-gp70 serum precipitated gp70, p15(E) and p12(E) (Fig. 4C); p15(E) and p12(E) coprecipitated with gp70 because of strong association with the latter protein (31). For the same reason anti-p15(E),p12(E) serum also precipitated gp70 (Fig. 4D). In addition, an unknown, probably cellular high molecular weight polypeptide (Marked * in Fig. 4D) was precipitated with the anti-p15(E),p12(E) serum. This polypeptide was never observed in isolated virions. Anti-p30 serum precipitated only p30 (Fig. 4E) and anti-p15 serum only p15 (Fig. 4F). None of the virus-specific polypeptides were precipitated with an anti-serum directed against bovine serum albumin (Fig. 4G).

The immunoprecipitation analysis of L-(^{35}S)methionine labeled R-MuLV virions and viral envelopes is shown in Fig. 5. Immunoprecipitations were performed under conditions that no precipitable virion polypeptides were left. It should be noted that in L-(^{35}S)methionine labeled R-MuLV virions no p15 and p10 can be detected, because these proteins lack methionine. gp70, p30, p15(E) and p12(E) were the only structural proteins that could be detected with anti-R-MuLV serum in L-(^{35}S)methionine labeled virions (Fig. 5B). The identity of these virion polypeptides was further confirmed in an immunoprecipitation analysis with monospecific antisera (not shown). In agreement with the results of the radioimmunoassays is the low amount of p30 in the viral envelope fractions (Table 1; Fig. 5A and 5D). In the viral envelope preparations variable amounts of gp70 relative to p15(E) and p12(E) were observed (not shown). However, the amount of p15(E) relative to p12(E) in the virions as well in the purified viral envelopes was always the same (Fig. 5). Both low molecular weight polypeptides in the envelope preparations were identi-

fied as p15(E) and p12(E), using anti-p15(E),p12(E) serum (Fig. 5C).

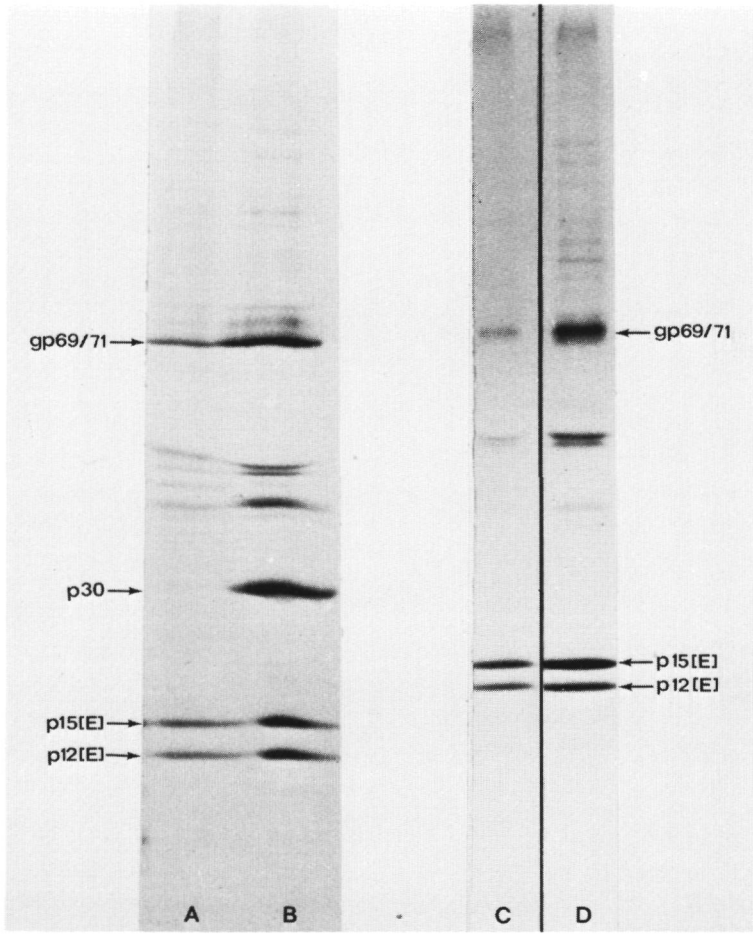

Fig. 5) Polypeptide composition of R-MuLV virions and envelopes. L-(^{35}S)-methionine labeled R-MuLV virions were used to study the env-gene encoded structural polypeptides in virions and purified viral envelopes. R-MuLV envelopes were isolated after treatment of the virions with Nonidet P-40 and ether. The viral polypeptides were characterized by immunoprecipitation with antisera as described in Materials and Methods.
A and D) R-MuLV envelope polypeptides immunoprecipitated with anti-R-MuLV serum.
B) R-MuLV virion polypeptides immunoprecipitated with anti-R-MuLV serum.
C) R-MuLV envelope polypeptides immunoprecipitates with anti-p15(E),p12(E) serum.

We have shown that R-MuLV disrupted virions after treatment with sodium dodecyl sulfate of Nonidet P-40 in combination with ether can be fractionated into a viral core fraction and a viral envelope fraction (Fig. 2 and 3). The fact that in preparations of disrupted virions, in contrast to mature virions, always intact immature virions are found is interesting and suggests that stripping of the viral envelope from immature virus particles is probably more difficult. In order to obtain pure preparations, isolation of viral envelopes has to be performed by flotation in a discontinuous sucrose gradient rather than by sedimentation. Purification of the viral envelopes by flotation was acequate since only a relative small amount of p30, a major component of the viral core (3,16), was present in the purified viral envelope preparations (Fig. 5). Furthermore, similar results were obtained for the internal structural polypeptides p30, p12 and p10 using sensitive competition racioimmunoassays (Table 1). However, a relatively high amount of the gag-gene product, p15, was found in the envelope preparations. According to Bolognesi et al. (3), p15 is not located in the viral envelope but is associated with the viral core or represents the inner coat. The polypeptide, however, is very hydrophobic and has the terdency to aggregate and to form specific complexes with hydrophobic membrane proteins (26). Therefore, p15 is possibly removed from the internal structure during treatment with detergent and ether and forms a complex with the hydrophobic proteins in the viral envelope. Alternatively, it might be possible that the association between p15 and the envelope is functional in that it links the N-terminus of the gag-precursor polypeptide to the envelope reflecting an early step in virion assembly. In a study with avian orcornaviruses (23) an association between the NH_2-terminal gag-polypeptide, p19, and the viral glycoprotein complex was observed. In the murine system, the primary env-gene product is a glycosylated precursor polypeptide, which is processed to gp70 anc p15(E) from which a polypeptice p12(E) is cleaved (13,31). From the present study it appeared that the amount of p15(E) relative to p12(E) in the virion and the viral envelope is approximately the same (Fig. 5). Apparently, there is no preferential loss of one of these components during the isolation procedure of the viral envelopes.

The amount of gp70, relative to p15(E) and p12(E), however, is variable; this is in agreement with the observation that by treatment with detergents gp70 can easily be removed from the surface of the virions (7). In the murine system the hydrophobic p15(E) component is embedded in the lipid bilayer while the glycosylated gp70 seems to be more exposed at the virion surface (3,14); gp70 is attached to p15(E) by disulfide bonds but the biological significance of this structure is not known at this time. In contrast to p15(E), p12(E) seems not to be linked by disulfide bonds to gp70 (3). Furthermore, in a previous study (30) we showed that p12(E) is formed at a later stage of viral assembly since at the restrictive temperature, in cells infected with ts-mutants of R-MuLV the envelope precursor polypeptide is cleaved into gp70 and p15(E), but p12(E) is not formed. Therefore, it is of interest to further characterize the synthesis and the biological of this low molecular weight viral envelope protein.

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CHAPTER III

EFFECT OF IMPAIRED GLYCOSYLATION ON THE SYNTHESIS OF ENVELOPE PROTEINS OF RAUSCHER MURINE LEUKEMIA VIRUS

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Effect of Impaired Glycosylation on the Synthesis of Envelope Proteins of Rauscher Murine Leukemia Virus

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The effects of 2-deoxy-D-glucose (deoxyglucose) and cytochalasin B on the synthesis of Rauscher murine leukemia viral envelope proteins were investigated JLS-V9 cells infected with and producing Rauscher murine leukemia virus (R-MuLV) were cultured in the presence of deoxyglucose (15 m*M*) or of varying concentrations of cytochalasin B (0 1–25 μg/ml) and the synthesis of virus-specific polypeptides was examined in pulse-chase experiments Overall protein synthesis was inhibited to some extent by cytochalasin B, at a concentration of 25 μg/ml an inhibition of about 70% was observed Incorporation of D-[1-^{3}H]glucosamine, however, was almost completely inhibited (97%) at the same concentration of the drug Newly formed virus-specific polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis after immunoprecipitation with polyvalent and monospecific antisera against R-MuLV proteins No specific effect on the synthesis of the *gag* gene product p30 was observed The synthesis of the precursor of the envelope polypeptides, *env*-pr82, however, was prevented in the presence of cytochalasin B Instead, the synthesis of a new glucosamine-deficient 70,000-molecular weight polypeptide (*env*-pr70*) was observed *Env*-pr70* could be immunoprecipitated with anti-gp69/71 serum and anti-p15(E),p12(E) serum and, therefore, probably represents the protein moiety of *env*-pr82 In pulse-chase experiments in the presence of cytochalasin B, *env*-pr70* was converted to a polypeptide with a molecular weight of about 75,000 which was slowly lost during the chase period Production of virus-specific gp69/71, p15(E), and p12(E) was inhibited under these conditions Similar results were obtained with deoxyglucose, except that the inhibition of glycosylation by the latter compound is irreversible

INTRODUCTION

Virus replication can be inhibited under conditions that interfere with viral glycoprotein synthesis (for review see Scholtissek, 1975). Interference with glycoprotein biosynthesis, caused by elevated concentrations of glucosamine or by sugar analogs such as 2-deoxy-D-glucose (deoxyglucose), 2-deoxy-2-fluoro-D-glucose, or 2-deoxy-2-fluoro-mannose, was shown to inhibit the virion synthesis in several virus-host cell systems (Kaluza *et al.*, 1972; Courtney *et al.*, 1973; Schmidt *et al.*, 1976). For example, a glucosamine concentration of 20 m*M* inhibits the production of fowl plague, Sindbis, and Semliki Forest virus almost completely (Kaluza *et al*, 1972). On the other hand, herpes simplex virus particles are still produced after treatment with deoxyglucose but these particles are not infectious (Courtney *et al.*, 1973). In the case of avian RNA tumor viruses, inhibition of virus replication by elevated concentrations of glucosamine has been reported (Hunter *et al.*, 1974; Hayman *et al.*, 1976); Lewandowski *et al.* (1975) found that under these conditions an incompletely glycosylated 70,000-molecular weight polypeptide was formed instead of the major glycoprotein of Rous sarcoma virus, gp85. Similar alterations in glycosylation of viral glycoproteins have often been ob-

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served after treatment with glucosamine or with sugar analogs (Klenk *et al* , 1972, Courtney *et al* , 1973, Courtney, 1976, Shapiro *et al* , 1976) and, therefore, the described inhibition of virus replication is probably caused by an altered glycosylation

An even more pronounced interference with glycosylation can be obtained with cytochalasin B (Dix and Courtney, 1976), a metabolite produced by the fungus *Helminthosporium dematioideum* In contrast to sugar analogs, cytochalasin B interferes with glycosylation mainly through inhibition of the transport of certain monosaccharides across the plasma membrane (Kletzien and Perdue, 1973)

In this study, we have investigated the effects of cytochalasin B and deoxyglucose on the synthesis and processing of the glycosylated precursor polypeptide *env*-pr82 of Rauscher murine leukemia virus (R-MuLV) (Van Zaane *et al* , 1976, Shapiro *et al* , 1976, Arcement *et al* , 1976, Naso *et al* , 1976) *Env*-pr82 is the precursor of the viral envelope polypeptides gp69/71, p15(E), and p12(E)

MATERIALS AND METHODS

Materials L-[^{35}S]Methionine (sp act, 200 Ci/mmol) and D-[1-^{3}H]glucosamine (sp act, 3 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, England The glucose analog deoxyglucose was obtained from Sigma Chemical Co , St Louis, Mo Cytochalasin B was bought from Aldrich Chemical Co , Milwaukee, Wis , and dimethyl sulfoxide (DMSO) from Merck, Darmstadt, Germany Stock solutions of cytochalasin B in DMSO (10 mg/ml) were stored at −20°

Cells, virus, and media JLS-V9 cells, derived from bone marrow cells of BALB/c mice (Chopra and Shibley, 1967) infected with and producing R-MuLV were cultured as monolayers in *maintenance medium* consisting of Eagle's minimal essential medium supplemented with 10% calf serum Cells are labeled in *labeling medium* consisting of Hanks' basic salt solution supplemented with dialyzed calf serum and amino acids For experiments with L-[^{35}S]methionine, labeling medium lacking methionine was used For experiments with D-[1-^{3}H]glucosamine, labeling medium lacking glucose was used

Treatment of JLS-V9 cells with cytochalasin B was performed in medium containing the desired concentration of cytochalasin B and a final DMSO concentration of 1%

Labeling of cells and preparation of lysates Labeling of JLS-V9 cells with L-[^{35}S]methionine or D-[1-^{3}H]glucosamine in the presence of DMSO and cytochalasin B was performed as follows Subconfluent monolayers of JLS-V9 cells infected with R-MuLV and growing in plastic tissue culture flasks (Falcon Plastics, 25 cm^2), were preincubated at 37° in 5 ml of Dulbecco's phosphate-buffered saline, pH 7 2 (DPBS) (Dulbecco *et al* , 1954), for 4 hr in order to reduce intracellular sugar pools Then DPBS was replaced by 4 ml of maintenance medium, containing 1% DMSO and an indicated concentration of cytochalasin B, and the cells were incubated for another 2 hr Thereafter, the cells were starved for 10 min in 3 ml of labeling medium lacking methionine or glucose but containing DMSO and cytochalasin B Subsequently, L-[^{35}S]methionine or D-[1-^{3}H]glucosamine was added and the cells were incubated for appropriate times Following pulse-labeling, the cells were washed once and 4 ml of maintenance medium containing DMSO and cytochalasin B were added, thereafter, the cells were either lysed immediately or radioactivity was chased for various times In the absence of DMSO and cytochalasin B the same labeling procedure was used

Pulse-chase experiments in the presence of 15 m*M* deoxyglucose were performed according to the same procedure but preincubation in DPBS was omitted

After pulses as well as after chases, the cells were lysed by the addition to the cultures (i e , cells and medium) of 1 ml of a buffer containing 50 m*M* sodium phosphate, pH 7 2, 4 5% sodium chloride, 5 0% Triton X-100, 2 5% deoxycholate, and 0 5% sodium dodecyl sulfate [fivefold-concentrated *immunoprecipitation buffer* (Van Zaane *et al* , 1976)] After clearing of the lysates through centrifugation at 220,000 *g* for 10 min in a Ti-50 rotor of a Spinco L-50 centrifuge, the lysates were stored at −80°

To study the effect of various concentra-

tions of cytochalasin B on the total incorporation of L-[^{35}S]methionine or D-[1-^{3}H]glucosamine, cells were labeled according to the procedure described above except that labeling with L-[^{35}S]methionine was performed in maintenance medium instead of labeling medium, in order to prevent exhaustion of methionine during long incubations. Incorporation of L-[^{35}S]methionine and D-[1-^{3}H]glucosamine was determined after lysis of pulse-labeled cells. Aliquots of lysates labeled with L-[^{35}S]methionine or with D-[1-^{3}H]glucosamine were precipitated with trichloroacetic acid (TCA). Before TCA precipitation, lysates labeled with L-[^{35}S]methionine were preincubated with 1 ml of 0 1 *N* NaOH for 15 min at 37°. The TCA-insoluble material was collected on Millipore filters and radioactivity was counted. Protein concentrations were determined according to Lowry *et al.* (1951).

Immunoprecipitation and polyacrylamide-gel electrophoresis. Detection of R-MuLV-specific polypeptides in cell lysates was performed with polyvalent and monospecific antisera against R-MuLV proteins according to the immunoprecipitation procedure described by Van Zaane *et al.* (1976). Anti-gp69/71 serum was a generous gift of M. Strand and J T. August. Characteristics of this antiserum were described by Strand and August (1976). The other antisera were prepared and described by Van Zaane *et al.* (1976) Analysis of the immunoprecipitation was performed by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) according to Laemmli (1970) on 7-18% polyacrylamide gradient slab gels. Radioactivity was visualized by scintillation autoradiography (Bonner and Laskey, 1974).

RESULTS

Effect of Cytochalasin B on the Incorporation of L-[^{35}S]Methionine and D-[1-^{3}H]Glucosamine

The effect of cytochalasin B on the incorporation of L-[^{35}S]methionine and D-[1-^{3}H]glucosamine into TCA-precipitable material was examined in R-MuLV-infected JLS-V9 cells. The results are summarized in Table 1. Labeling of the cells for 2 hr with L-[^{35}S]methionine revealed that, at increasing concentration of the drug, the incorporation of L-[^{35}S]methionine decreases. A reduction of the incorporation to about 30% was observed at a concentration of 25 μg/ml. The reduction of incorporation was maintained throughout a labeling period of several hr (Fig. 1a). The inhibitory effect of cytochalasin B on the overall incorporation of D-[1-^{3}H]glucosamine was much more pronounced (Fig. 1b). Even at low concentrations of the drug, inhibition of overall incorporation was drastic (Table 1).

TABLE 1

EFFECT OF CYTOCHALASIN B ON THE INCORPORATION OF L-[^{35}S]METHIONINE AND D-[1-^{3}H]GLUCOSAMINE IN JLS-V9 CELLS INFECTED WITH R-MuLV[a]

Treatment	L-[^{35}S]Methionine		D-[1-^{3}H]Glucosamine	
	Counts per minute per microgram of protein[b]	Percentage of control	Counts per minute per microgram of protein[b]	Percentage of control
None	860	100	2865	100
DMSO (1%)	660	77	2118	74
Cytochalasin B				
1 μg/ml	600	70	935	33
5 μg/ml	440	51	293	10
10 μg/ml	320	37	106	4
25 μg/ml	280	31	78	3

[a] Subconfluent monolayers of JLS-V9 cells infected with R-MuLV were preincubated in DPBS for 4 hr and thereafter cultured for 2 hr in maintenance medium or maintenance medium containing 1% DMSO and cytochalasin B Cells were labeled either with L-[^{35}S]methionine (3 μCi/ml) for 2 hr or with D-[1-^{3}H]glucosamine (20 μCi/ml) for 20 hr, lysed, and treated with trichloroacetic acid as described in Materials and Methods, then overall incorporated radioactivity was determined

[b] Incorporation is expressed as count per minute per microgram of protein present in the lysate In the case of glucosamine, the incorporated label may be present in lipopolysaccharides as well as in glycoproteins.

Effect of Cytochalasin B on the Synthesis of Virus-Specific Precursor Polypeptides

The effect of cytochalasin B on the synthesis of virus-specific precursor polypep-

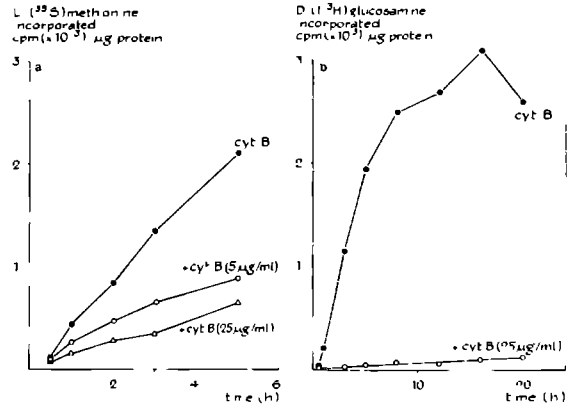

FIG 1 Effect of cytochalasin B on the incorporation of L [35S]methionine and D [1 3H]glucosamine into TCA precipitable material Subconfluent monolayers of JLS V9 cells infected with R MuLV were preincubated in DPBS for 4 hr and thereafter cultured for 2 hr in maintenance medium or maintenance medium containing 1% DMSO and cytochalasin B (5 or 25 µg/ml) Cells were labeled either with L-[35S]methionine (3 µCi/ml) (a) or with D-[1 3H]glucosamine (20 µCi/ml) (b) as described in Materials and Methods At indicated intervals, incorporated radioactivity was determined

tides was studied in pulse-chase experiments After preincubation in DPBS, R-MuLV-infected JLS-V9 cells were cultured in the presence of various concentrations of cytochalasin B and pulse-labeled with L-[35S]methionine, lysates were prepared for radioimmunoprecipitation with polyvalent and monospecific antisera The solubilized precipitates were subjected to gel electrophoresis

The control experiments (Fig 2A and B) showed that the precursor polypeptides *env*-pr82, *gag*-pr75, and *gag*-pr65 had been synthesized [the identification of these products is based on analysis with monospecific antisera and tryptic digests (cf Van Zaane *et al* , 1976)] At increasing concentrations of cytochalasin B a progressive disappearance of *env*-pr82 was observed (Fig 2C–H) Concomitantly, polypeptides with greater electrophoretic mobility appeared (Fig 2F–H) At a concentration of 25 µg/ml, cytochalasin B prevented the synthesis of *env*-pr82 completely while a new polypeptide of 70,000 molecular weight was formed (Fig 2H) The same polypeptide was precipitated with both monospecific anti-gp69/71 serum and anti-p15(E),p12(E) serum (Fig 2I and J), indicating that it carried antigenic determinants of the virus-specific polypeptides gp69/71, p15(E), and p12(E), the same set of antigenic determinants is present in *env*-pr82 (Van Zaane *et al* , 1976) The 70,000-molecular weight polypeptide is not precipitated in control experiments using bovine serum albumin antiserum (Fig 3F) Since the precursor polypeptide *env*-pr82 is glycosylated (Van Zaane *et al* , 1976, Shapiro *et al* , 1976, Arcement *et al* , 1976, Naso *et al* , 1976), the effect of cytochalasin B on the incorporation of D-[1-3H]glucosamine into virus-specific polypeptides was investigated Therefore, radioimmunoprecipitation analysis with anti-R-MuLV serum was performed on lysates from cells cultured in the presence or absence of cytochalasin B and labeled with D-[1-3H]glucosamine for 20 hr

The results of this experiment were compared with those obtained under the same conditions except that labeling was performed with L-[35S]methionine for 30 min In the absence of the drug, the glycosylated virus-specific polypeptides *env*-pr82 and gp69/71, labeled with D-[1-3H]glucosamine, were synthesized as could be seen after immunoprecipitation with

anti-R-MuLV serum (Fig. 3A and B). In a control experiment none of these polypeptides was precipitated with antiserum to bovine serum albumin (Fig. 3G).

In the presence of cytochalasin B no D-[1-^{3}H]glucosamine-labeled polypeptides could be precipitated with anti-R-MuLV serum (Fig. 3C). Comparison with L-[^{35}S]methionine-labeled immunoprecipitates revealed that the failure of D-[1-^{3}H]glucosamine to be incorporated into the virus-specific glycoproteins *env*-pr82 and gp69/71 was accompanied by the appearance of the 70,000-molecular weight polypeptide and the absence of *env*-pr82 (Fig. 3D). From these data we conclude that the 70,000-molecular weight polypeptide, synthesized in the presence of cytochalasin B (25 μg/ml) is an uncompletely glycosylated or nonglycosylated form of *env*-pr82. Therefore, this polypeptide will be indicated as *env*-pr70*, the asterisk indicating that it is formed under abnormal conditions (notably in the presence of an inhibitor of glycosylation). In contrast to the effects on *env* polypeptides, cytochalasin B had no effect on the electrophoretic mobility in SDS-PAGE of the precursor polypeptides *gag*-pr75 and *gag*-pr65 (Fig. 3D and E).

Effect of Deoxyglucose on the Synthesis of Virus-Specific Precursor Polypeptides

Shapiro *et al.* (1976) also reported the synthesis of a 70,000-molecular weight polypeptide with gp69/71 antigenic determinants in cells infected with R-MuLV when glycosylation was inhibited with deoxyglucose. In order to compare the 70,000-molecular weight polypeptide synthesized in the presence of cytochalasin B with the one synthesized in the presence of

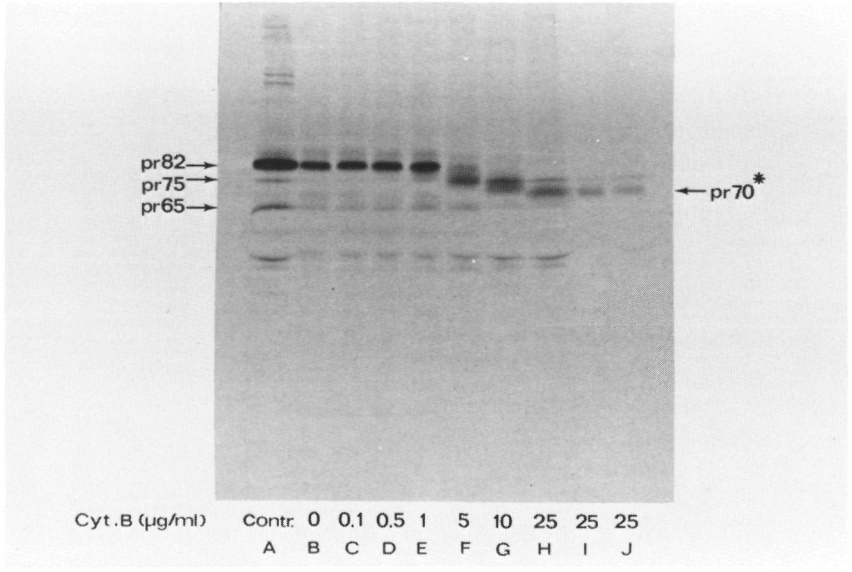

FIG. 2. Effect of various concentrations of cytochalasin B on the synthesis of R-MuLV-specific precursor polypeptides. Subconfluent monolayers of JLS-V9 cells infected with R-MuLV were preincubated in DPBS for 4 hr and thereafter cultured for 2 hr in maintenance medium (A) or maintenance medium containing 1% DMSO and various concentrations of cytochalasin B as indicated (B–J). After labeling for 90 min with L-[^{35}S]methionine (25 μCi/ml) as described in Materials and Methods, cells were lysed and virus-specific polypeptides were analyzed by SDS-PAGE and scintillation autoradiography after immunoprecipitation with anti-R-MuLV serum (A–H), anti-gp69/71 serum (I), or anti-p15(E),p12(E) serum (J).

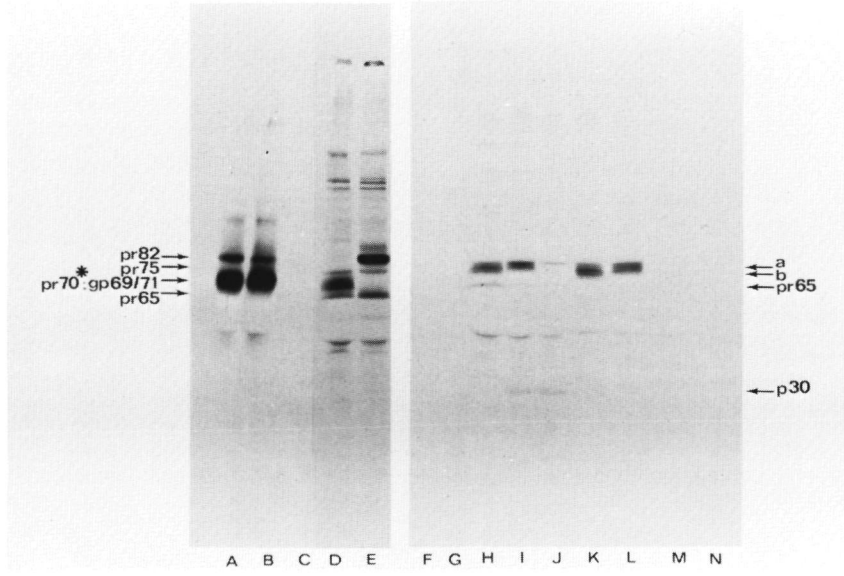

FIG. 3. Comparison of the effects of cytochalasin B (cyt. B) (25 μg/ml) and deoxyglucose (15 mM) on the synthesis of R-MuLV-specific precursor polypeptides. In pulse-chase experiments JLS-V9 cells infected with R-MuLV were labeled with L-[^{35}S]methionine (25 μCi/ml) for 30 min. In other experiments, cells were labeled with D-[1-^{3}H]glucosamine (20 μCi/ml) for 20 hr. After lysis of the cells, immunoprecipitation was performed as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE and scintillation autoradiography. Experimental details are listed in the following columns.

Lane	Label used	Chase (hr)	Treatment during experiment	Antiserum
A	[^{3}H]Glucosamine	–	None	Anti-R-MuLV
B	[^{3}H]Glucosamine	–	1% DMSO	Anti-R-MuLV
C	[^{3}H]Glucosamine	–	1% DMSO, cyt. B	Anti-R-MuLV
D	[^{35}S]Methionine	0	1% DMSO, cyt. B	Anti-R-MuLV
E	[^{35}S]Methionine	0	None	Anti-R-MuLV
F	[^{35}S]Methionine	0	1% DMSO, cyt. B	Anti-BSA
G	[^{3}H]Glucosamine	–	None	Anti-BSA
H	[^{35}S]Methionine	0	Deoxyglucose	Anti-R-MuLV
I	[^{35}S]Methionine	2	Deoxyglucose	Anti-R-MuLV
J	[^{35}S]Methionine	20	Deoxyglucose	Anti-R-MuLV
K	[^{35}S]Methionine	0	Deoxyglucose	Anti-gp69/71
L	[^{35}S]Methionine	2	Deoxyglucose	Anti-gp69/71
M	[^{35}S]Methionine	0	Deoxyglucose	Anti-BSA
N	[^{35}S]Methionine	2	Deoxyglucose	Anti-BSA

deoxyglucose, the latter compound was used in parallel experiments. Radioimmunoprecipitation analysis with anti-R-MuLV serum of lysates of cells cultured in the presence of 15 mM deoxyglucose and pulse-labeled with L-[^{35}S]methionine for 30 min revealed that the synthesis of *env*-pr82 was inhibited (Fig. 3H). Simultane-

ously, not one but several new polypeptides of about 70,000 molecular weight were immunoprecipitated with monospecific anti-gp69/71 serum (band b in Fig 3H and K). There is a slight difference in the electrophoretic mobilities of these polypeptides and the 70,000-molecular weight polypeptide, synthesized in the presence of cytochalasin B (Fig. 3D and H).

Effect of Cytochalasin B on the Synthesis of the env Gene Products gp69/71, p15(E), and p12(E)

The *env* gene products gp69/71, p15(E), and p12(E) are formed after proteolytic cleavage of the glycosylated precursor polypeptide *env*-pr82 (Van Zaane *et al*., 1976, Arcement *et al.*, 1976; Shapiro *et al.*, 1976; Naso *et al.*, 1976). The effect of cytochalasin B on the production of these structural polypeptides was studied in pulse-chase experiments described in Fig 4. Synthesis and processing of the virus-specific polypeptides *env*-pr82, *gag*-pr75, and *gag*-pr65 in JLS-V9 cells cultured in the presence or absence of 1% DMSO (the solvent for cytochalasin B) are shown in Fig. 4A, B, D, and E. In the presence of cytochalasin B, *env*-pr70* was synthesized (Fig. 4C). After a chase period of 2 hr, still in the presence of cytochalasin B, *env*-pr70* had disappeared and a new polypeptide of about 75,000 molecular weight was precipitated with anti-R-MuLV serum (Fig. 4F). In a separate experiment (not shown) the 75,000-molecular weight polypeptide was also precipitated with anti-gp69/71 serum and anti-p15(E),p12(E) serum but not with anti-p30 serum. It therefore has the same serological properties as *env*-pr82. After longer chase periods the new polypeptide disappeared gradually (Fig. 4G and H). Furthermore, production of the *env*-gene products gp69/71, p15(E), and p12(E) was inhibited [traces of p15(E) and p12(E) were visible in the original autoradiogram]. The 75,000-molecular weight polypeptide was not synthesized *de novo* during the chase period because inhibition of protein synthesis with cycloheximide did not prevent its formation (not shown). In another experiment, cytochalasin B was present during the pulse but absent during a chase period of 2 and 20 hr; under these conditions *env*-pr70* disappeared and polypeptides varying in molecular weight between 75,000 and 82,000 were formed (Fig 4N and O). These polypeptides carried gp69/71 antigenic determinants (not shown). Furthermore, production of the *env* gene-related structural viral proteins gp69/71, p15(E), and p12(E) was observed (Fig. 4N and O). Production of these polypeptides was also observed when DMSO or cytochalasin B (Fig. 4I and J) was present during the chase period only.

Very similar results were obtained with 15 m*M* deoxyglucose instead of cytochalasin B (Fig. 3H–N). Polypeptides with gp69/71 antigenic determinants, formed during the chase period in the presence of deoxyglucose, were only slightly higher in molecular weight than 70,000 (band a in Fig 3I) and these polypeptides also disappeared during a chase period (Fig. 3I–J). In that case, however, no virus-specific gp69/71, p15(E), and p12(E) were formed (Fig. 3I, J, and L).

DISCUSSION

Cytochalasin B interferes with various cellular functions (Carter, 1972; Spooner *et al.*, 1971; Zigmond and Hirsch, 1972), including the transport of certain monosaccharides across the plasma membrane (Kletzien and Perdue, 1973), and in this way depresses glycoprotein synthesis in cultured cells (Sanger and Holtzer, 1972). The sugar analog deoxyglucose inhibits glycoprotein synthesis in a different way. It enters the cell and becomes incorporated into glycoproteins (Kaluza *et al.*, 1973). Once incorporated, elongation and/or branching of the carbohydrate chain of the glycoprotein is irreversibly blocked (Scholtissek, 1975) apparently because glycosyl transferases fail to recognize the deoxysugar (Dix and Courtney, 1976). In studying the effect of cytochalasin B on protein synthesis in JLS-V9 cells, the observed reduction in L-[^{35}S]methionine incorporation (Fig. 1a) is in agreement with the results of previous studies (Koch and Opperman,

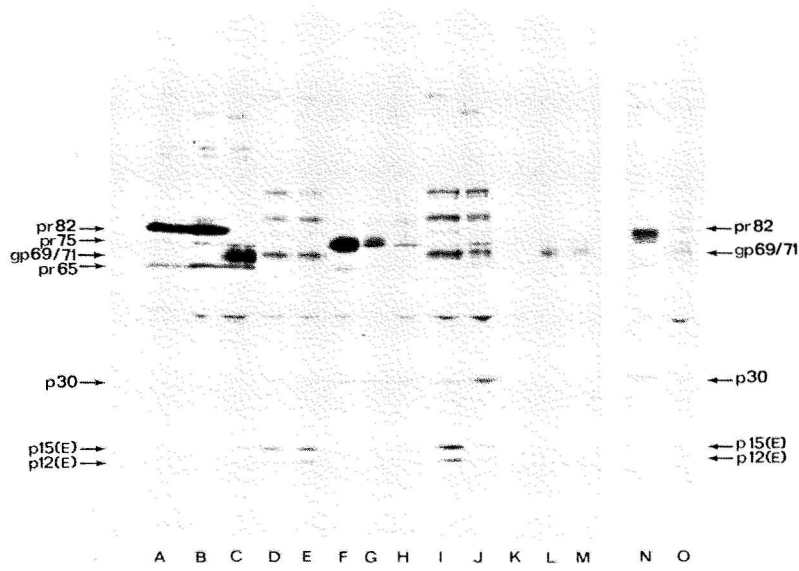

FIG. 4. Effect of cytochalasin B (cyt. B) (25 μg/ml) on the synthesis of the viral envelope proteins. Subconfluent monolayers of JLS-V9 cells infected with R-MuLV were pulse labeled for 30 min with L-[^{35}S]methionine (25 μCi/ml) and radioactivity was chased for various times. After lysis of the cells immunoprecipitation was performed as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE and scintillation autoradiography. Experimental details are listed in the following columns.

Lane	Chase (hr)	Treatment during pulse	Treatment during chase	Antiserum
A	0	None	–	Anti-R-MuLV
B	0	1% DMSO	–	Anti-R-MuLV
C	0	1% DMSO, cyt. B	–	Anti-R-MuLV
D	20	None	None	Anti-R-MuLV
E	20	1% DMSO	1% DMSO	Anti-R-MuLV
F	2	1% DMSO, cyt. B	1% DMSO, cyt. B	Anti-R-MuLV
G	7	1% DMSO, cyt. B	1% DMSO, cyt. B	Anti-R-MuLV
H	20	1% DMSO, cyt. B	1% DMSO, cyt. B	Anti-R-MuLV
I	20	None	1% DMSO	Anti-R-MuLV
J	20	None	1% DMSO, cyt. B	Anti-R-MuLV
K	0	1% DMSO, cyt. B	–	Anti-BSA
L	0	1% DMSO, cyt. B	–	Anti-gp69/71
M	0	1% DMSO, cyt. B	–	Anti-p15(E), p12(E)
N	2	1% DMSO, cyt. B	None	Anti-R-MuLV
O	20	1% DMSO, cyt. B	None	Anti-R-MuLV

1975). Nevertheless, the effect of cytochalasin B on the incorporation of labeled amino acids can vary considerably with the cell line used; for instance, Koch and Opperman (1975) reported a 55–60% inhibition of incorporation in poliovirus-infected HeLa cells at a concentration of the drug of 50 μg/ml; Dix and Courtney (1976)

on the other hand found no significant inhibition of incorporation in HSV-1-infected human embryonic lung fibroblasts at the same concentration of the drug Yet, in the latter case, the strong inhibitory effect of cytochalasin B on the overall incorporation of D-[1-^{3}H]glucosamine, as found in this study, was also observed

In pulse-labeling experiments, the effects of cytochalasin B on the synthesis of virus-specific precursor polypeptides *gag*-pr75, *gag*-pr65, and *env*-pr82 was investigated (Fig 2) While cytochalasin B did not affect the synthesis of *gag*-pr75 and *gag*-pr65 very much it strongly inhibited the synthesis of the glycosylated precursor polypeptide *env*-pr82 (Fig 2H) Instead of *env*-pr82, polypeptides with higher electrophoretic mobility appeared (Fig 2) From this kinetic relationship as well as from their serological properties we conclude that these intermediate bands between 70,000 and 82,000 molecular weight are *env*-pr82-related, partly glycosylated polypeptides We have not attempted to confirm this result by analysis of (chymo)tryptic digests because of the low amounts of radioactivity incorporated in these polypeptides *Env*-pr70* (formed at 25 μg/ml of cytochalasin B) is probably not glycosylated at all since, even at a cytochalasin B concentration of 50 μg/ml, no smaller *env*-pr82-related polypeptides were synthesized (result not shown) A minor degree of glycosylation of *env*-pr70*, however, cannot be excluded, since carbohydrate components for limited glycosylation may be available from intracellular sources

Other evidence in favor of the assumption that *env*-pr70* represents the bare protein moiety of *env*-pr82 follows from our earlier work 22 S virus-specific mRNA from cells infected with R-MuLV was translated in a rabbit reticulocyte lysate and gave rise to the synthesis of a 70,000-molecular weight polypeptide that reacted with anti-R-MuLV serum (Gielkens *et al*, 1976) The 70,000-molecular weight polypeptide was not analyzed with monospecific antisera. In a later study with oocytes of *Xenopus laevis*, it was established that the 22 S mRNA directs the synthesis of *env*-pr82 and its cleavage products gp69/71 and p15(E) (Van Zaane *et al*, 1977) Therefore, the 70,000-molecular weight polypeptide formed in the reticulocyte cell free system is presumably identical or almost identical to *env*-pr70* described in this paper

In the presence of 15 m*M* deoxyglucose, several similar carbohydrate-deficient polypeptides were formed with molecular weights of approximately 70,000 (Fig. 3H), they also possessed gp69/71 antigenic determinants (Fig 3K) Furthermore, the molecular weights of these polypeptides were slightly higher than the molecular weight of *env*-pr70* formed in the presence of cytochalasin B (Fig 3D and H) The small difference in molecular weight may be due to difference in glycosylation, since inhibition of glycosylation with deoxyglucose becomes effective only after incorporation of the sugar analog

Cytochalasin B did not affect the proteolytic cleavage of the virus-specific precursor polypeptides *gag*-pr75 and *gag*-pr65 (Fig 4F-H) [The only detectable *gag* gene-related mature viral protein is p30; neither p15 nor p10 were detected because they lack methionine (Ikeda *et al*, 1975), furthermore, p12 and p10 were not detected in our immunoprecipitation assay]. In contrast, cytochalasin B had an inhibitory effect on the production of the envelope proteins (Fig 4F-H) This inhibitory effect of cytochalasin B, however, is reversible After removal of the drug, glycosylated *env*-pr70*-related polypeptides of higher molecular weight (MW, 75,000-82,000) are formed as well as the *env* gene products gp69/71, p15(E), and p12(E) (Fig 4N-O)

The inhibitory effect of deoxyglucose of the synthesis of the envelope precursor polypeptide and the production of the envelope proteins is in agreement with the observations of Shapiro *et al*, (1976) They reported the synthesis of a 70,000-molecular weight polypeptide and no cleavage to specific smaller proteins The relationship between this 70,000-molecular weight polypeptide, the envelope precursor polypeptide, and gp69/71 was confirmed by these authors with tryptic digest analysis

In contrast to cytochalasin B, deoxyglucose irreversibly inhibits glycosylation (Kaluza *et al*, 1973), since its incorporation inhibits further addition of carbohydrate moieties to the oligosaccharide chain.

The present data show the effect of inhibitors of glycosylation on the synthesis and processing of glycosylated R-MuLV-specific precursor polypeptides A nonglycosylated precursor polypeptide of the envelope proteins, as synthesized in the presence of cytochalasin B or deoxyglucose, has not been detected under normal tissue culture conditions. Probably, the nascent chain of *env*-pr82 is the normal substrate for glycosylation We have no direct proof for this assumption but in a study on vesicular stomatitis virus a similar suggestion was recently made by Hunt and Summers (1976). Furthermore, Molnar (1975) has shown in a general model for glycosylation in the liver that glycosylation of plasma glycoproteins starts with the addition of *N*-acetylglucosamine to the nascent polypeptide chain on the membrane-bound ribosomes. Other authors have concluded that correct glycosylation of certain precursor polypeptides is required for a correct processing (Shapiro *et al*, 1976, Shapiro and August, 1976) Our results seem to support that conclusion

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CHAPTER IV

IMPAIRED PROCESSING OF PRECURSOR POLYPEPTIDES OF TEMPERATURE-SENSITIVE MUTANTS OF RAUSCHER MURINE LEUKEMIA VIRUS

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Impaired Processing of Precursor Polypeptides of Temperature-Sensitive Mutants of Rauscher Murine Leukemia Virus

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The synthesis and processing of virus-specific precursor polypeptides in NIH/3T3 cells infected at the permissive temperature (31°C) with temperature-sensitive (*ts*) mutants of Rauscher murine leukemia virus was studied in pulse-chase experiments at the permissive and nonpermissive (39°C) temperatures. The newly synthesized virus-specific polypeptides were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis after immunoprecipitation with polyvalent and monospecific antisera against Rauscher murine leukemia virus proteins. In cells infected with *ts* mutants defective in early replication steps (the early mutants *ts*17 and *ts*29), and *ts* mutants defective in postintegration steps (the late mutants *ts*25 and *ts*26), the processing of the primary *gag* gene product was impaired at the nonpermissive temperature. *gag*-pr75 of all four mutants was converted into *gag*-pr65, however, *gag*-pr65 accumulated at the nonpermissive temperature, and the main internal virion polypeptide p30 was not formed. Therefore, the proteolytic cleavage is blocked beyond *gag*-pr65. Concomitantly, the formation of the *env* gene-related polypeptide p12(E) of all four mutants was blocked at the restrictive temperature. In contrast, cells infected with the late mutant *ts*28, which produced noninfectious virions at 39°C, showed a normal turnover of the *gag* and *env* precursor polypeptides.

In studies on the molecular biology of type C RNA tumor viruses, the isolated conditional lethal mutants of avian (6, 9, 22, 26) and mammalian (13, 15, 17, 25) RNA tumor viruses provide useful markers for the analysis of the viral genome. Temperature-sensitive (*ts*) mutants of murine leukemia virus (MuLV) have been derived from the Kirsten (17), Rauscher (15), and Moloney (25) strains. The mutants of the Rauscher strain (R-MuLV) have been divided into three different classes based upon the stage at which virus replication is impaired at the restrictive temperature (15). Through the courtesy of J. R. Stephenson, we have obtained representatives of the *ts* mutants of class I (at 39°C, defective in replication functions before the synthesis of viral group-specific antigens), class II (at 39°C, defective in functions between the synthesis of viral group-specific antigens and production of virions), and class III (noninfectious virions produced at 39°C).

The virus-specific structural polypeptides of the RNA tumor viruses are formed through proteolytic cleavage of precursor polypeptides (for a review see 14). Thus, in the case of R-MuLV, the primary *gag* gene product, *gag*-pr75, is converted into *gag*-pr65, which is split into certain intermediary products (with molecular weights between 40,000 and 50,000) and, subsequently, into the polypeptides of the viral core, p15, p12, p30, and p10 (2). Likewise, gp69/71 and p15(E), the virus-specific polypeptides located in the viral envelope as well as in the plasma membrane of infected cells, are derived from the primary *env* gene product *env*-pr82. Finally, p12(E), found in the viral envelope, is presumably derived from p15(E) (12, 24, D. van Zaane, thesis, University of Nijmegen, 1977). Using gel filtration columns that did not resolve the two *gag* precursor polypeptides *gag*-pr75 and *gag*-pr65, Stephenson et al. (18) showed that two *ts* mutants of R-MuLV, *ts*25 and *ts*26, possessed temperature-sensitive defects in the proteolytic cleavage of the *gag* gene-related precursor polypeptides. These studies were undertaken to localize the defects more precisely and to investigate whether the other thermosensitive replication mutants of R-MuLV also possess thermosensitive defects in gene expression. Therefore, the gene expressions of two early (*ts*17 and *ts*29) and one late (*ts*28) *ts* mutants

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were studied and the results were compared with those obtained with the *ts* mutants *ts*25 and *ts*26 and with a clonal stock of wild type (WT) R-MuLV.

MATERIALS AND METHODS

Materials. L-[^{35}S]methionine (specific activity, 880 Ci/mmol) and a ^{14}C-labeled amino acid mixture (specific activity, 54 mCi/g-atom) were obtained from the Radiochemical Centre, Amersham, England. Anti-gp69/71 and anti-p15 sera were the generous gift of M. Strand and J. T. August. Characteristics of these antisera were described by Strand and August (20). Anti-R-MuLV serum, anti-p30 serum, anti-p15(E)-p12(E) serum, and an antiserum raised against bovine serum albumin (anti-BSA serum) were prepared and described by van Zaane et al. (24).

Cells and viruses. NIH/3T3 cells (21) were cultured as monolayers in Dulbecco's modification of Eagle medium supplemented with 10% fetal calf serum (Grand Island Biological Co. Bio-Cult Ltd.).

The viruses used included a clonal isolate of WT-R-MuLV (WT-248) and five R-MuLV *ts* mutants, *ts*1, *ts*25, *ts*26, *ts*28, and *ts*29 (15). NIH/3T3 cells were infected with virus as described by Stephenson and Aaronson (15). Infection with *ts*-R-MuLV was performed at 31°C, and that with WT-R-MuLV was performed at 37°C.

BLS-V9 cells, derived from bone marrow cells of BALB/c mice (4), infected with, and producing R-MuLV (V9-R-MuLV), were cultured as monolayers in Eagle minimal essential medium supplemented with 10% calf serum. V9-R-MuLV labeled with L-[^{35}S]methionine was isolated as described by Duesberg and Robinson (5), and was used as a reference.

Labeling of cells and preparation of lysates. Subconfluent monolayers of cells growing in plastic tissue culture flasks (Falcon Plastics, 25 cm^2) were starved for 10 min in labeling medium (Hanks basic salt solution supplemented with 10% dialyzed calf serum and amino acids except for the radioactive ones). Then the medium was replaced by labeling medium with a radioactive amino acid(s), and the cells were incubated for 30 min. After being pulse labeled, the cells were washed once, and 4 ml of normal culture medium was added thereafter. The cells either were lysed immediately or radioactivity was chased for various times. In some experiments the temperature was shifted during the chase period. When indicated, the temperature shift from 39 to 31°C was performed 2 h after the beginning of the chase period; the temperature shift from 31 to 39°C was performed immediately after pulse labeling of the cells.

After pulses as well as after chases, the cells were lysed by the addition to the cultures (of cells and medium) of 1 ml of a buffer containing 50 mM sodium phosphate (pH 7.2), 1.5% sodium chloride, 5.0% Triton X-100, 2.5% deoxycholate, and 0.5% sodium dodecyl sulfate (fivefold concentrated immunoprecipitation buffer [24]). After clearing the lysates by centrifugation at 220,000 × *g* for 10 min in a Ti-60 rotor of a Spinco L-50 centrifuge, the lysates were stored at −80°C.

Immunoprecipitation and polyacrylamide gel electrophoresis. Detection of R-MuLV-specific polypeptides in cell lysates was performed with polyvalent and monospecific antisera against R-MuLV proteins according to the immunoprecipitation procedure described by van Zaane et al. (24). Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (10) on 7 to 18% polyacrylamide gradient slab gels. Radioactivity was visualized by scintillation autoradiography (3).

RESULTS

Synthesis and identification of virus-specific polypeptides in NIH/3T3 cells infected with WT R-MuLV (WT-248). The synthesis of virus-specific polypeptides in NIH/3T3 cells infected with WT-R-MuLV was examined in pulse-chase experiments at 37°C (Fig. 1A–G). Cells were pulse labeled for 30 min with a ^{14}C-labeled amino acid mixture and thereafter radioactivity was chased for 0 and 20 h. The viral structural polypeptides were identified through immunoprecipitation analysis with well-defined (see Materials and Methods) monospecific antisera and a polyvalent antiserum against R-MuLV polypeptides. Radioimmunoprecipitation analysis of pulse-labeled cells with anti-R-MuLV serum revealed that, in addition to the virus-specific precursor polypeptides *gag*-p75, *gag*-pr65, and *env*-pr82 (cf. references 1, 24), small amounts of two high-molecular-weight polypeptides (indicated with ●) were precipitated (Fig. 1A). Upon analysis with monospecific antisera it appeared that both high-molecular-weight polypeptides carried antigenic determinants of the *gag*-related structural polypeptides p30 and p15, but not of the *env*-related structural polypeptide gp69/71 (not shown). These high-molecular-weight polypeptides are therefore probably virus-specific polypeptides, which in addition to the antigenic determinants of the *gag* precursor contain other virus-specific antigenic determinants (possibly of reverse transcriptase [8]). They might represent virus-specific precursor polypeptides whose synthesis is due to an occasional readthrough at the *gag* gene termination site on a viral mRNA (14).

Moreover, after pulses as well as after chases, some additional high-molecular-weight polypeptides (>90,000) were precipitated with the antisera used, especially with the anti-R-MuLV serum (Fig. 1A–O) and the anti-p15(E),p12(E) serum (Fig. 1D, indicated with *). Probably many of these polypeptides are not virus-specific and represent cross-reacting host-cell polypeptides. However, some of them might be degradation products of the high-molecular-weight precursor polypeptides synthesized according to a readthrough mechanism. We will not discuss the significance of these products here, because

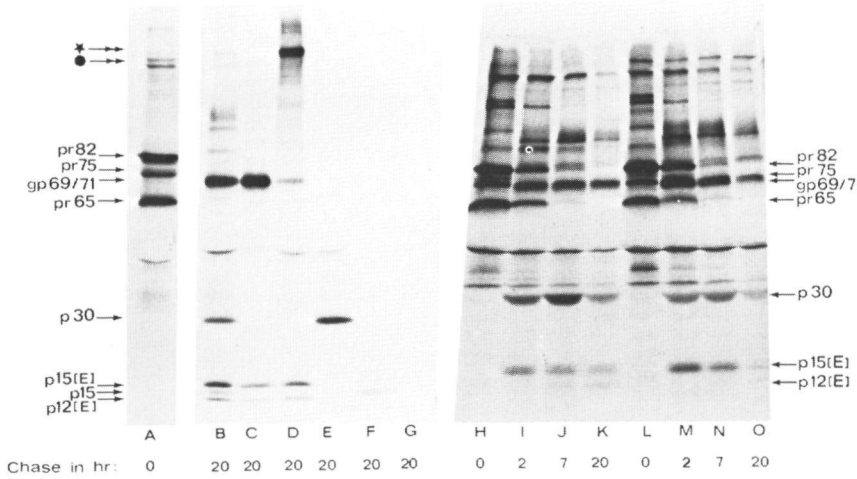

FIG. 1. *Normal processing of gag and env products of WT R-MuLV (WT-248). Subconfluent monolayers of NIH/3T3 cells infected with WT R-MuLV were pulse-labeled for 30 min with 100 μCi of a* ^{14}*C-amino acid mixture (A–G) or with 75 μCi of* L-[^{35}S]*methionine (H–O), and radioactivity was chased for 0, 2, 7, and 20 h as indicated. The pulse-chase experiments were performed at 31°C (H–K), 37°C (A–G), or 39°C (L–O). After lysis of the cells as described in the text, virus-specific polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and scintillation autoradiography after immunoprecipitation with: anti-R-MuLV serum (A, B, H–O); anti-gp69/71 serum (C); anti-p15(E),p12(E) serum (D); anti-p30 serum (E); anti-p15 serum (F); or anti-BSA serum (G). Bands marked with ● and ⋆ are discussed in the text.*

their synthesis does not seem to be affected in any of the mutants we have investigated. After a chase period of 20 h, radioimmunoprecipitation analysis of labeled cultures (i.e., cells and medium) with anti-R-MuLV serum showed that the *gag* and *env* precursor polypeptides had disappeared and that the viral structural polypeptides gp69/71, p30, p15(E), and p12(E) were formed (Fig. 1B). These viral structural polypeptides were identified with monospecific antisera (Fig. 1C–F). In a control experiment none of the polypeptides could be precipitated with anti-BSA serum (Fig. 1G). It should be noted that the *gag* products p12 and p10 are not recognized by the anti-R-MuLV serum used.

As a control for the analysis of the *ts* mutants, Fig. 1(H–O) shows the results of a pulse-chase experiment at 31°C (Fig. 1H–K) and 39°C (Fig. 1L–O) with NIH/3T3 cells infected with WT R-MuLV (WT-248). Cells were pulse-labeled with L-[^{35}S]methionine for 30 min, and, thereafter, radioactivity was chased for 0. 2, 7, and 20 h. Radioimmunoprecipitation analysis was performed with anti-R-MuLV serum. It appeared that at both temperatures the precursor polypeptide *env*-pr82, as well as *gag*-pr75 and *gag*-pr65, was cleaved into virus-specific structural polypeptides (Fig. 1H–O). It should be noted that p15 does not contain methionine (7) and consequently is not detected when cells are labeled with L-[^{35}S]methionine.

Localization of the blockage in the cleavage of the *gag* gene-related precursor polypeptides of *ts*25 and *ts*26. The cleavage of the *gag* gene-related precursor polypeptides of *ts*25 and *ts*26 was shown to be blocked at the nonpermissive temperature (2, 19). To localize the blockage in the cleavage scheme, the synthesis and processing of virus-specific precursor polypeptides was examined in NIH/3T3 cells infected at 31°C with *ts*25 and *ts*26. Pulse-chase experiments were performed at 31 and 39°C, and the virus-specific polypeptides were immunoprecipitated with anti-R-MuLV serum. The results obtained with *ts*25 are presented in Fig. 2. At 31°C no differences in the synthesis and processing of the precursor polypeptides of *ts*25 and WT-248 could be observed (cf. Fig. 2A–D and Fig. 1H–K). In accordance with the work of Stephenson et al. (19), Fig. 2(E–H) shows that the processing of the *gag* gene precursor polypeptides was impaired at the nonpermissive

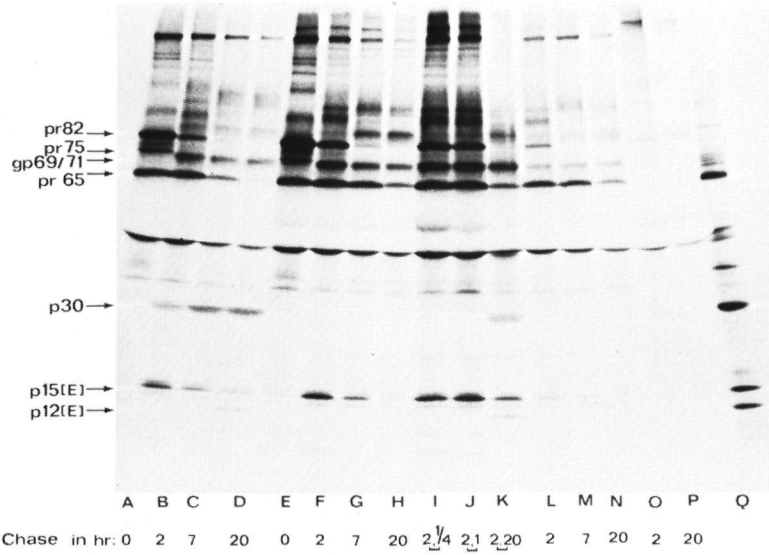

FIG. 2. *Thermosensitive processing of gag products of ts25. Subconfluent monolayers of NIH/3T3 cells infected at 31°C with ts25 were pulse-labeled for 30 min with 75 μCi of L-[35S]methionine, and radioactivity was chased for various times as indicated (A–P). Pulse-chase experiments were performed at 31°C (A–D, O–P) or 39°C (E–H). In some cases the temperature was shifted for a chase at 31°C (I–K) or 39°C (L–N), as described in the text. Thus, "chase in h 2¼" indicates a 2-h chase at 39°C followed by a 0.25-h chase at 31°C, etc. Virus-specific polypeptides were analyzed as described in the legend to Fig. 1. Immunoprecipitation with: anti-R-MuLV serum (A–N); anti-BSA serum (O–P); reference V9-R-MuLV labeled with L-[35S]methionine (Q).*

temperature; *gag*-pr65 of *ts*25 is accumulated and no p30 is formed. On the other hand, like WT *gag*-pr75, the *gag*-pr75 of *ts*25 is converted into *gag*-pr65. When the temperature was shifted to 31°C 2 h after pulse-labeling the cells at 39°C (Fig. 2I–K), the proteolytic cleavage was resumed and p30 was produced. When, in another experiment, the temperature was shifted from 31°C (during the pulse) to 39°C (during the chase), the proteolytic cleavage was blocked (Fig. 2L–N).

The results of parallel experiments with NIH/3T3 cells infected with *ts*26 (not shown) were similar to those observed with *ts*25; again, at 39°C *gag*-pr65 was accumulated, but the mutation was slightly leaky: cells infected with *ts*26 produced small amounts of p30 at the restrictive temperature (Fig. 3G–H).

A more detailed analysis of the blockage in the proteolytic cleavage of the *gag* precursor polypeptides of *ts*25 and *ts*26 was obtained after radioimmunoprecipitation analysis with monospecific antisera (Fig. 3). Cells infected with *ts*25 or *ts*26 were labeled for 30 min with a ^{14}C-amino acid mixture at 31 or 39°C, and radioactivity was chased for 20 h. Figure 3 shows that at the nonpermissive temperature *gag*-pr65 was accumulated in cells infected with *ts*25 (Fig. 3M–O) or *ts*26 (Fig. 3G–I); furthermore, the production of the *gag*-related structural polypeptides p30 and p15 was inhibited. In addition to the impaired processing of the *gag* precursor polypeptides of *ts*25 and *ts*26, a blockage in the production of an *env* gene-related structural polypeptide was also observed at the restrictive temperature. Radioimmunoprecipitation analysis with anti-R-MuLV serum (Fig. 2H and N; Fig. 3G and M), anti-gp69/71 serum (Fig. 3K and Q), and anti-p15(E),p12(E) serum (Fig. 3J and P) revealed that production of p12(E) was inhibited. Production of the structural envelope polypeptides gp69/71 and p15(E), however, seemed to be normal (Fig. 2F–H and Fig. 3G, J, K, M, P, and Q). It should be noted that some-

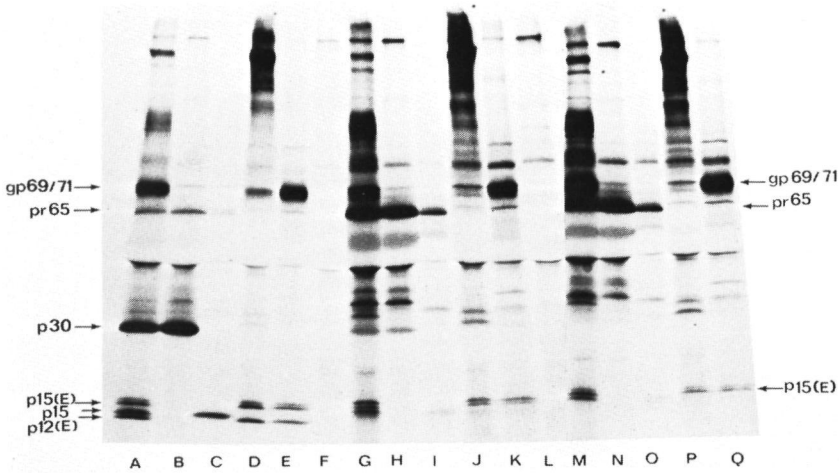

FIG. 3. *Leaky thermosensitive processing of gag products of ts26; thermosensitive production of the env product p12(E) in addition to the thermosensitive processing of gag products of ts25 and ts26. NIH/3T3 cells infected at 31°C with ts26 (A–L) or ts25 (M–Q) were pulse-labeled for 30 min with 100 μCi of a ^{14}C-amino acid mixture, and radioactivity was chased for 20 h. Pulse-chase experiments were performed at 31°C (A–F) or 39°C (G–Q). Virus-specific polypeptides were analyzed as described in the legend to Fig. 1. Immunoprecipitation with: anti-R-MuLV serum (A, G, M); anti-p30 serum (B, H, N); anti-p15 serum (C, I, O); anti-p15(E),p12(E) serum (D, J, P); anti-gp69/71 serum (E, K, Q); anti-BSA serum (F, L).*

times p15(E) seemed to be composed of two components (Fig. 1, 2, and 3). Both polypeptides were immunoprecipitated with anti-R-MuLV serum (Fig. 1, 2, and 3A, G, and M), anti-gp69/71 serum (Fig. 3E, K, and Q), and anti-p15(E),p12(E) serum (Fig. 3D, J, and P). [Incidentally, the precipitation of p15(E) and p12(E) with anti-gp69/71 serum is presumably due to the presence of a complex between gp69/71, p15(E), and p12(E) as discussed by van Zaane et al. (24) and Leamnson et al. (11).]

Synthesis of virus-specific polypeptides in NIH/3T3 cells infected with *ts*17, *ts*28, and *ts*29. Two early temperature-sensitive mutants of R-MuLV, *ts*17 and *ts*29, were shown to be defective in one or more functions before the synthesis of group-specific antigens (15). To examine the possibility that these mutants also had a defect in the proteolytic cleavage of their precursor polypeptides, the synthesis of virus-specific polypeptides was studied at 31 and 39°C, after infection at the permissive temperature. Cells were labeled with L-[^{35}S]methionine, and radioimmunoprecipitation was performed with anti-R-MuLV serum. The results obtained with *ts*17 and *ts*29 are presented in Fig. 4 and 5, respectively. In both cases, *gag*-pr75 was processed to *gag*-pr65 at the nonpermissive temperature. However, proteolytic cleavage was blocked beyond *gag*-pr65, since no p30 was formed. When the temperature was shifted from 39 to 31°C, cleavage was resumed, as could be concluded from the production of p30. Furthermore, the inhibition of the production of p12(E) at the restrictive temperature, as found in cultures infected with *ts*25 and *ts*26, was also observed. On the other hand, the late mutant *ts*28, similarly analyzed in parallel experiments, was not defective for the processing of its precursor polypeptides. The relevant pulse-chase experiments at 31 and 39°C are shown in Fig. 6.

DISCUSSION

Some temperature-sensitive mutants of R-MuLV are partially characterized (15, 16, 18, 19, 23, 27). Among them are the mutants *ts*17, *ts*25, *ts*26, *ts*28, and *ts*29, which were used in this study.

At 39°C, some of these mutants are defective in either early replication steps (class I represented by *ts*17 and *ts*29) or postintegration steps (class II represented by *ts*25 and *ts*26, and class

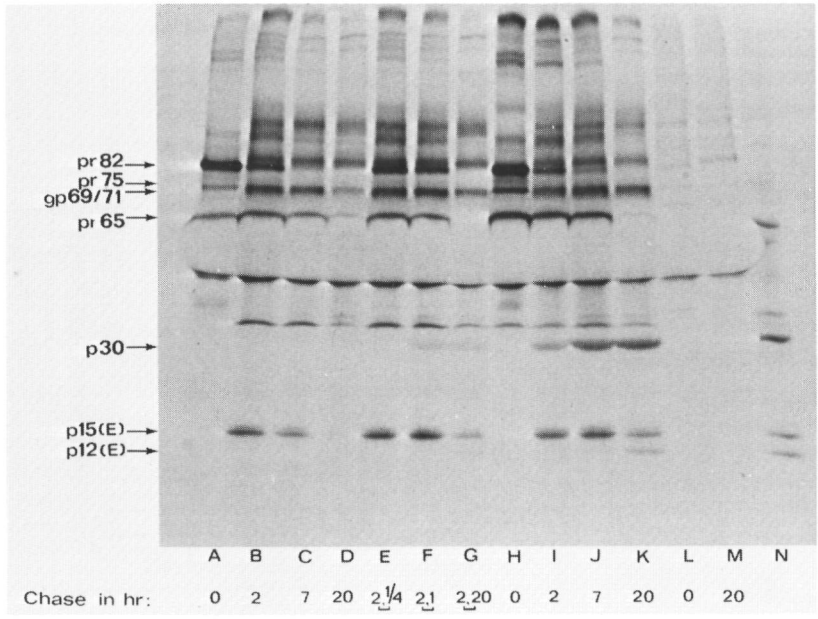

FIG. 4. *Thermosensitive processing of gag products of ts17 and concomitant failure to produce the env product p12(E). NIH/3T3 cells infected at 31°C with ts17 were pulse-labeled with 75 μCi of L-[^{35}S]methionine for 30 min, and radioactivity was chased for various times as indicated (A–M). Pulse-chase experiments were performed at 39°C (A–D) or 31°C (H–M). The temperature was shifted from 39 to 31°C (E–G), as described in the legend to Fig. 2). Virus-specific polypeptides were analyzed as described in the legend to Fig. 1. Immunoprecipitation with: anti-R-MuLV serum (A–K); anti-BSA serum (L–M); reference V9-R-MuLV labeled with L-[^{35}S]methionine (N).*

III represented by *ts*28) (15). *ts*25 and *ts*26 are postintegration defective mutants whose defect is known to involve the cleavage of the *gag* gene-related precursor polypeptides (2, 19). Mutant *ts*29 possesses a thermolabile reverse transcriptase (23) and *ts*28 has an unidentified *ts* defect at a late step in its replication (15, 27).

In this study cells were infected with these mutants at the permissive temperature (31°C), and subsequently the synthesis and processing of virus-specific precursor polypeptides were examined at the permissive and nonpermissive temperatures. At 31°C no differences in the processing of the *gag* and *env* gene-related precursor polypeptides were observed in cells infected with either WT R-MuLV or one of the *ts* mutants (Fig. 1, 2, 4, 5, and 6). However, at 39°C, a blockage in the processing of a *gag* gene-related precursor polypeptide of the mutants *ts*17, *ts*25, *ts*26, and *ts*29 was observed (Fig. 2, 3, 4, and 5). When the temperature was shifted down to 31°C after pulse-labeling the cells at 39°C, processing of the *gag* precursor polypeptides to the internal structural virion polypeptides p30 (Fig. 2, 3, 4, and 5) and p15 (Fig. 3) was observed. The impaired processing of the *gag* precursor polypeptides at the restrictive temperature was localized beyond *gag*-pr65, since *gag*-pr75 disappeared but *gag*-pr65 accumulated and p30 was not produced (Fig. 2, 3, 4, and 5). In all analyses, impaired production of p12 and p10 escaped detection, since these virus-specific polypeptides were not recognized by the anti-R-MuLV serum. However, presumably none of the *gag* gene-related structural polypeptides is produced at 39°C since all four subgene

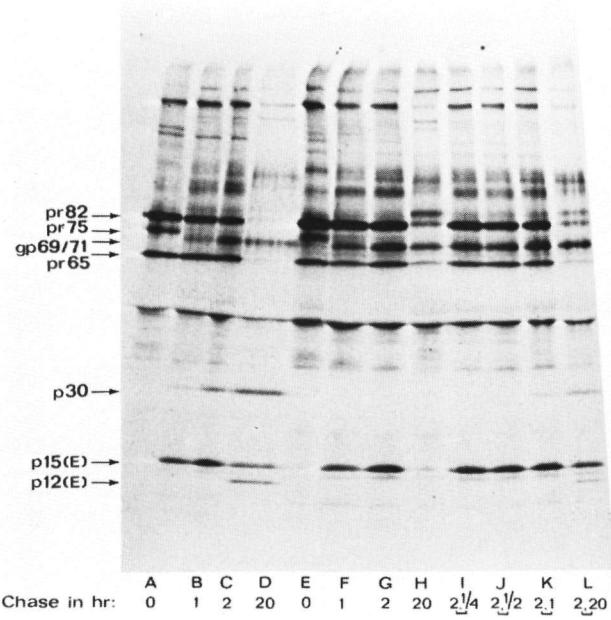

FIG. 5. *Thermosensitive processing of gag products of ts29 and concomitant failure to produce the env gene-related product p12(E). NIH/3T3 cells infected at 31°C with ts29 were pulse-labeled for 30 min with 75 μCi of L-[^{35}S]methionine, and radioactivity was chased for various times as indicated (A–L). Pulse-chase experiments were performed at 31°C (A–D) or 39°C (E–H). The temperature was shifted from 39 to 31°C (I–L), as described in the text (see also legend to Fig. 2). Virus-specific polypeptides were analyzed as described in the legend to Fig. 1. Immunoprecipitation with: anti-R-MuLV serum (A–L).*

products, p15, p12, p30, and p10, are present in the smaller *gag* precursor polypeptide *gag*-pr65 (van Zaane, thesis). This conclusion does not exclude the possibility that eventually *gag*-pr65 is degraded and may give rise to an incomplete set of *gag* subgene products (cf. Barbacid et al. [2]). The impaired processing of the *gag* precursor polypeptide of *ts*25 and *ts*26 is consistent with the observations of Stephenson et al. (19). The presence of the same defect in the mutants *ts*17 and *ts*29 is quite unexpected, since both were characterized as early mutants (class I). In the case of *ts*29 the early replication defect is associated with a thermolabile reverse transcriptase (23). Further evidence for a second replication defect in *ts*29 is obtained from an electron microscopic study by Yeger et al. (27).

Upon a shift to the nonpermissive temperature after infection at the permissive temperature, these authors found an accumulation of immature virions at the cell surface. The immature virions were in an early stage of budding, suggesting a replication defect at an early stage of virion assembly. Whereas cultures infected with *ts*17, *ts*25, *ts*26, and *ts*29 revealed an impaired processing of the *gag* precursor polypeptides at the nonpermissive temperature, production of the *env* gene-related polypeptide p12(E) was also inhibited (Fig. 2, 3, 4, and 5). It is not yet clear whether or not there is a relation between these defects. For instance, the defect could be primarily in virion assemblage, and, as a consequence, the cleavage of different precursor polypeptides could be blocked. As discussed above, two of the mutants of this phenotype (*ts*17 and *ts*29) possess an additional early defect on the basis of which they were listed as class I mutants. Again, it is not known whether they are double (or triple) mutants or single pleiotropic mutants.

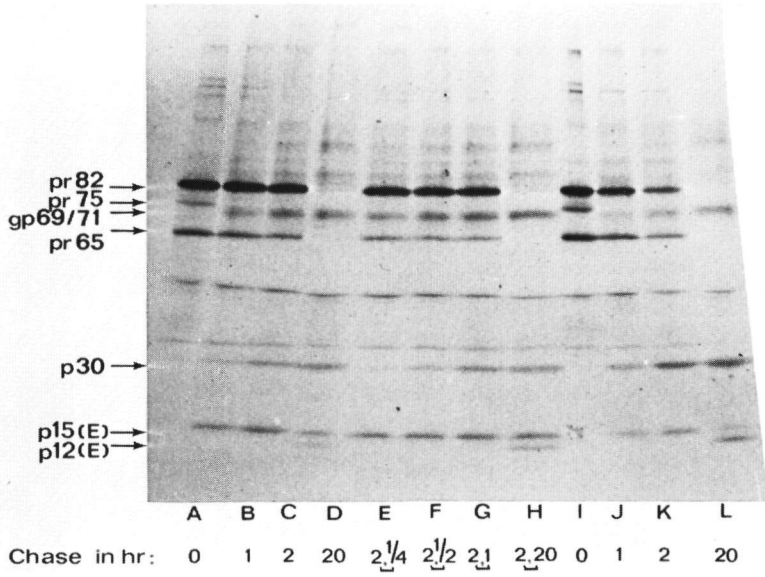

FIG. 6. *Normal processing of gag and env products of ts28. NIH/3T3 cells infected at 31°C with ts28 were pulse-labeled for 30 min with 75 μCi of L-[^{35}S]methionine, and radioactivity was chased for various times as indicated (A–L). Pulse-chase experiments were performed at 39°C (A–D) or 31°C (I–L). The temperature was shifted from 39 to 31°C (E–H), as described in the text. Virus-specific polypeptides were analyzed as described in the legend to Fig. 1 (see also legend to Fig. 2). Immunoprecipitation with anti-R-MuLV serum (A–L).*

Finally, the structural polypeptides of *ts*28 are produced normally at the restrictive temperature (Fig. 6). However, the distorted morphology of the virions formed at 39°C (27) suggests a defect in a late stage of the assembly.

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CHAPTER V

TRANSLATION OF TYPE-C VIRAL RNAs IN *XENOPUS LAEVIS* OOCYTES: EVIDENCE THAT THE 120,000 MOLECULAR WEIGHT POLYPROTEIN EXPRESSED IN ABELSON LEUKEMIA VIRUS TRANSFORMED CELLS IS VIRAL CODED

TRANSLATION OF TYPE-C VIRAL RNAs IN *XENOPUS LAEVIS* OOCYTES: EVIDENCE THAT THE 120,000 MOLECULAR WEIGHT POLYPROTEIN EXPRESSED IN ABELSON LEUKEMIA VIRUS TRANSFORMED CELLS IS VIRAL CODED

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SUMMARY

The genomic RNA of Abelson leukemia virus (AbLV) has been purified and translated in *Xenopus laevis* oocytes. The primary AbLV-specific protein synthesized is a polyprotein corresponding in molecular weight (Mr) and immunologic properties to a previously-described, p15 and p12 containing 110,000-130,000 Mr polyprotein expressed in AbLV-transformed cells. *In vitro* translation of woolly monkey sarcoma virus (WSV) genomic RNA resulted in synthesis of a 55,000 Mr polyprotein analogous to a protein expressed in WSV-transformed cells containing woolly helper-virus p30, p15 and

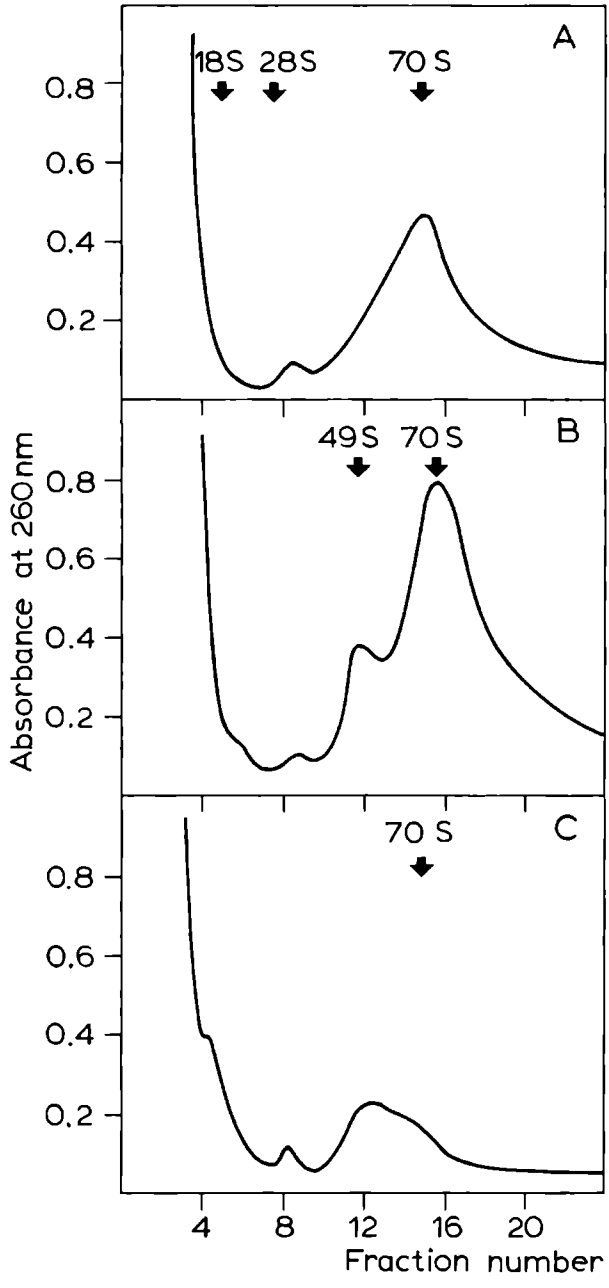
p12. These findings establish the viral-coded nature of the nonstructural component of the polyprotein expressed in AbLV-transformed cells and indicate that a polyprotein containing structural and nonstructural components is either not present in WSV-transformed cells or is subject to rapid post-translational cleavage.

Mammalian RNA type-C transforming viruses induce tumors of a variety of histologic type in vivo, frequently cause morphologic alteration of embryo fibroblasts in cell culture, and require leukemia helper viruses for completion of their replication cycle (1). These replication-defective viruses appear to represent recombinants between a portion of the leukemia helper virus genome and cellular genes coding for malignant transformation (15, 20). In view of the possibility that the cellular-acquired transforming sequences of such viruses may be etiologically involved in spontaneous tumors of their natural hosts, efforts have been undertaken to identify and develop immunologic assays for their translational products. Cell lines nonproductively-transformed by each of several such viruses, including one isolate of cat origin, namely feline sarcoma virus (FeSV) (6, 18) and two mouse-derived viruses, Abelson murine leukemia virus (AbLV) (9) and the T-8 MCF AKR-derived leukemia virus (13), express polyproteins of 100,000-130,000 Mr containing the two amino terminal gag-gene proteins, p15 and p12. In the feline system, the FeSV-associated 130,000 Mr polyprotein is efficiently immunoprecipitable by select FeLV-absorbed cat sera with high-titered antibody directed against the feline oncornavirus-associated cell membrane antigen (FOCVA) (17, 18) raising the possibility that this polyprotein may contain transformation (src)-specific sequences. In an effort to resolve whether the nonstructural components of such polyproteins are coded in entirety by the transforming virus genome, we have undertaken to translate AbLV genomic RNA in Xenopus laevis oocytes. A similar approach is used for characterization of translational product(s) of a sarcoma virus isolate of woolly monkey origin.
Viruses used were sucrose gradient-purified preparations obtained through the courtesy of Dr. R.V. Gilden (Frederick Cancer Research Center) and included Rauscher murine leukemia virus (R-MuLV) grown on JLS-V9 cells and the WB334 strain of woolly monkey virus (WSV) propagated on normal rat kidney (NRK) cells. This latter virus consists of approximately equal

titers of replication-defective WSV and amphotropic mouse helper virus (16). Superinfection of an AbLV nonproductively transformed mouse cell line, ANN-1 (14) with wild mouse amphotropic type-C virus, 4070-A, generously provided by J.W. Hartley, NIAID (5), led to production of virus stocks containing approximately equal infectious titers of leukemia helper virus and pseudotype virions containing the AbLV-genome in the amphotropic virus coat. A rat cell line nonproductively transformed by WSV, designated WSV-NRK in the present study, was provided by E.M. Scolnick, NCI.

Viral genomic RNA was extracted by resuspension of 5-10 mg pelleted virus in TNE buffer (0.01 M Tris-HCl, pH 8.0, 1% NaCl, 0.001 M EDTA) containing 1% sodium dodecyl sulfate (SDS), 0.5 mg/ml pronase (Calbiochem, La Jolla, Calif.) and 1% β-mercaptoethanol. The sample was incubated at 37° for 30 min prior to sedimentation through a 15-35% (w/w) sucrose gradient in TNE at 40,000 rpm for 5 hrs at 4° in a SW-41 rotor. Gradients were fractioned using an ISCO Density Gradient Fractionator Model 640 (Lincoln, Neb.) and absorbance at 260 nm was recorded by an ISCO Model UA-5 Absorbance Monitor. Appropriate fractions were pooled, and precipitated twice with 2 volumes of ethanol and 0.1 volume of 2.0 M sodium acetate, pH 5.0, at -20°. RNA was pelleted from the alcohol by centrifugation at 8,000 rpm at -20° in a Sorvall SS-34 rotor. After removal of the alcohol, the pellet was dried briefly in a dessicator under vacuum before resuspension of the RNA in H_2O to a concentration of 1 mg/ml.

Female Xenopus laevis frogs were acquired from C.W. Fletcher, Hampstead, Md. After removal from the frog, oocytes were maintained in Barth's media (4) and injections were performed using an MM 358 Special Microposition er (Bunton Instruments, Rockville, Md.) and a 10 µl syringe attached to a micrometer screw. Oocytes were injected with 40 nl of the RNA solution, incubated for 20 hrs at 20° in 5 µl Barth's media containing 1 mCi/ml (^{3}H)-leucine (New England Nuclear, Boston, Mass.) (>110 Ci/mmol), washed 3 times with unlabeled Barth's media and dounced in 250 µl PBSTDS (10 mM $NaPO_4$, pH 7.2, 1.0% Triton X-100, 0.9% NaCl, 0.5% deoxycholate and 0.1% SDS) buffer containing 0.1% leucine. Following clarification of the extracts to remove lipid and cell debris, 2 x 10^6 trichloroacetic acid precipitable counts were incubated with 5 µl antiserum in order to immunoprecipitate viral-specific translational products. After 12-16 hrs, 50 µl of a 20% suspension of Protein A-Sepharose CL-4B (Pharmacia, Piscataway,


A
18S
28S
70 S
0.8
0.6
0.4
0.2
B
49S
70S
0.8
0.6
0.4
0.2
Absorbance at 260nm
C
70 S
0.8
0.6
0.4
0.2
4
8
12
16
20
Fraction number

(N. J.) in PBSTDS was added to absorb the immune complexes. Sepharose-bound protein A immune complexes were washed three times in PBSTDS buffer, resuspended in 20 µl sample buffer (0.0625 M Tris-HCl, pH 6.7, 1% SDS, 10% glycerol, 2.5% β-mercaptoethanol and 0.1% bromophenol blue), heated for 2 min at 90°, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (7) or 5 to 20% polyacrylamide gradient gels. Radioactivity was visualized by scintillation autoradiography (3).

Competition immunoassays for woolly monkey leukemia virus (WLV) structural proteins including p30, p15, p12 and p10 were performed according to previously-described methods (19). These assays measure the ability of unlabeled viral antigens, titrated at serial twofold dilutions, to compete with ^{125}I-labeled viral proteins for binding limiting amounts of antiserum directed against detergent-disrupted WLV.

Initial studies were undertaken to purify and characterize R-MuLV, WB334 and AbLV(4070-A) genomic RNA. The RNA profile obtained from SDS-pronase treatment of density-gradient purified R-MuLV and subsequent sedimentation through a 15-35% (w/w) sucrose gradient (Fig. 1A) shows a single prominent peak of absorbancy at 260 nm in the 70S region of the gradient. WB334 RNA similarly purified gave a major peak of absorbancy at around 70S in addition to a slower sedimenting peak of absorbancy in the 45-50S region of the gradient (Fig. 1B). This latter peak has been shown by Scolnick et al. (16) to contain genetic sequences corresponding to those of the woolly sarcoma virus genomic RNA, while the 70S peak represents the amphotropic helper virus RNA. While AbLV(4070-A) pseudotype virion RNA was also characterized by a peak of absorbancy in the 45-50S region and in the 70S region of the gradient this was not as well resolved as with R-MuLV or WB334 RNA (Fig. 1C).

Fractions from the 70S region of the R-MuLV RNA gradient were alcohol-precipitated, redissolved in water at a concentration of 1 mg/ml, and injected into Xenopus laevis oocytes as described in Methods. After in-

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Fig. 1) Sedimentation profiles of RNA extracted from A) R-MuLV; B) WB334; C) AbLV(4070-A) density gradient-purified virus. The RNA was subjected to centrifugation through 15-35% (w/w) sucrose gradients at 40,000 rpm in a Beckman SW 41 rotor for 5 hrs at 4°. Gradients were collected from the top by displacement with 60% (w/v) sucrose in 0.5 ml fractions. Fractions used for translation in the oocyte system were A) 13-17; B) 11-13 and 15-18; and C) 13-18.

cubation of the oocytes in (^{3}H)-leucine-labeling media, extracts were subjected to immunoprecipitation by various sera in order to separate the virus-specific proteins from the endogenous translation products. Immunoprecipitates were analyzed on SDS-polyacrylamide slab gels and the protein bands visualized by fluorography. As shown in Figure 2, the major product of R-MuLV RNA translation was a 65,000 Mr protein which was immunoprecipitated by antisera directed against disrupted R-MuLV p30, p15, p12 and p10 but not by either normal goat serum or by anti-R-MuLV gp70 (Fig. 2A-G), and therefore appears to represent the previously-described 65,000 Mr R-MuLV gag-gene coded precursor polyprotein (10,13).

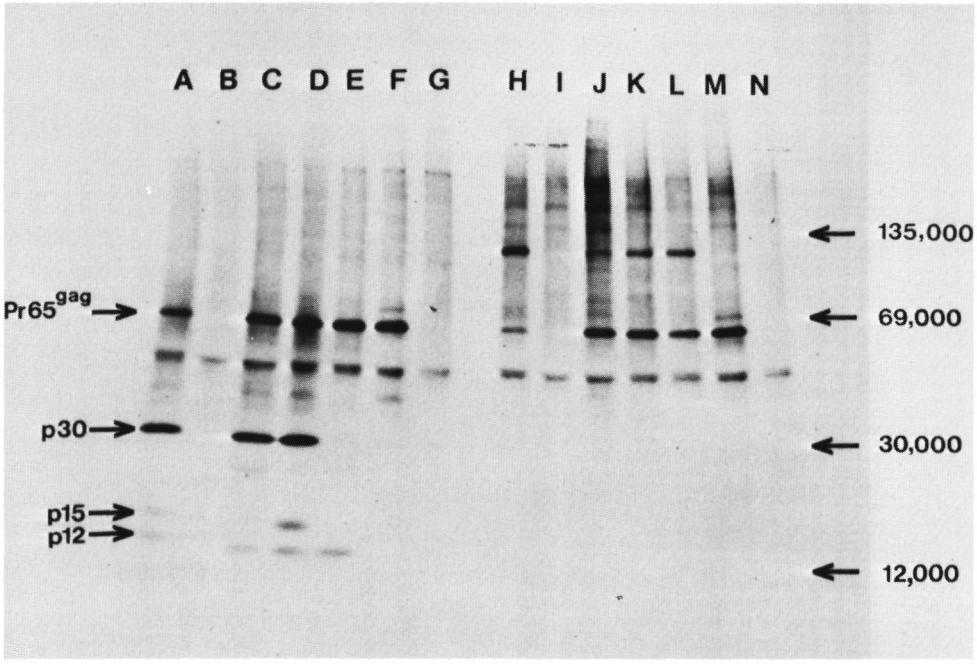
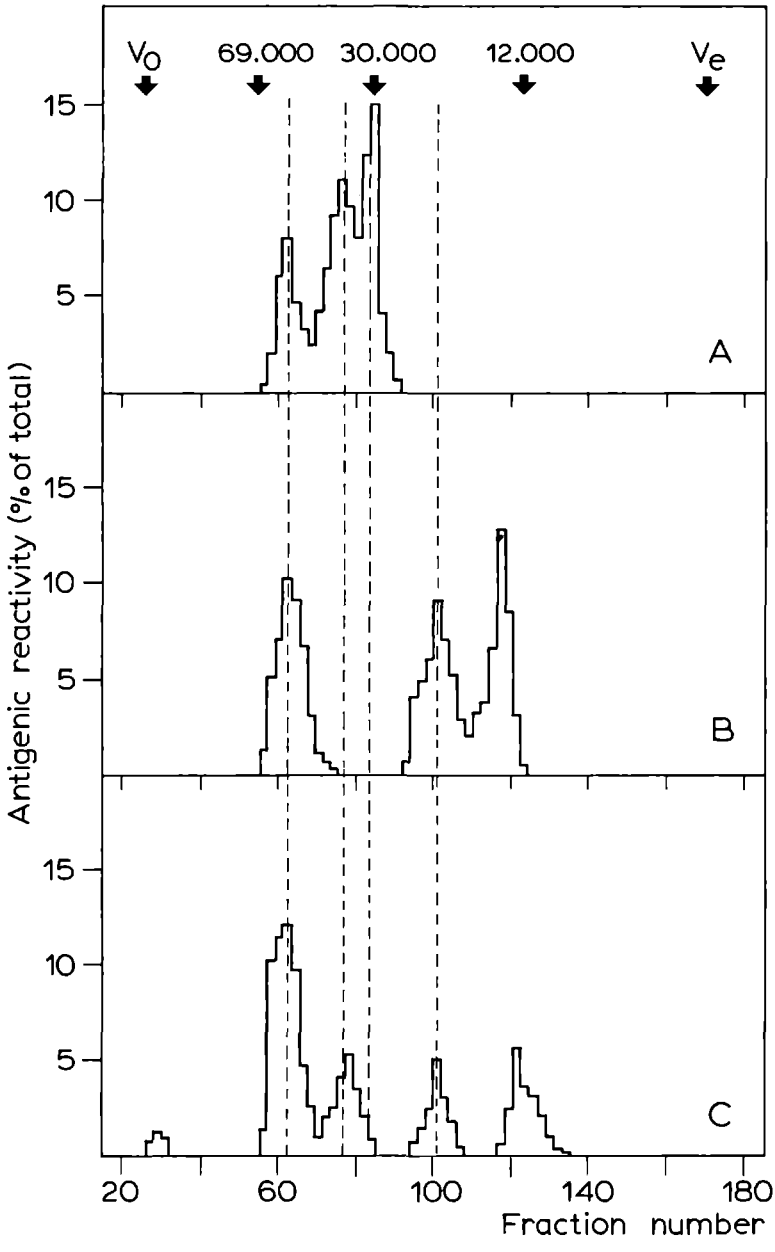


Fig. 2) Immunoprecipitation and SDS-PAGE analysis of (^{3}H)-leucine-labeled viral proteins translated in Xenopus laevis oocytes from R-MuLV (A-G) and AbLV (4070-A) pseudotype (H-N) genomic RNA. Sera included goat anti-R-MuLV (A, H); anti-R-MuLV gp70 (B, I); anti-R-MuLV p30 (C, J); anti-R-MuLV p15 (D, K); anti-R-MuLV p12 (E, L); anti-R-MuLV p10 (F, M) and normal goat sera (G, N). ^{125}I-labeled molecular weight standards included β-galactosidase (135,000) bovine serum albumin (69,000) and R-MuLV p30 (30,000).

The appearance of protein bands at 30,000, 15,000 and 12,000 Mr, which were efficiently immunoprecipitable by antisera directed against R-MuLV p30, p15 and p12, respectively (Fig. 2C-E), demonstrates post-translational cleavage of a portion of the Pr65 into its compenent structural proteins and defines the specificities of the antisera used. For instance, while p30 was the major protein precipitated by anti-R-MuLV p30, a smaller amount of p12 (Fig. 2C) was also observed. Anti-R-MuLV p15 was the least specific of the sera used in that it precipitated not only p15, but also p30 and p12 (Fig. 2D). In contrast, anti-R-MuLV p12 only recognized p12 (Fig. 2E), while anti-R-MuLV p10 did not precipitate p30, p15 or p12 to significant extents (Fig. 2F). While this latter serum did precipitate p10, the band was not sufficiently intense to be seen in the gel shown in Figure 2.

In both mink and mouse cells nonproductively-transformed by AbLV, we have previously reported the expression of a 110,000-130,000 Mr polyprotein containing MuLV p15 and p12 as well as a possible transformation-specific nonstructural component (9). In addition, a 25,000 Mr intermediate cleavage product containing p15 and p12 as well as lesser amounts of p15 and p12 in a fully processed form were observed (9). Translation of AbLV genomic RNA, in the present study resulted in synthesis of a 120,000 Mr polyprotein which was efficiently immunoprecipitated by antisera to detergent-disrupted R-MuLV, anti-R-MuLV p15 and anti-R-MuLV p12 but not by anti-R-MuLV p10 or gp70 (Fig. 2H-N). This latter protein coelectrophoresed with a high Mr (^{35}S)-methionine-labeled polyprotein immunoprecipitable from AbLV-transformed mink cells by either anti-R-MuLV p12 of p15 (data not shown). While the AbLV-specific polyprotein was also immunoprecipitable, although to a lesser extent by anti-R-MuLV p30, this latter serum also contained significant anti-p12 reactivity (Fig. 2C). Thus the question as to whether the AbLV-specific polyprotein contains a portion of MuLV p30 remains unresolved. In addition to the 120,000 Mr polyprotein, translation of genomic RNA from AbLV(4070-A) pseudotype virions in the oocytes system resulted in synthesis of a 65,000 Mr precursor polyprotein analogous to that obtained from translation of R-MuLV genomic RNA and presumably representing a translational product of the 4070-A amphotropic helper virus genome. In contrast to the extensive processing of the R-MuLV gag-gene coded precursor polyprotein, Pr65, no significant post-translational cleavage of the 120,000 Mr AbLV-specific polyprotein was


V_O
69.000
30.000
12.000
V_e
15
10
5
A
B
C
Antigenic reactivity (% of total)
20
60
100
140
180
Fraction number

observed. These findings thus indicate that in the oocyte in vitro translation system, AbLV genomic RNA codes for a polyprotein similar in molecular weight and immunologic properties to that expressed in AbLV-transformed cells.

Studies were next extended to analysis of translational products of WSV, the only primate-derived mammalian transforming virus isolate presently available. Molecular size analysis of rat cells nonproductively-transformed by WSV revealed a major peak of antigenic reactivity at 55,000 Mr containing WLV p30, p15 and p12, a 40,000 Mr peak containing p30 and p12, and a 25,000 Mr peak containing p15 and p12 (Fig. 3). Lesser amounts of p30, p15 and p12 were observed in a fully cleaved form. Similarly, a major 55,000 Mr precursor polyprotein containing WLV gag-gene coded structural components was detected by SDS-PAGE analysis of (^{35}S)-methionine-labeled WSV-transformed rat cells (data not shown). Moreover, inoculation of the slower sedimenting 45S-50S WB334 RNA described in Figure 1B into Xenopus laevis oocytes resulted in synthesis of a major protein at around 55,000 Mr which was immunoprecipitable by antisera to detergent-disrupted R-MuLV, WLV, R-MuLV p30, and to a limited extent by high-titered anti-R-MuLV p10 (Fig. 4F-I). In contrast, translation of the more rapidly sedimenting (70S) viral RNA resulted in synthesis not only of the 55,000 Mr polyprotein, but also a 65,000 Mr protein which was precipitable by antisera to detergent-disrupted R-MuLV, WLV, R-MuLV p30, and R-MuLV p10, but

Fig. 3) Molecular size analysis of helper virus-specific antigens expressed in WSV transformed cells. Twenty mg cell extract, prepared by sonication of cells for 20 sec in an equal volume of 10 mM Tris-HCl, pH 7.8, buffer containing 100 mM NaCl, 0.5 mM EDTA, and 0.5% Triton X-100 was clarified by centrifugation in a Beckman type-30 rotor for 60 min, lyophilized, resuspended in 1 ml of 0.05 M Tris-HCl, pH 8.5, buffer containing 8M guanidine hydrochloride (GuHCl), 1 mM EDTA, and 20 mM dithiothreitol (DTT), heated at 45° for 30 min and applied to an A-15 m agarose (100-200) mesh, (BioRad) column (1.5 by 90 cm) in the presence of 6 M GuHCl, 0.01 M DTT and 0.02 M sodium phosphate buffer (pH 6.5). One ml fractions were collected and tested at serial twofold dilutions in competition immunoassays in which antibody to density gradient purified WLV was used for precipitation of ^{125}I-labeled WLV A) p30; B) p15; and C) p12. Results are expressed as the percentage of total antigenic reactivity in each fraction and are based on the degree of displacement of competition curves relative to known standards. ^{125}I-labeled molecular weight markers included tracer amounts of bovine serum albumin (69,000), R-MuLV p30 (30,000) and R-MuLV p12 (12,000). The vertical dotted lines represent the elution positions of Pr55 (p30, p15 and p12), Pr42 (p30 and p12) and Pr25 (p15 and p12) WSV-coded gag-gene precursors.

not by normal goat serum (Fig. 4A-E) indicating it to represent an amphotropic gag-gene coded polyprotein analogous to that shown in Figure 2. No evidence for a high (>55,000) Mr polyprotein containing WLV structural compenents was obtained either by analysis of WSV transformed cells (Fig. 3) or by in vitro translation of the WSV genome (Fig. 4).

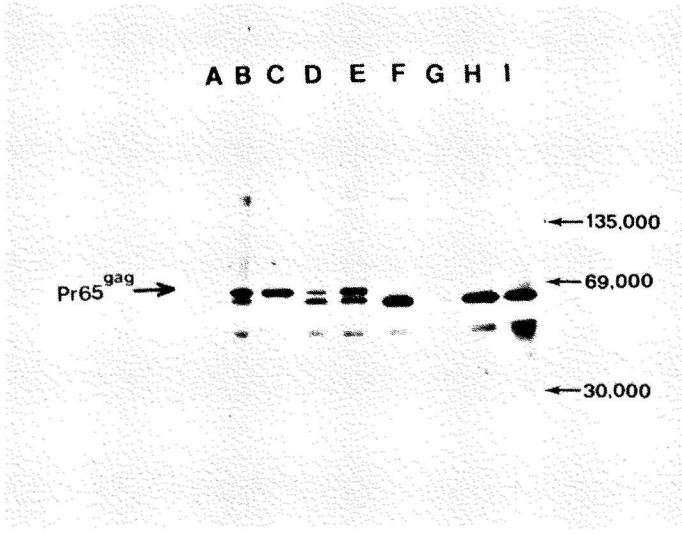


Fig. 4) Immunoprecipitation and SDS-PAGE analysis of (^{3}H)-leucine-labeled viral proteins translated in Xenopus laevis oocytes from WB334 70S RNA (A-E) and WB334 45-50S RNA (F-I). Sera included normal goat (A); anti-R-MuLV (B, F); anti-R-MuLV p10 (C, G); anti-R-MuLV p30 (D, H) and anti-WLV (E, J). ^{125}I-labeled molecular weight standards include β-galactosidase (135,000), bovine serum albumin (69,000) and R-MuLV p30 (30,000).

In the present study we report in vitro translation of the genomic RNA of two representative replication-defective mammalian transforming viruses, one (AbLV) of mouse, and the second (WSV) of primate origin. AbLV-transformed cells express a 110,000-130,000 Mr precursor polyprotein containing MuLV p15 and p12 and nonstructural component(s) containing possible transformation (src)-specific sequences (9, 12). Suggestive evidence that the nonstructural component(s) of this latter polyprotein is AbLV-specific was obtained by the demonstration of its expression in both mouse and mink cells non-productively transformed by AbLV (9). More direct evidence for the viral-coded nature of this polyprotein was obtained in the present study by its in vitro translation following inoculation of AbLV ge-

nomic RNA into Xenopus laevis oocytes. An alternative, although less likely possibility, is that the AbLV polyprotein represents a translational product of a spliced mRNA species containing genetic sequences corresponding to the 5' terminus of the AbLV gag-gene covalently linked to cellular sequences located adjacent to the integrated viral genome. Precedent for spliced type-C viral messenger RNA species containing non-contiguous RNAs has been recently reported (8, 11). If the latter were the case, however, it would be necessary to postulate either the incorporation of this spliced mRNA species into pseudotype virions with equal efficiency as the amphotropic helper virus genomic RNA or, alternatively, less efficient incorporation but more efficient translation of the spliced mRNA.

As in the case of AbLV, type-C transforming viruses such as FeSV (17, 18) and T8-MCF (12) also express polyproteins which contain both structural and nonstructural components. In contrast to these viruses, however, the WSV genome codes both in vivo and in vitro for a protein of around 55,000 containing WLV p15, p30 and p12. The fact that this latter protein was weakly immunoprecipitable by anti-R-MuLV p10, but failed to compete in a highly sensitive p10 competition immunoassay suggests that it may contain a small portion of WLV p10 and thus, presumably the p30-p10 cleavage site. In this respect, WSV closely resembles the S^+L^- strain of Moloney murine-sarcoma virus (2, data not shown). The inability to demonstrate a precursor polyprotein either in WSV-transformed cells or by in vitro translation of the WSV genome may indicate either that such a protein does not exist, or alternatively, that it is subject to rapid post-translational processing and that its nonstructural component, presumably containing transformation-specific sequences, is not recognized by presently available antisera. If the latter model were correct, the translation of a nonstructural WSV-coded protein in Xenopus laevis oocytes, at levels coordinate with those of the WSV gag-gene proteins, would be predicted. Such a protein should be detectable in a fully cleaved form in the event that appropriate sera for its recognition become available. The possibility, however, that the WSV-transforming protein is coded by a spliced mRNA species, analogous to that coding for type-C viral envelope glycoprotein (8, 11) which does not become incorporated into mature virions, cannot be excluded.

The results of the present study thus provide direct evidence favoring

the possibility that the nonstructural portion of the 110,000-130,000 Mr polyprotein expressed in AbLV-transformed cells is viral-coded. The ability both in the present, and in previous studies, to identify transforming virus-coded precursor polyproteins containing structural and nonstructural components has been limited to those transforming viruses which code only for p15 and p12. The *in vitro* translation system described should facilitate further analysis of such viral-coded polyproteins and may provide a means of testing sera from various sources for antibody directed against their nonstructural components.

ACKNOWLEDGEMENT

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CHAPTER VI

CELLS INFECTED WITH A TEMPERATURE-SENSITIVE MURINE LEUKEMIA VIRUS AND TRANSFECTED WITH FRAGMENTS OF WILD-TYPE MURINE LEUKEMIA VIRUS PROVIRAL DNA RELEASE PROGENY VIRUS INFECTIOUS AT THE NONPERMISSIVE TEMPERATURE

CELLS INFECTED WITH A TEMPERATURE-SENSITIVE MURINE LEUKEMIA VIRUS AND TRANSFECTED WITH FRAGMENTS OF WILD-TYPE MURINE LEUKEMIA VIRUS PROVIRAL DNA RELEASE PROGENY VIRUS INFECTIOUS AT THE NONPERMISSIVE TEMPERATURE

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SUMMARY

The isolation is described of infectious progeny murine leukemia virus (MuLV) from cells infected with temperature-sensitive MuLV and transfected with wild-type MuLV proviral DNA fragments synthesized *in vitro*. DNA fragments were synthesized in the presence of actinomycin D by priming the endogenous reverse transcriptase reaction of purified Moloney MuLV with added random oligodeoxynucleotides obtained from DNAse digested

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calf thymus DNA. Recipient cultures of mouse embryo fibroblasts were treated with DNA fragments after infection with an early temperature-sensitive mutant of Rauscher MuLV (Mutant ts29) defective in reverse transcriptase and in the processing of the gag-precursor polypeptides. Infectious progeny virus replicating at the nonpermissive temperature was isolated.

INTRODUCTION

In 1972, Hill and Hillova demonstrated that susceptible chicken cells undergo morphological transformation and produce infectious progeny Rous sarcoma virus upon treatment with DNA isolated from Rous sarcoma virus infected cells (1,2). Extensive studies have been carried out on transfection with DNA extracted from cells infected with oncoviruses of several groups (for reviews see Graham (3) and Hill and Hillova (4)). With sensitive methods available for detection and characterization of progeny viruses, the transfection technique appeared to be an useful tool for the study of the properties of integrated proviral DNA and a number of aspects of the interaction between cells and viruses. However, experiments designed in this way are limited to the study of DNA that contains intact viral genomes, since subgenomic viral DNA fragments did not give rise to the release of infectious virus (5,6). Cooper and Castellot (7,8) overcame this limitation in marker rescue experiments. In these experiments, they transfected recipient cells with subgenomic viral DNA fragments isolated from cells infected with wild-type Rous sarcoma virus and, after infection with ts mutants and a subsequent shift to the nonpermissive temperature, they selected for virus of the wild-type phenotype.

Using the same approach, we have investigated whether treatment of cells with proviral DNA fragments of wild-type Moloney MuLV synthesized *in vitro* in combination with infection with a thermosensitive mutant of Rauscher MuLV could give rise to the production of progeny virus replicating at the restrictive temperature.

Cells and viruses

NIH-3T3 cells (the clonal line NCL5611P3) were grown in Dulbecco's modified Eagle's medium or in Eagle's basal medium supplemented with 10% fetal calf serum (Grand Island Biological Co, Biocult, Ltd).
Clonal isolates of wild-type Moloney MuLV, wild-type Rauscher MuLV and temperature-sensitive Rauscher murine leukemia virus (mutant ts29) were used. Ts29 is an early mutant, which is defective in polymerase and processing of the gag-precursor polypeptice (9,10,11).

Preparation of DNA

To obtain high molecular weight DNA from cells infected with murine leukemia virus, the DNA extraction procedure of Gross-Bellard et al. (12) was used. After extraction, the DNA was precipitated with two volumes of ethanol, sterilized by submerging the precipitate in 70% ethanol overnight at 4°C, dissolved in 0.1 x Standard Saline Citrate and stored at 4°C. The DNA preparation had an $A_{260}:A_{280}$ ratio of 1.8 to 1.9 and a molecular weight of about 50 million daltons as determinec by centrifugation in a neutral sucrose gradient (13).
DNA fragments of murine leukemia virus were synthesized in the presence of actinomycin D according to the procedure of Fan and Baltimore (14) using the endogenous reverse transcriptase reaction of purified murine leukemia virus with added random oligodeoxynucleotides from DNAse-digested calf thymus DNA as a primer (15). The purified reaction product was precipitated with two volumes of ethanol and stored at -20°C. As determined by agarose gel electrophoresis (16), the average molecular weight of the DNA fragments was about 2×10^5 daltons.

Transfection and virus rescue assay

In transfection experiments, NIH-3T3 cells were plated in Dulbecco's modified Eagle's medium at a density of 10^6 cells per tissue culture flask (Falcon Plastics, 25 cm^2). Twenty four hours later the medium was removed and 4 ml of freshly prepared Eagle's basal medium (pH 7.05) was added to the cells, immediately followed by inocculation of 1.0 ml of a

DNA-calcium phosphate suspension (17), which was freshly prepared according to the procedure of Graham and Van der Eb (18). Unless otherwise noted, DNA (with or without carrier calf thymus DNA) was adjusted to 100 µg/ml in HEPES buffer (pH 7.05) (18) and sheared by forcing it in and out 10 times through a 21 gauge needle, both before and after calcium phosphate precipitation. Fifteen hours after DNA inoculation, the cells were washed twice and medium was replaced by Dulbecco's modified Eagle's medium containing polybrene (2µg/ml). When cells became confluent, they were passed to 75 cm^2 tissue culture flasks. When these cells became confluent they were passed at a dilution of 1:10 for the first subculture and, thereafter, subcultures were made at the same dilution every five days. Release of virus was assayed by reverse transcriptase assay using poly(rA) and $oligo(dT)_{12-18}$ (Collaborative Research) as described by Stephenson et al. (19).

In virus rescue assays, cells, plated 24 hours before in Dulbecco's modified Eagle's medium at a density of 10^6 cells per tissue culture flask (Falcon Plastics, 25 cm^2), were infected at 31°C with Rauscher murine leukemia virus ts29 at a multiplicity of infection of about one. After incubation for 90 minutes the cells were treated with DNA fragments as described above and incubation was continued at 31°C. After six hours tissue culture medium was replaced and cell culturing was continued in Dulbecco's modified Eagle's medium. Twenty four hours later, the temperature was shifted to 39°C. Cells were subcultured as described above and virus production at 39°C was assayed by the reverse transcriptase assay as already described. Supernatant media harvested from cells growing at 39°C and producing virus at that temperature, were assayed on fresh cells at 39°C to select for progeny able to initiate infection at the nonpermissive temperature.

Immunoprecipitation and polyacrylamide gel electrophoresis

Murine leukemia virus-specific polypeptides were detected by the radioimmunoprecipitation procedure as described previously (11). Antiserum directed against bovine serum albumin (BSA), against disrupted Rauscher MuLV or Rauscher MuLV p30 were prepared and described by Van Zaane et al. (20). Analysis of the immunoprecipitates were performed by polyacrylamide gel electrophoresis according to Laemmli (21) on a sodium dodecyl sulfate

containing polyacrylamide slab gel gradient. Visualization of radioactivity was performed by scintillation autoradiography according to Bonner and Laskey (22).

Radioimmunoassay

Competition immunoassays for the structural polypeptides p12 and gp70 of AKR virus, Rauscher MuLV and Moloney MuLV were performed according to the methods described by Barbacid et al. (23) and Reynolds et al. (24). These assays measure the ability of unlabeled viral antigens to compete with ^{125}I-labeled viral proteins for binding limiting amounts of antiserum against homologous detergent disrupted murine leukemia virus.

RESULTS AND DISCUSSION

Although transfection with oncoviral DNA has already been described (3), we have performed pilot experiments in order to determine the optimal conditions for the transfection procedure and to test the specificity of the transfection assay. Based upon the studies of Graham and Van der Eb (18) we have used the calcium phosphate precipitation procedure as described in Materials and Methods and according to this procedure we have treated recipient cultures of NIH-3T3 cells with integrated proviral DNA derived from murine leukemia virus infected cells. The results are listed in Table 1. Treatment of recipient NIH-3T3 cell cultures with 100 µg of oncoviral DNA isolated from cells producing high titers of either wild-type Rauscher MuLV or wild-type Moloney MuLV, resulted in the production of progeny virus as determined by assaying the tissue culture medium of the second subculture for sedimentable reverse transcriptase activity.

In other experiments, we have tested lower amounts of oncoviral DNA in the transfection assay, namely 5 and 10 µg. In these experiments oncoviral DNA isolated from NIH-3T3 cells infected with a temperature-sensitive mutant of Rauscher MuLV (mutant ts29) was used and transfection was performed at 31°C. By assaying the tissue culture medium for reverse transcriptase activity production of progeny virus was observed in both

TABLE 1

REVERSE TRANSCRIPTASE ASSAY ON TISSUE CULTURE MEDIUM OF CELLS TRANSFECTED WITH DNA FROM MURINE LEUKEMIA VIRUS PRODUCER CELLS

Experiment	Source of DNA	Amount of DNA	Temperature	Virus production in subcultures 1	2	3	4	5
1	NIH-3T3 cells producing wild-type Rauscher virus	100 µg	37°C	NT	60			
2	NIH-3T3 cells producing wild-type Moloney virus	100 µg	37°C	NT	60			
3	NIH-3T3 cells producing ts29 Rauscher virus	5 µg	31°C	NT	0.02	9	NT	70
4	NIH-3T3 cells producing ts29 Rauscher virus	10 µg	31°C	NT	6	60	NT	72
Controls								
A	uninfected NIH-3T3 cells	100 µg	37°C	NT	<0.02	<0.02	NT	<0.02
B	Calf thymus DNA	100 µg	37°C	NT	<0.02	<0.02	NT	<0.02
C	No DNA	-	37°C	NT	<0.02	<0.02	NT	<0.02

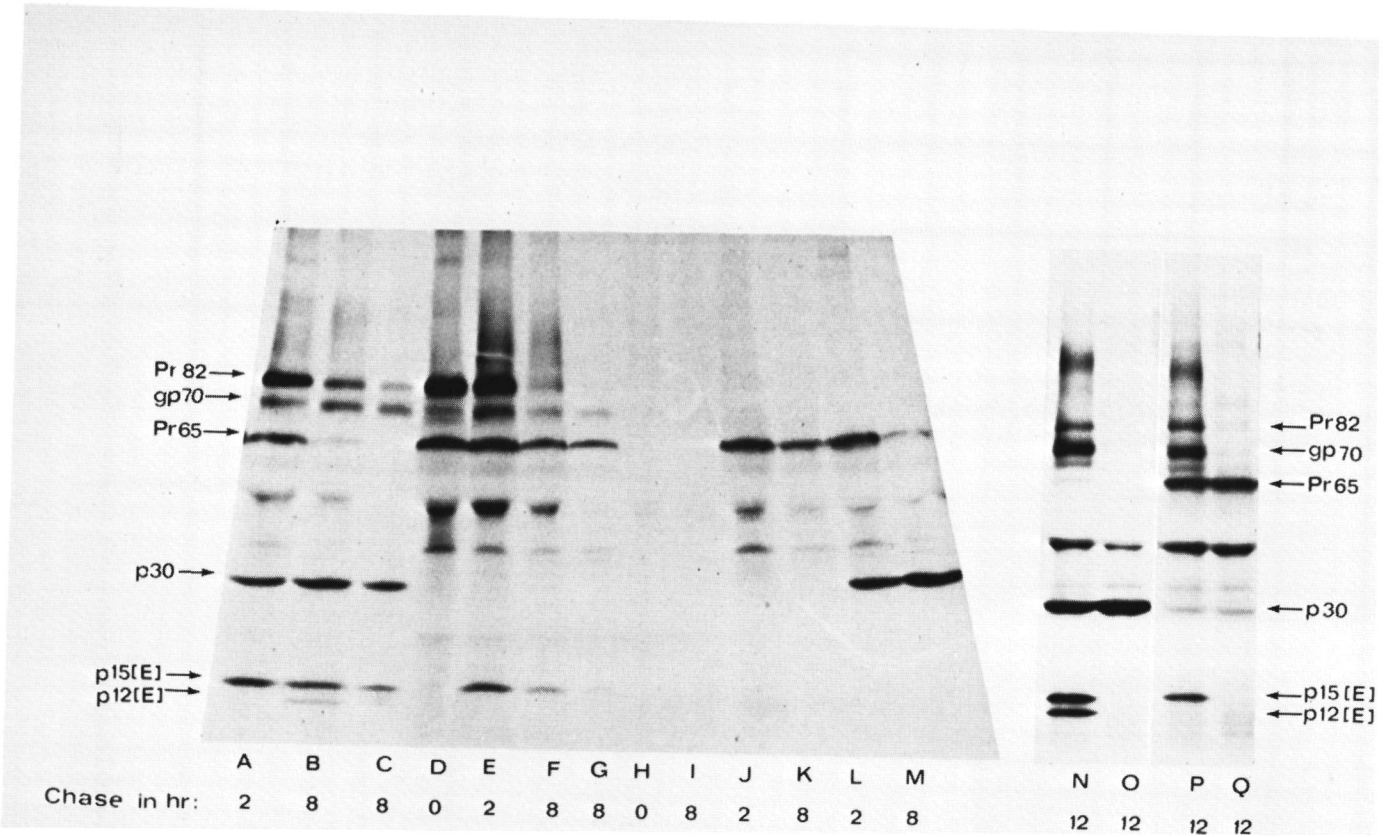
At the temperature indicated, NIH-3T3 cells were transfected with DNA using the calcium precipitation technique and reverse transcriptase assays were performed on tissue culture medium of subcultured cells. Results are expressed as pmole ^{3}H-TMP (6000 cpm/pmole) incorporated per ml tissue culture fluid. All DNA samples were assayed in duplicate.

cases. Furthermore, as can be seen in Table 1, there is a clear relationship between the amount of DNA transfected (5 or 10 µg) and the amount of progeny virus released in the successive subcultures. Control experiments with cells transfected with DNA from uninfected NIH-3T3 cells, or transfected with calf thymus DNA as well as control experiments with cells treated with a calcium precipitate without DNA gave negative results.

The use of DNA preparations from Rauscher MuLV ts29 infected cells provided a marker for assessing the specificity of transfection. We have studied this specificity of transfection by comparing the oncoviral gene expression in cells either infected with Rauscher MuLV ts29 or transfected with DNA isolated from cells producing high titers of the temperature-sensitive mutant. Therefore, pulse-chase experiments were performed at the permissive and nonpermissive temperature and the newly synthesized virus-specific polypeptides were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis after immunoprecipitation with antisera raised against Rauscher MuLV structural polypeptides. The results of the immunoprecipitation analysis are depicted in Figure 1.

In NCL5611P3 cells infected with mutant ts29 the synthesis and processing of virus-specific precursor polypeptides at the permissive and nonpermissive temperature (Figure 1, A-M) was in accordance with our previous observations using another cell line (11). In NCL5611P3 cells transfected with DNA from NIH-3T3 cells producing high titers of mutant ts29, we have observed at the nonpermissive temperature the same impaired processing of the gag-precursor polypeptide and the lack of formation of p12(E) (Figure 1, N-Q). Furthermore, at 39°C no sedimentable reverse transcriptase activity could be detected in the tissue culture medium of these cells (results not shown).

From studies of Chattopadhyay et al. (25) it appeared that the NIH-Swiss mouse from which the NIH-3T3 line was derived do not contain a complete ecotropic oncoviral genome. That might be a reason why transfection of DNA isolated from uninfected NIH-3T3 cells did never give rise to virus production (Table 1). The latter observation is in agreement with the results of Lowy et al. (26) who used DNA isolated from uninfected NIH-3T3 cells in control transfection experiments. In recent studies the transfectability of integrated proviral DNA of endogenous oncoviruses was examined. From these studies, it appeared that DNA isolated from


Pr 82
gp70
Pr65
p30
p15[E]
p12[E]
A B C D E F G H I J K L M
Chase in hr: 2 8 8 0 2 8 8 0 8 2 8 2 8
N O P Q
12 12 12 12
Pr82
gp70
Pr65
p30
p15[E]
p12[E]

LEGEND TO FIGURE 1

NIH-3T3 cells were either infected at 31°C with Rauscher MuLV ts29 or transfected with integrated proviral DNA isolated from ts29 infected cells. The synthesis and processing of virus-specific precursor polypeptides were examined in pulse-chase experiments at the permissive and nonpermissive temperature. The synthesized virus-specific polypeptides were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and scintillation autoradiography after immunoprecipitation with a polyvalent or a monospecific antiserum directed against Rauscher MuLV polypeptides. To facilitate the interpretation of the figure we summarize the conditions in the following table:

Lane	treatment	temp.	label	antiserum	Lane	treatment	temp.	label	antiserum
A	infection	31°	met	αMuLV	J	infection	39°	met	αp30
B	"	"	"	"	K	"	"	"	"
C	"	"	leu*	"	L	"	31°	"	"
D	"	39°	met	"	M	"	"	"	"
E	"	"	"	"	N	transfection	"	"	αMuLV
F	"	"	"	"	O	"	"	"	αp30
G	"	"	leu*	"	P	"	39°	"	αMuLV
H	"	31°	met	αBSA	Q	"	"	"	αp30
I	"	"	"	"					

*Note: leucine was used in two experiments instead of methionine in order to analyse the formation of p15 which does not contain methionine.

avian (27), feline (28), or baboon (29) cells gave rise to progeny virus only when DNA was derived from cells that were releasing high titers of these oncoviruses. Progeny virus production was never observed when DNA was derived from cells that were not releasing high titers of these oncoviruses. Apparently, transfection of repressed integrated proviral DNA does not give rise to release of progeny virus either.

From transfection studies with integrated proviral DNA, it appeared that only intact oncoviral genomes were infectious and could give rise to progeny virus release (5,6). To answer the question whether treatment of cells with noninfectious proviral DNA fragments of a murine leukemia virus in combination with infection with a thermosensitive mutant could give rise to the production of progeny virus replicating at the restrictive temperature, we have performed virus rescue assays. In these experiments, we have used two different oncoviruses, namely wild-type Moloney MuLV for the in vitro synthesis of proviral DNA and Rauscher MuLV ts29 as the temperature-sensitive mutant. In transfection experiments, we have first examined the biological activity of the in vitro synthesized proviral DNA. Under the reaction conditions used for their synthesis, mainly low molecular weight single stranded DNA fragments (about 400 nucleotides in lenght) were formed (result not shown). These proviral DNA fragments were approximately representative for the whole genome of the virus (Dr. A.J.M. Berns, personal communication; see also reference 30). Recipient cultures treated with these in vitro synthesized DNA fragments of Moloney MuLV were never shown to release progeny oncoviruses (Table 2). Apparently, the proviral DNA fragments alone were biologically inactive. The observation that proviral DNA synthesized in the presence of actinomycin D is biologically inactive was also reported by Rothenberg et al. (31). Despite the fact that they used reaction conditions to synthesize high molecular weight DNA, it appeared from their studies that only DNA synthesized in the absence of actonimycin D was biologically active and that the biologically inactive proviral DNA synthesized in the presence of actinomycin D was 0.6 kilobases shorter and missed some genome sequences (32). In virus rescue assays, recipient cultures of NIH-3T3 cells were infected at 31°C with Rauscher MuLV ts29 and, subsequently, cells were treated with fragments of in vitro synthesized DNA of Moloney MuLV. In these cases calf thymus DNA was added as carrier DNA. The results of these experiments and of the control experiments are summarized in Table 2.

TABLE 2

VIRUS RESCUE ASSAY WITH FRAGMENTS OF MOLONEY MURINE LEUKEMIA VIRUS DNA SYNTHESIZED IN VITRO

Experiment	Virus	Calf thymus DNA	Molorey virus DNA fragments	Virus production at 39°C a)	Released progeny virus infectious at 39°C b)
1	none	none	none	<0.02	<0.02
2	ts29	none	none	<0.02	<0.02
3	none	50 µg	none	<0.02	<0.02
4	ts29	50 µg	none	<0.02	<0.02
5 ǂ	none	50 µg	0.8 µg	<0.02	<0.02
6	ts29	50 µg	0.8 µg	39	NT
7	ts29	50 µg	0.8 µg	21	13
8	ts29	50 µg	0.8 µg	41	29
9	ts29	50 µg	0.8 µg	40	32
10	ts29	50 µg	0.8 µg	2 *	5

NIH-3T3 cells were mock infected or infected with Rauscher MuLV ts29 at 31°C. Ninety minutes after infection, cultures were treated with DNA as indicated in the Table according to the procedure as described in Materials and Methods. After the temperature was shifted to 39°C, cells were subcultured as described in Materials and Methods.

a) Production of progeny virus was assayed by reverse transcriptase assay after three subcultures. Results are expressed as pmole ^{3}H-TMP (6000 cpm/pmole) incorporated per ml tissue culture fluid and represent mean value from two separate determinations.

b) Infectivity of the progeny virus was tested at 39°C on fresh NIH-3T3 cells and virus production was assayed by reverse transcriptase assay, after three subcultures.

*) Production of progeny virus was assayed by reverse transcriptase assay after one subculture.

ǂ) Experiment performed in triplicate.

No progeny virus replicating at 39°C was produced by control cultures (Table 2, experiments 1-5). Apparently, reversion of the parental ts29 mutant did not occur (Table 2, experiments 2 and 4). It should be noted that mutant ts29 is probably a double mutant (9,10,11) and even in long term cultures we have never observed any reversion of this mutant (unpublished observations). In contrast to the control cultures, all cell cultures infected with mutant ts29 and subsequently treated with DNA fragments of Moloney MuLV (Table 2, experiments 6-10) released progeny virus replicating at 39°C, as was determined by assaying the culture fluids for sedimentable DNA polymerase activity. Supernatant media of these cultures contained virus infectious at 39°C as appeared upon infection of fresh NIH-3T3 cells (Table 2). Analysis of lysates of these cells in competition radioimmunoassays for the gag-gene related structural polypeptide p12 and the env-gene related structural polypeptide gp70 revealed the presence of Moloney MuLV specific antigenic determinants. In all five lysates about equal amounts of either Moloney MuLV p12 or Moloney MuLV gp70 were found and these values were similar to those observed in NIH-3T3 cells productively infected with Moloney MuLV (Moloney MuLV infected NIH-3T3 cells contain about 3000 ng p12 and 5000 ng gp70 per mg total cellular protein; uninfected NIH-3T3 cells contain less than 30 ng p12 and 40 ng gp70 per mg total cellular protein). No reactivity was observed in similar assays for the same structural polypeptides of AKR virus or Rauscher MuLV. This result indicates that in all five cultures at least the sequences coding for the type-specific antigenic determinants of p12 and gp70 were derived from the large excess of Moloney MuLV proviral DNA fragments added in these experiments. Since the Moloney MuLV proviral DNA fragments alone did not give rise to any progeny virus release, factors required for the observed oncovirus production were apparently introduced by mutant ts29. The present data do not allow any conclusion on a possible mechanism involved. It cannot be excluded that the progeny virus is derived from minor amounts of genome lenght proviral DNA which, although not infectious alone, became infectious with the aid of an infecting oncovirus. In view of the observations of Rothenberg et al. (31,32) synthesis of genome lenght proviral DNA in the presence of actinomycin D is rather unlikely. However, the biological inactivity of the proviral DNA alone may also be a consequence of its

single stranded character, if at least partial double strandedness were required for infectivity. Furthermore, it cannot be excluded that the progeny virus release occurred as a result of recombination between Moloney MuLV sequences and Rauscher MuLV sequences. To study the latter possibility, virus rescue experiments with defined proviral restriction fragments are presently under investigation. In this way we hope to develop a method for the study of the biological activity of subgenomic DNA fragments.

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Oncoviruses constitute a group of widely spread oncogenic RNA viruses. They can induce tumors of various histological types when inoculated into appropriate hosts. Upon infection oncoviruses convert their genomic RNA into cellular genes with the aid of the virus-specific RNA-dependent DNA polymerase or reverse transcriptase. Thus, the newly acquired genes are passed from parent to progeny cell. Oncoviral genes can also be present latently in the genomes of a large number of species, their expression apparently being subjected to the same regulatory mechanisms that affect cellular genes. Although normally repressed, such endogenous viral genes can be activated by a variety of factors and can sometimes escape from host control. Released infectious oncoviruses can be transmitted horizontally to animals of the same species. During the course of evolution they can even become stably integrated in the germ line of species that are only remotely related phylogenetically. Consequently, oncoviruses enable the cell to donate or to receive genetic sequences encapsidated into viral particles. A survey of these and other basic properties of oncoviruses is given in the introductory chapter of this thesis.

In chapter II we describe a new method to fractionate virions into cores and viral envelopes. In contrast to older methods, viral envelopes could now be obtained without detectable amounts of core material. Flotation rather than sedimentation appeared to be a crucial step in the purification procedure. New data on the structure of the virions were revealed by an electronmicroscopic and biochemical analysis of the purified fractions.

Chapter III focuses on the precursor to the viral envelope polypeptides. This precursor polypeptide is the primary translation product of the viral env-gene. Its post-translational cleavage is dependent upon its correct glycosylation at the level of the nascent protein as could be shown with the aid of a reversible and an irreversible inhibitor of glycosylation. It appeared from these experiments that the protein moiety of the envelope precursor polypeptide has a molecular weight of about 70,000 dalton.

Chapter IV deals with temperature-sensitive mutants of Rauscher murine leukemia virus. Apart from other phenotypical characteristics some of them appeared to be defect in the processing of the precursor to the internal viral polypeptides, $Pr65^{gag}$, at the restrictive temperature. Concomitantly, the same mutants did not produce p12(E), an envelope polypeptide coded for by the env-gene.

Many replication-defective mammalian oncoviruses are able to transform fibroblasts in cell culture, a property which presumably closely correlates with the induction of tumors by these viruses. They apparently have acquired this property as a result of genetic recombination between an autonomously replicating oncovirus and genetic information present in the host cell. Translation experiments are the most direct way to study the coding properties of the genome of these viruses. In chapter V we describe translation experiments with oocytes of Xenopus laevis, a protein synthesizing system known for its faithful translating and processing capacity. Thus, we showed that the polyprotein expressed in Abelson murine leukemia virus-transformed cells was viral-coded and that it contained p15 and p12 moieties derived from leukemia helper virus covalently linked to an unknown polypeptide sequence. An analogous polyprotein either was absent in woolly monkey sarcoma virus-transformed cells or was subjected to rapid post-translational cleavage.

Finally, in chapter VI we report that by transfection oncoviral genes can be introduced into appropriate recipient cells. Furthermore, using non-infectious proviral DNA we could rescue oncovirus when recipient cells were coinfected with a temperature-sensitive oncovirus. In a continuation of this study we hope to discover exactly how virus rescue took place so that we will be able to apply this type of experiments as a biological assay.

W.J.M. van de Ven was born on May 22, 1947 at Veghel, The Netherlands. In 1967, he graduated from the "St. Willibrord Gymnasium" at Deurne. In September of the same year he started his chemistry studies at the University of Nijmegen, Nijmegen, The Netherlands. He obtained his bachelor's degree in May 1970. In October 1973 he obtained his doctoral degree in biochemistry (Prof. Dr. H. Bloemendal), pharmacology (Prof. Dr. J.M. van Rossum) and submicroscopic morphology (Dr. A.M. Stadhouders). In September 1973 he was appointed as an assistant in the Department of Biochemistry (Head: Prof. Dr. H. Bloemendal) at the University of Nijmegen, where he participated in teaching students and performed some of the research activities described in this thesis. He performed part of the studies for his dissertation at the Frederick Cancer Research Center, National Cancer Institute, Frederick, Maryland, USA, in collaboration with the laboratory of Dr. J.R. Stephenson (Head: Viral Genetics Section).
From September 1978, he is appointed at the National Cancer Institute of the United States as a visiting fellow in order to study in the laboratory of Dr. J.R. Stepherson recombinant and replication defective transforming oncoviruses.

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9. W.J.M. van de Ven, A.J.M. Vermorken, C. Onrekink, H.P.J. Bloemers and H. Bloemendal (1978).
Structural studies on Rauscher murine leukemia virus: Isolation and characterization of viral envelopes.
J. Virol. (in press).

10. Roberta K. Reynolds, Wim J.M. van de Ven and John R. Stephenson (1978).
Translation of type-C viral RNAs in Xenopus laevis oocytes: Evidence that the 120,000 molecular weight polyprotein expressed in Abelson leukemia virus transformed cells is viral coded.
Submitted for publication.

11. W.J.M. van de Ven, F.A.M.J. van der Hoorn, H.J. Dodemont and H.P.J. Bloemers (1978).
Cells infected with a temperature-sensitive murine leukemia virus and transfected with fragments of wild-type murine leukemia virus proviral DNA release progeny virus infectious at the nonpermissive temperature.
Submitted for publication.

ABSTRACTS OF PAPERS PRESENTED AT INTERNATIONAL MEETINGS.

1. Xth meeting on the European Tumor Virus Group, October 1976, Grindelwald, Switzerland. Impaired processing of precursor polypeptides of temperature-sensitive mutants of Rauscher leukemia virus.

2. Oncornavirus Workshop, March 1977, Basel, Switzerland. Impaired processing of env-related gene products.

3. Meeting on RNA Tumor viruses, May 1977, Cold Spring Harbor, New York, USA. Impaired expression of the env- gene of Rauscher leukemia virus.

4. VIIIth International Symposium on Comparative Research on Leukemia and Related Diseases, August 1977, Amsterdam, The Netherlands. Impaired gene expression of Rauscher murine leukemia virus.

5. Meeting on RNA Tumor Viruses, May 1978, Cold Spring Harbor, New York, USA. Biological assay of in vitro synthesized subgenomic DNA fragments of murine leukemia virus by marker rescue.

CURRICULUM VITAE.

W.J.M. van de Ven werd op 22 mei 1947 in Veghel geboren. In 1967 behaalde hij op het St. Willibrord Gymnasium te Deurne het diploma Gymnasium β. In september van datzelfde jaar begon hij zijn studie scheikunde aan de Katholieke Universiteit in Nijmegen. Het kandidaatsexamen (S_2) werd afgelegd in mei 1970. In oktober 1973 werd het doktoraalexamen scheikunde afgelegd met als hoofdvak biochemie (Prof. Dr. H. Bloemendal) en bijvakken farmacologie (Prof. Dr. J.M. van Rossum) en submicroscopische morfologie (Dr. A.M. Stadhouders). Vanaf september 1973 was hij als wetenschappelijk medewerker verbonden aan het Laboratorium voor Biochemie (Hoofd: Prof. Dr. H. Bloemendal) van de Katholieke Universiteit alwaar hij betrokken was bij het onderwijs aan studenten en waar hij een gedeelte van het hier beschreven promotieonderzoek verrichtte. Een bepaald gedeelte van zijn promotieonderzoek voerde hij uit in de Verenigde Staten op het Frederick Cancer Research Center, National Cancer Institute, Frederick, Maryland in samenwerking met het laboratorium van Dr. J.R. Stephenson (Hoofd: Viral Genetics Section).
Vanaf september 1978 zal hij als "visiting fellow" verbonden aan het "National Cancer Institute" in de Verenigde Staten, op het laboratorium van Dr. J.R. Stephenson onderzoek doen aan recombinant en replicatie defectieve transformerende oncovirussen.

STELLINGEN

I

Virus specifieke polyproteinen in nonproducer cellen geinfecteerd met replicatie-defectieve oncovirussen lijken in het algemeen opgebouwd te zijn uit structurele en niet-structurele componenten. Door karakterisering van dergelijke polyproteinen kan de subgenvolgorde in het gag-gen van aviaire oncovirussen definitief bepaald worden.

V.M. Vogt, R. Eisenman and H. Diggelmann (1975) J. Mol. Biol. 96, 471-493.
D.J. Shealy and R.R. Rueckert (1978) J. Virol. 26, 380-388.

II

Karakteristieke eigenschappen van de 5'-terminale nucleotide sequenties in het genoom van type-C helper virussen maken conservering van deze sequenties in gerelateerde recombinant oncovirussen waarschijnlijk.

L.C. Waters, B.C. Mullin, T. Ho and W.K. Yang (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2155-2159.
W.A. Haseltine, A.M. Maxam and W. Gilbert (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 989-993.
E. Rothenberg, D.J. Donoghue and D. Baltimore (1978) Cell 13, 435-451.

III

Het is betreurenswaardig dat Kirkwood zijn beschouwing getiteld "Evolution of ageing" in de laatste alinea ontsiert door de volgende ongefundeerde veronderstelling:
"...we may hope to gain insight into the abnormalities represented by (....) the apparently immortal transformed cells of malignant tumours. It may, for example, be possible that oncogenic viruses interfere with these processes (commitment of cells to limited growth) to enhance the accuracy of their own replication in a way that restores some error-committed cells to stability".

T.B.L. Kirkwood (1977) Nature 270, 301-304.

IV

De juistheid van de door Okasinski en Velicer geponeerde translatie volgorde voor de interne structurele eiwitten van katte-leukemie virus moet betwijfeld worden.

G.F. Okasinski and L.E. Velicer (1977) J. Virol. 22, 74-85.

V

Uit de qualitatieve variatie in expressie van het gag-gen in nonproducer cellen getransformeerd door verschillende sarcomavirus-isolaten mag men niets afleiden omtrent de eventuele afwezigheid van bepaalde leukemievirus specifieke sequenties in de sarcomavirus-genomen.

K.C. Robbins, H. Okabe, S.R. Tronick, R.V. Gilden and S.A. Aaronson (1978) J. Virol. 25, 471-478.

VI

De lymfocyt is als testsysteem voor het meten van de genetisch bepaalde gevoeligheid voor longkanker door sigarettenrook niet zeer geschikt.

B. Paigen, H.L. Gurtoo, J. Minowad and K. Paigen In: Microsomes and drug oxidations. Proceedings of the Third International Symposium, Berlin, July 1976. Eds.: V. Ullrich, I. Roots, A. Hildebrandt, R.W. Estabrook and A.H. Conney. Oxford, Pergamon Press (1977).

VII

Dysplastische veranderingen van het bronchusepitheel na toediening van carcinogeen worden snel opgespoord door rasterelectronenmicroscopisch onderzoek van het oppervlak. Dit type onderzoek maakt een goed inzicht mogelijk in de driedimensionale opbouw en expansieve groei van toekomstige tumoren.

VIII

De synthese van het variabele gedeelte van de lichte keten

(V_L) van het muize myeloma IgA protein 315 door Gavish et al. wordt onvoldoende gesteund door hun experimentele gegevens; bovendien wekken de auteurs ten onrechte de indruk een semi-synthetisch antilichaam te hebben bereid.

M. Gavish, R. Zakut, M. Wilchek and D. Givol (1978) Biochemistry 17, 1345-1351.

IX

In verband met de datering van Korinthisch aardewerk, verdient het aanbeveling nader te onderzoeken in hoeverre de datering van Kirua's nederlaag in 696 voor Christus kan gelden als datering voor de invallen van de Cimmeriers in Anatolië en daardoor als datering voor het Middel Protokorinthisch aardewerk, gezien de afwezigheid ervan in Smyrna en Sardes.

Hanfmann, G.M.A. Türk Arkeoloji Dergisi 24, 115-124 (1977)
Andersen, J.K. Annual of the British School at Athens 53/54, 138-151 (1958-1959)

X

Het zoeken van cellulaire kankerverwekkende genen met behulp van oncovirussen is als het zoeken van een speld in een hooiberg met behulp van een magneet: een niet volstrekt onmogelijke opgave.

6 september 1978 W.J.M. van de Ven