EXPRESSION AND CHARACTERIZATION OF THE ROUS SARCOMA VIRUS GAG GENE PRODUCT IN MAMMALIAN CELLS USING AN SV40 LATE REGION REPLACEMENT VECTOR

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PREFACE

Classical retrovirology had its birth in 1908 with Peyton Rous studying spontaneous sarcomas (tumors of the connective tissue) in chickens. He found that the sarcomas were transmissible and that the tumors showed greater virulence with each passage. He tested cell free filtrates for the presence of the etiological agent, and for the first time demonstrated that the entity involved in the transmissible sarcomas was a virus (Rous, 1911). This virus now bears his name, Rous sarcoma virus.

Since Rous's famous discovery of a filterable entity that could induce tumor formation in infected chickens, countless other viruses have also been discovered that induced a variety of neoplastic growths in the infected host. With the new sophisticated techniques of today we have been able to probe the molecular events that occur during a retroviral infection in hopes of elucidating how these viruses not only induce their neoplastic growth but also the molecular events that must occur to allow viral replication. At the time of this thesis, a major obstacle inhibiting detailed molecular studies of Rous sarcoma virus replication is the lack of adequate immortalized avian cell lines and expression vector systems. This thesis therefore is written in two parts. The first part addresses the block of RSV replication in mammalian cells. The idea was to devise a system to study retroviral assembly in immortalized cell lines. If we can elucidate the differences between the avian and murine retroviruses that allow

the murine viruses but not the avian viruses to replicate in mammalian cells, we might be able to better understand the molecular requirements for viral replication and assembly.

In order to study this block to replication of avian retroviruses in mammalian cells, I chose to use the simian virus 40 (SV40) as a vector to express the RSV *gag* gene (or the viral polyprotein $Pr76^{gag}$) in mammalian cells.

The second part of this thesis arose from a problem encountered while using the SV40 virus as an eukaryotic expression vector. The strategy for using this virus as an expression vector is quite simple. The sequence of choice to be expressed, *gag*, is cloned into the late region of the SV40 virus genome in place of the late genes. The expression of the foreign gene is then driven from the late SV40 promoter. This recombinant vector can then be propagated with the help of a second SV40 virus that supplies the late gene products *in trans*. The problem was encountered while trying to propagate the recombinant vector. We were able to successfully express Pr76 but unable to passage any newly assembled recombinant virions to fresh monolayers. Instead of then abandoning the project, I chose to pursue the problem in hopes of finding the molecular block in SV40 replication. This is important since finding a sequence or protein that inhibits SV40 replication might prove to be a valuable tool for elucidating the mechanisms of viral replication.

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Above all, I express my deepest thanks and love to my wife Sally Kay, for her endless support and belief in my abilites, to her I dedicate this thesis.

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CHAPTER I

RETROVIRAL CHARACTERISTICS AND REPLICATIVE STRATEGIES

General Characteristics

Taxonomy of Retroviruses

As with any new discovery of a living entity, the first scientific observations performed are to classify the organism into a genetic phylum or family. For a virus to be included in the viral family Retroviridae, it must meet the following criteria (Teich, 1982):

The nucleic acid must be composed of two identical molecules of positive-sense single-stranded RNA (60S-70S) joined at or near the 5′ termini (Beemon et al., 1976; Bender et al., 1976). The molecule must also have a 5′ "capped" structure ($m^7G^5ppp^{5'}NmpNp$) (Rose et al., 1976) and a polyadenylated 3′ terminus (Bender et al., 1976) and two large repeated sequences (LTR) at each the 3′ and 5′ ends of the proviral DNA (Teich, 1984). The genome must have a $tRNA^{trp}$ (or some other related tRNA) base-paired to the genome at a site approximately 100 nucleotides from the 5′ end (Bishop, 1978). This tRNA molecule is important in initiating proviral cDNA synthesis which is required for the replicative life cycle of all retroviruses.

The virion must be composed of 60-70% protein. These proteins are all synthesized from three major coding sequences in the viral

RNA; *gag*, group associated antigens or structural proteins; *pol*, reverse transcriptase; and *env*, envelope glycoproteins.

Another major criterion for classification of a virus into the retroviridae family is the physical structure of the virion which can be classified as either A-, B-, C-, or D-type (see below).

Early electron micrographs of tumor cells depicted a structure budding from the surface membranes (Bernard et al., 1958). The budding virus was roughly spherical with a diameter of 90-100 nm. The inner electron dense core or nucleoid was surrounded by an outer lipid-containing membrane with projecting spikes (see Figure 2 for a schematic representation of such as a particle).

Since these early studies, microscopists have classified retroviruses into four distinct categories according to their gross morphology and site of assembly. A-type particles are generally 60-90 nm in diameter and have a doughnut-shaped internal spherical core. These intracellular particles are further classified into intracisternal and intra-cytoplasmic forms. The intracytoplasmic forms are thought to be immature type B particles or endogenous B- or D-type particles (see below). The destination of the intracisternal forms on the other hand is not known.

B-type particles include the prototype mouse-mammary tumor virus (MMTV). These spherical particles are 125-130 nm in diameter with an electron dense core (75-80 nm diameter) located off center and enveloped by a lipid membrane with long projecting spikes. Fully formed nonenveloped particles can be seen in the cytoplasm of infected cells.

C-type particles are most common of the retroviruses with Rous sarcoma virus being the prototype virus. The most striking characteristic of this group of viruses is the absence of intracellular particles. It is not until the budding process begins at the surface membrane that the particles become visible. After budding, the newly released immature virions are distinguished by their loosely-packed cores. Upon maturation, the cores condense into a crescent-shaped electron-dense structure similar to the core of B-type particles except that the cores are centrally located within the outer envelope of the virion.

D-type particles are quite different from A, B, and C-type particles. Mason-Pfizer monkey virus (MPMV) is the prototype virus of the group. Like the B-type particles, both intracellular and extracellular forms can be seen. The major morphological difference between D- and B-type particles is that D-type particles do not have a spherical core but instead have an elongated ring-like structure. Also, the outer-membrane spikes are shorter than those of the B- and C-type viruses.

The Retroviridae can also be divided into three subfamilies based on their pathogenic properties. The **oncovirinae**, including all the oncogenic and related nononcogenic viruses, are classified according to their host range and the type of neoplasm they induce (avian leukemia-leukosis virus, avian sarcoma virus, mouse mammary tumor virus etc.). The **lentiviruses**, or slow viruses, are cytopathic and induce a slow chronic degenerative disease (*ie.* visna virus). The third family of viruses are the **spumavirinae**, or foamy viruses. These viruses are symbiotic and do not even elicit a host defense response

(Teich, 1982).

Genomic Organization

As mentioned earlier, the packaged viral RNA is modified with both 5′ cap structures and poly (A) tails (Rose et al., 1976; and Bender er al., 1876). Therefore, the viral RNA can also serve as a functional mRNA (see below). Because the most detailed information about the genomic structure has been worked out using the Rous sarcoma and avian leukosis viruses (RSV and ALV respectively) and the murine leukemia virus (MLV), this discussion will be limited to these prototype viruses.

Figure 1 shows the genomic structure of three replication proficient retroviruses. RSV is the only known retrovirus to contain all the sequences and genes needed for replication (two LTRs, *gag*, *pol*, *env*, and etc.) and a viral oncogene, *src*. Other retroviruses are known to carry viral or cellular oncogenes, but they do so at the expense of an essential region. These viruses are "replication deficient" and can only be propagated with a helper virus supplying the missing factors *in trans*.

The 5′ regulatory regions are required for transcription initiation and cDNA synthesis. The "R" region is a short repeated sequences (20-80 nucleotides) at both ends of the genome that are apparently required for replication of the viral RNA. The "U_5" is a 5′ unique sequence (approx. 100 nucleotides) that separate the R region and the tRNA binding site. This tRNA binding is the "PB$^-$" region. The tRNA serves as the primer for DNA synthesis via reverse transcriptase. The sequence is complementary to the 3′ termini (16-19 nucleotides) of

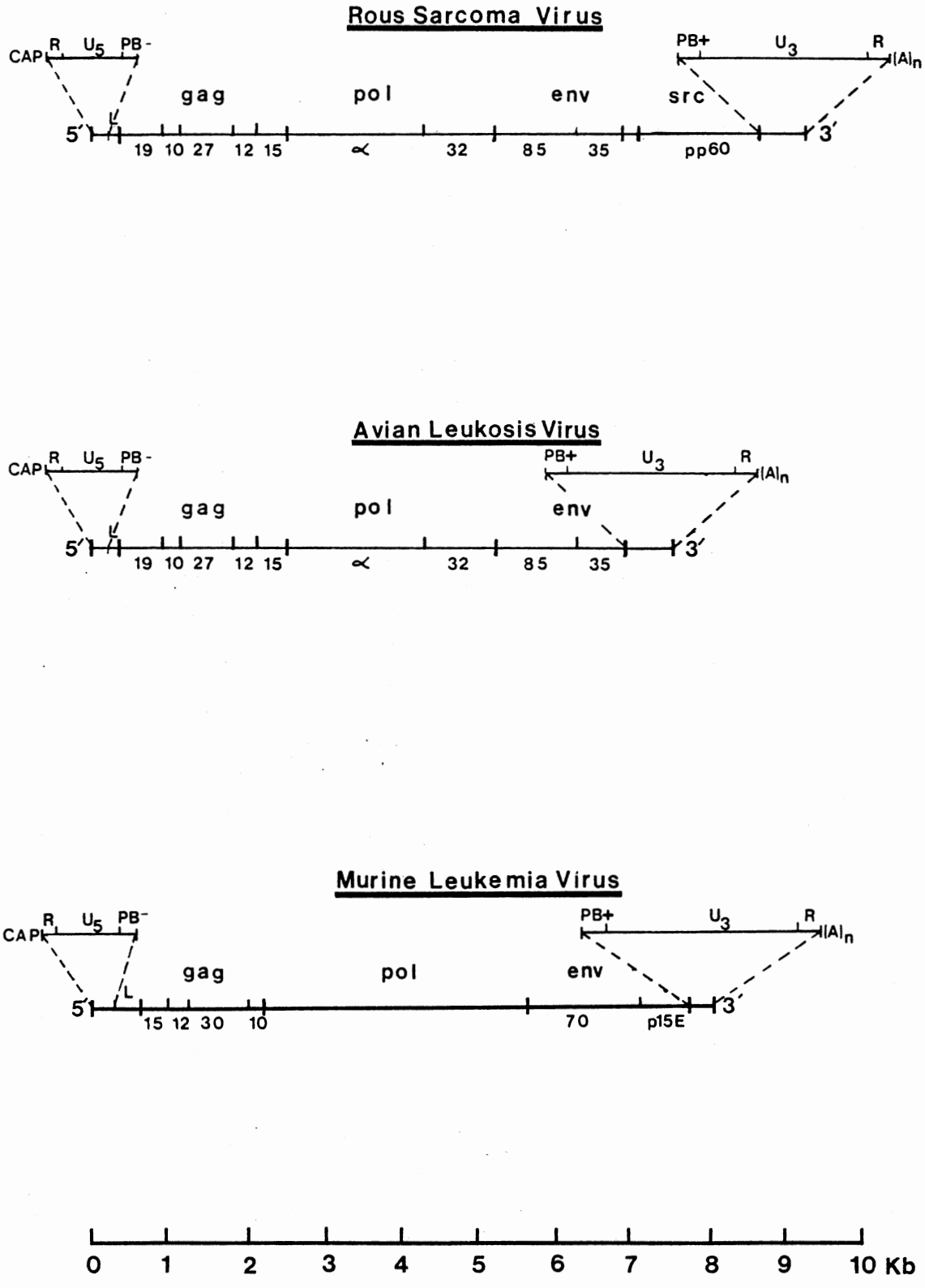

Figure 1. Genomic map of three different retroviruses.

the $tRNA^{trp}$ molecule. "L" is a long untranslated region (approx. 250 nucleotides) which contains the sequences required for viral RNA (vRNA) packaging as well as those sequences needed for efficient transcriptional initiation.

The translated regions or structural genes are always in the order *gag*, *pol*, *env*, (and *src* in the case of RSV). The *gag* gene (approx. 2000 nucleotides) codes for the structural proteins associated with the internal core. The products of the *gag* coding region are also responsible for the group-specific antigens that were once used to distinguish the different groups of retroviruses. The product of the *gag* coding region is a precursor polypeptide that must undergo proteolytic processing to generate the structural proteins. The *pol* coding region (approx. 3000 nucleotides) encoding the RNA-dependent DNA polymerase or reverse transcriptase. The active enzyme (either one or two peptides depending on the particular virus) is a processed product from a precursor polypeptide. This processing event is required to give the active polymerase along with an indispensable endonuclease and RNAse H activity. Figure 2 shows the location of reverse transcriptase in the virion. Even though this diagram depicts only two reverse transcriptase molecules associated with the virion, it is not meant to imply there are only two. In fact there are over 50 copies present per virion in MMTV (Panet et., 1975a). The *env* coding region (approx. 2000 nucleotides) codes for the surface glycoproteins. These glycoproteins, or spikes, are required for viral attachment to host-specific cell surface receptors and confer subgroup antigenicity. The product is also synthesized as a polyprotein precursor and at least one of the the processed proteins is glycosy-

lated before it is incorporated into the viral envelope. The *src* coding region (approx. 2000 nucleotides) codes for the protein kinase responsible for the rapid transformation of infected cells. The 60 kilodalton (kDa) phosphoprotein is also translated from a spliced mRNA. RSV is the only replication profecient retrovirus to carry a viral oncogene.

The 3′ regulatory regions are primarily required for cDNA synthesis and addition of the poly(A) tail. The "PB^+" region is the counter part to PB-. The function of the site is not known but it is probably important since this sequence is highly conserved among the members of the retroviral family. "U_3" is a unique region near the 3′ end between PB+ and R. It is known that U_3 contains the strong enhancers required for initiation of transcription (Coffin, 1984).

Architecture of the Virion

All retroviruses have a similar physical structure. Both copies of the viral RNA are coated by viral specific proteins. This nucleoprotein complex along with 20-70 copies of reverse transcriptase (Panet et al., 1975; Dickson et al., 1984) is packaged into internal cores. These cores are surrounded by an outer membrane with protruding viral glycoproteins (see Fig. 2). The outer membrane is derived from the host cell during the process of viral budding. Since most of the information about the physical structure of retroviruses has been obtained through studies using RSV, the following discussion will be limited to RSV.

The outer membrane of RSV is derived from the host cellular membrane and is studded with numerous glycoprotein projections consisting of the two structural peptides encoded by the *env* gene. The membrane anchoring protein, gp37 is covalently attached via disulfide bonds to the larger glycoprotein, gp85. This larger peptide is responsible for recognizing the host cell-surface receptors (Varmus and Swanstrom, 1982, and Weiss, 1982).

The amino-terminal *gag* peptide, p19, is found associated with the viral envelope along with p15 and the newly identified p10. The major capsid protein is p27. Within the capsid are the viral RNA molecules tightly associated with P12, reverse transcriptase, and $tRNA^{trp}$.

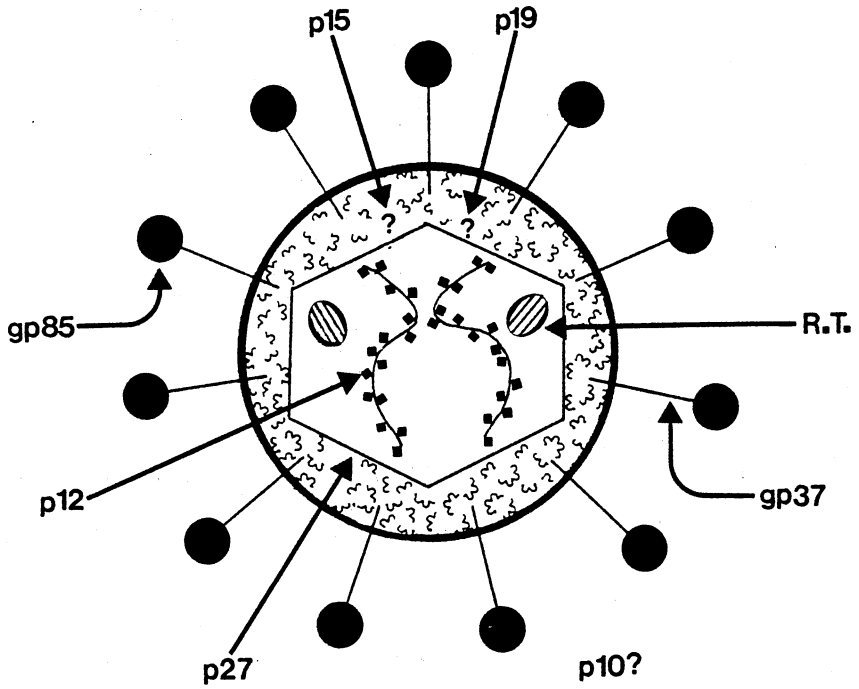

Figure 2. Schematic representation of the structural organization of a type-C retrovirus (Rous sarcoma virus). The location of the different viral proteins is indicated with arrows. Question mark indicates that the precise location between the core and envelope of viral proteins, p10, p19, and p15 is not known. R.T. is reverse transcriptase.

Replicative Cycle

The replicative cycles of retroviruses are quite complex yet very similar. Therefore, the following discussion will again be limited to RSV unless otherwise indicated.

As discussed by Varmus and Swanstrom (1982), the first step of infection is the entry of the virus into the host cell. This requires attachment of the virus to the host outer-membrane to allow adsorption of the virus possibly by an endocytotic event. It has been hypothesized that the capsid then makes its way to the nucleus by some unidentified mechanism while making a cDNA copy of the viral RNA (Varmus et al., 1974; Aboud et al., 1979; Jolicoeur and Baltimore, 1876a and 1976b; and Krontinis et al., 1973). Once the capsid has reached the nucleus, the cDNA is released there thus allowing the DNA to become integrated into the host chromosome. The provirus can then be passed through the germ line or undergo a productive infection using the host RNA and protein synthesizing machinery to produce progeny virus. After all viral RNAs and proteins have been synthesized, the virus is assembled into infectious progeny particles (Varmus and Swanstrom, 1982).

Entry of the Virus

The first step toward a productive infection is adsorption of the virus to the host cell via viral glycoproteins and specific host-cell surface receptors (Varmus and Swanstrom, 1982; Weiss, 1982). Most nonpermissive cells can block infection at this step (Duff and

Vogt, 1969). Other nonpermissive cells allow viral attachment and penetration but block viral replication at other stages of the viral life cycle (Weiss, 1982).

The steps required for transport of the viral nucleic acid from the cell surface to the nucleus are not clear. There is very little information about the structure of the virion after penetration and the onset of cDNA synthesis. Varmus et al. (1974) suggest that viral DNA synthesis commences in the cytoplasm 3 hours after infection. Since deoxyribonucleotide triphosphate (dNTP) concentrations are low in the cytoplasm, one would expect DNA synthesis to occur at a slow rate. Their data shows that ^{32}P-labeled dNTPs begin to be incorporated into viral DNA in the cytoplasm after 3 hours and does not arrive in nucleus till 10 hours post-infection. This data along with the fact that viral DNA can be made in enucleated cells is strong evidence that DNA synthesis does indeed occur in the cytoplasm. Indirect evidence has also accumulated that suggests that uncoating of the core may begin in the cytoplasm as early as two hours after infection when assayed by reverse transcriptase (R.T.) activity in pelleted cell lysates (Aboud et al., 1979). They suggest that in these experiments, uncoating of the viral particles in the cytoplasm of the infected cells can be followed by the disappearance of R.T. activity.

Since the rate of viral DNA synthesis is so slow (30 nucleotides per minute), the DNA-RNA hybrid molecule must somehow be protected from nucleases to allow for a full length genomic DNA molecule to be made (Varmus et al., 1978). One explanation is that after viral attachment, the core is released into the cytoplasm. Here the core not only protects the viral RNA but also harbors reverse transcriptase in

its active form. Once dNTPs appear in the core, reverse transcription begins. It would seem plausible that the core also serves as a shuttle for the genetic material from the cytoplasmic membrane to the nucleus. In fact many inestigators have shown that certain mouse cells can restrict the appearance of MLV cDNA in the nucleus by the action of the *Fv-1* allele (Jolicoeur and Baltimore, 1976a and 1976b; Sveda and Soeiro, 1976; Rassart and Jolicoeur, 1980 and Krontinis, Soeiro, and Feilds, 1973: Boone et al., 1983; and Des Groseillers and Jolicoeur, 1983). It was further shown by nucleotide sequencing of revertant viruses, which had overcome the effects of the *Fv-1* allele, that the only changes that had occured were single amino acid changes at positions 109 and 159 in p30 (Ou et al., 1983). These results suggest that the capsid protein is important in shuttling the genetic information to the nucleus.

Viral DNA Synthesis

Once all the appropriate factors for cDNA synthesis have accumulated in the core, reverse transcription begins. The first molecule needed by reverse transcriptase besides the RNA template is $tRNA^{trp}$. Biochemical studies have shown that the $tRNA^{trp}$ serves as the primer for reverse transcriptase by annealing the 3′ end of the tRNA molecule to the PB- region on the viral RNA just down stream from the U_5 region. (Cordell et al., 1976 and 1978). DNA synthesis begins at the U_5 region and proceeds through the U_5 and R regions to the 5′ terminus creating a "strong-stop" DNA-RNA molecule. The RNAse H activity of reverse transcriptase is then thought to remove the 5′ R region of the viral RNA leaving a "sticky" R region overhang (Varmus, 1977; Myers et al.,

1980; and Olsen and Watson, 1980). The overhang can now base pair with the 3′ R region of the second viral RNA or to the original template RNA. In any case, reverse transcriptase can use the strong-stop DNA as a primer for continued DNA synthesis through the 3′ R and U_3 regions then through the structural genes. The transcriptase again reads through the U_5 and R creating another genomic length strong-stop DNA. RNAse H again digests away one strand of the 5′ R allowing the 5′ R overhang to base pair with the original strand's 3′ R. This allows for 3′ R and U_3 again to be transcribed into the new cDNA. RNAse H then digests the RNA from the DNA-RNA hybrid molecule (for review see Varmus and Swantrom, 1982). The question how the second plus sensed DNA is synthesized still remains a mystery (Varmus and Swanstrom, 1984). It has been suggested that RNAse H might cleave the viral RNA into fragments that can be used as primers by the host DNA polymerase for the second DNA strand synthesis (Leis et al., 1973). In any case, the resulting complementary DNA (cDNA) has the following genomic structure depicted in Figure 3.

After the double-stranded linear cDNA molecule has been made, it circularizes to form a circle junction between the two LTRs. Whether or not this event actually occurs inside the viral core or the nucleus has not been determined (Bishop, 1978). Once the circular DNA transverses the nuclear membrane, it may integrate into the host chromosome (Varmus et al., 1973b and 1976; Khoury and Hanafusul, 1976; and Varmus and Swanstrom, 1984) by means not fully understood. It seems that the two LTRs are essential along with the endonuclease activity of reverse transcriptase for correct integration (Panganiban and Temin, 1984; Donehover and Varmus, 1984). The sites for integration seem to be

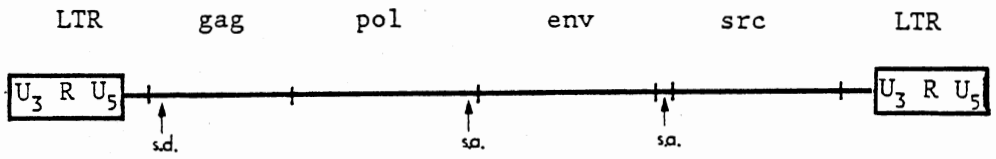

Figure 3. Rous sarcoma virus proviral structure. The splice donor (s.d.), and splice acceptor (s.a.), required to make the different mRNAs for each gene are listed. The *gag* and *pol* genes are transcribed as a single message.

some what random (Hughes et al., 1978). However, whether or not the integrated provirus can induce tumorogenesis or effeciently express its own gene products semms to be, in part, determined by the site of integration (Varmus and Swanstrom, 1984). Many investigators have also tried to implicate DNA methylation, chromatin configuration, and chromosomal position with this efficiency (Groffer et al., 1983; Groudine et al., 1981; Chriswell et al., 1982; Jaenisch et al., 1983; and Katz et al., 1983).

Retroviral Expression

After the provirus has integrated, viral RNA can be synthesized. However, there is no clear evidence to suggest that integration is an absolute requirement for transcription of viral RNA.

Synthesis of Viral mRNA.

Viral mRNA synthesis is not only dependent upon the presence of host RNA polymerase II but upon also functional promoters and enhancers. Numerous studies on retroviral promoters have mapped the promoters to the LTRs and more specifically to U_3. Sequence and genetic analysis has confirmed the presence of a conserved "TATA" box 24-30 b.p. before the "cap" site in RSV (Yamamoto et al., 1980; Ostrowski et al., 1981; and Fuhrman et al., 1981). Deletion of this TATA sequence dramatically reduces transcriptional initiation both *in vivo* and *in vitro* (Mitsialis et al., 1983b). Mitsialis et al. (1983a) has demonstrated the importance of the enhancer elements by demonstrating that the removal of the probable enhancer sequence (position -114 to -299) markedly decreases but not abolishes the ability of the promoter to initiate transcription *in vivo*. This region also is dispensible for transcription initiation *in vitro*. Luciw (1983) determined that the enhancer element probably lies at least 140 b.p. upstream from the transcription initiation site (Varmus and Swanstrom, 1984). Arrigo et al. (1987) have also identified another enhancer-like sequence within the p10 coding region. This enhancer is unique in that it seems to have orientation polarity. The biological significance of this enhancer is not known since the enhancer elements that drive transcription of the viral RNAs are located in the LTRs.

Examination of the mature viral specific mRNAs found in the cytoplasm of RSV infected permissive cells reveals three major classes of mRNA. The largest species has a sedimentation coefficient of 38S

and is made up of approximately 10,000 nucleotides with a molecular weight of 31.3 x 10^6. The second largest species, 28S, has a molecular weight of 1.8 x 10^6 (5400 nucleotides) while the smallest, 21S, has a molecular weight of 1.2 x 10^6 and is made up of approximately 3600 nucleotides (Bishop, 1978). Both Weiss et al. (1977) and Howard et al. (1977) used stringent DNA-RNA hybridization techniques to reveal the probable function of each of the three classes of RNA. They probed with several cDNAs that corresponded to either; (a) the 3′ terminus, (b) specific internal sequences (*gag, pol, env,* or *src*), and (c) the 5′ terminus. They found that all three species annealed with the 3′ terminal probe (see Fig. 4). The largest 38S species annealed with all the probes suggesting that this RNA was a full-length genomic copy. The 28S RNA annealed with the 5′, *env*, *src*, and 3′ probes. The 21S RNA had similar annealing patterns but did not anneal to the *env* probe. These results strongly suggest that all three species of viral RNA must originate at the 5′ region of the viral genome and that some kind of RNA splicing event must take place within the genomic length mRNA to give rise to the RNA species that can translate the 5′ terminal sequence. These observations are consistent with the fact that eukaryotic ribosomes can only translate the 5′terminal open-reading frame given the correct ribosome binding sites and correct initiation codon. Direct sequence analysis of genomic cDNA has revealed the correct consensus sequences required for RNA splicing (Swartz et al., 1983). The splice donor and splice acceptor sites are also consistent with the size and sequence of the smaller classes of mRNAs. The only viral sequence that is expressed without being positioned at the 5′

end of a subgenomic viral RNA is the *pol* coding sequence. The mechanism used to synthesize reverse transcriptase will be described later.

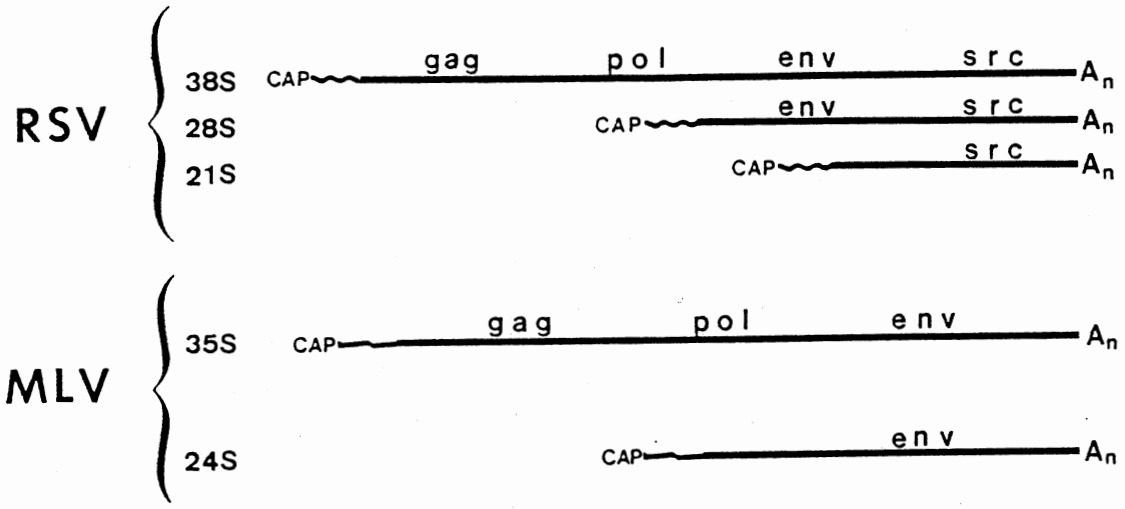

Figure 4. The major spliced mRNAs from Rous sarcoma virus and murine leukemia virus. The wavy lines at the 5′ termini represent the common nucleotide sequences present in the 28S or the 35S transcript are found in the smaller transcripts as a result of splicing (see Fig. 2 for the actual splice donor and acceptor sites). A_n represents the poly A tail and CAP represents the 5′ capped structures which are present on each transcript.

Structural examination of many retroviral RNAs have revealed that all the genomic and subgenomic RNAs have methylated 5′ caps as well as 3′ poly-A tails (Bishop, 1978). DNA sequence analysis has revealed a sequence that is commonly referred to as a polyadenylation signal (AAUAAA) 10-20 nucleotides 5′ of the poly-A tail. As described by Varmus and Swanstrom (1982), *in vitro* translation studies of each of the three RNA classes has confirmed the predicted products. The 38S RNA can probably be used for either progeny viral RNA or mRNA. The decision whether or not the RNA is to be used for mRNA is not understood. This 38S mRNA is translated into two large polypeptides in

RSV. The larger peptide has a molecular weight of 180 kDa and is referred to as Pr $180^{gag\text{-}pol}$. The smaller peptide has a molecular weight of 76 kDa and is referred to as $Pr76^{gag}$. The prefix "Pr" refers to the "precursor" polypeptide. As will be described later, both of these large polypeptides will undergo proteolytic processing to form the active viral proteins. The 28S RNA serves as the message for $Pr90^{env}$ and is usually found associated with polyribosomes bound to the rough endoplasmic reticulum. The samller 21S RNA gives rise to $pp60^{src}$ (or phosphprotein). $Pr180^{gag\text{-}pol}$, Pr 76^{gag}, and $pp60^{src}$ are found associated with free cytoplasmic polyribosomes.

Other type-C retrovirus transcription strategies examined to date are generally the same. Other retroviruses such as HIV and HTLV I have very different transriptions stratagies in order to regulate viral gene expression (Weiss, 1985) and will not be described here. Murine leukemia virus (MLV) infected permissive cells transcribe two major RNA classes with sedimentation coefficients of 35S and 22S-24S. The 22S-24S RNA is mainly found associated with membrane-bound polyribosomes and codes for a protein precursor for the *env* glycoprotein. The larger 35S RNA is found associated with free polyribosomes and has been shown to code for the precursor products of *gag* and *pol*. It has also been shown that the 5′ termini has sequence homology with the 5′ end of *gag* suggesting a splicing event occurs to give rise to the 22S-24S RNA.

Synthesis of Viral Proteins

As mentioned earlier, retroviruses not only require the RNA splicing capabilities of their host for expressing a relatively large array of virus-specific proteins from a limited information source, but, they also process large polyprotein precursors into smaller active proteins. By using both of these strategies, simple retroviruses can ultimately produce 8-9 or proteins from their open reading frames. Other retroviruses such as the human retroviruses HTLV I, HTLV II, and HIV, the feline leukemia virus (FeLV), and Bovine leukemia virus (BLV), may produce many more virus-specific proteins from alternative coding sequences generated from multiple splicing events, But the theme for expressing the major structural proteins from two or three large transcripts is generally the same. Therefore the following review of retroviral translational products will be limited to the more simple prototype viruses; RSV and MLV both C-type particles, and MMTV, a B-type particle.

Synthesis of the *Group-associated AntiGens (gag)*. The primary translated product of the full genomic-length 38S mRNA is a polyprotein precursor (Pr76 in RSV, Pr65 in MLV, and Pr77 in MMTV). This precursor protein undergoes proteolytic cleavage into four or five polypeptides that are associated with the viral core. The final cleaved *gag* products are listed in Table 1. Of the three retroviruses listed, RSV is the only virus which synthesis its protease as part of the *gag* polyprotein precursor. This protease is found as part of the *gag-pol* fusion peptide in the murine viruses (Dickson et al, 1985).

TABLE I

AVIAN AND MURINE VIRION PROTEINS
AND THEIR POLYPROTEIN PRECUSORS

Virus	Precursor Polyprotein	Lipid-binding Protein	Genome-binding Protein	Capsid Protein	Nucleoprotein	Protease
RSV	Pr76	p19	*p10	p27	p12	p15
MLV	Pr65	p15	pp12	p30	p10	---**
MMTV	Pr77	p10	pp21	p28	p14	---**

* p10 has only recently been identified and its function has not yet been elucidated (Dickson et al, 1982).

** The murine viruses do harbor a viral encoded protease but it is not synthesized as part of the gag polyprotein precursor.

In RSV, p19 is located at the amino-terminus of Pr76. In its cleaved form p19 is found associated with the viral envelope. It has also been reported that p19 exists in both a phosphorylated or unmodified form. It is not clear yet what the important functions of the phosphorylations are (Erikson et al., 1977).

It was at one time suggested that p19 also had a high specificity for binding to viral RNA (Darlix and Spahr, 1982). It was thought since p19 associated with both the viral RNA and viral envelope, that p19 played some important role in RNA packaging and RNA splicing (Sen and Todaro, 1977; Leis et al., 1978, 1980). It has since been determined that p12 and not p19 that binds to RSV RNA (Meric et al., 1984).

Schlesinger et al. (1976) and Montelaro et al. (1978) propose that the cytoplasmic tail of gp37 associates with p19 in some manner in order to align Pr76 in the right orientation at the site of budding. In fact, Gebhardt et al. (1984) were able to chemically cross-link p19 and gp37 with dithiobispropionimidate (DTBP). The equivalent proteins in MLV, FeLV (feline leukemia virus), BLV (Bovine leukemia virus), and the D-type MPMV, are also found associated with the viral envelope. There is a major difference however between the mammalian and avian lipid-binding proteins. In the mammalian system, this protein is myristylated near the amino-terminus (Henderson et al., 1983; Dickson et al., 1982). This modification must be important to the mammalian system since it is conserved among the mammalian retroviruses. The avian retroviral proteins on the other hand are not myristylated but rather acylated with an acetyl group (Palmer et al.,

1978). It has been proposed that these post-translational modifications might be important in targeting the precursor polypeptide to the site of viral assembly (J. Wills, pers. comm.).

The second protein of Pr76, carboxy proximal to p19, is p10. This peptide has only recently been identified (Pepinski and Vogt, 1983 and Hunter et al., 1983). It has been shown that this protein is soluble in methanol and trichloroacetic acid, labels poorly with the commonly used radioactive amino acids, and migrates with peculiar mobilities in SDS-gels. Therefore this protein escaped detection in SDS-polyacrylamide gels. The presence of the p10 coding sequence in *gag* was identified after DNA sequence analysis (Schwartz et al., 1983). This protein was later found between the viral envelope and the nucleocapsid and determined to comprise 7% of the total protein of the virion (Pepinsky and Vogt, 1983). The function of p10 is not known but amino acid sequence analysis exhibits a high degree of similarity with p12 of MLV.

The major capsid protein in RSV is p27. Biochemical data has shown that p27 can form multimers when chemically cross-linked with dimethyl suberimidate (DMS) or dithiobispropionimidate (DTBP) suggesting that the monomers are in close association with one another to possibly for the viral core (Pepinsky et al., 1980). Burnette et al. (1976) demonstrated that the MLV equivalent, p30, can self associate in solution. These observations are consistent with the idea that these proteins can associate with one another to form the viral core.

The major polypeptide of the ribonucleoprotein complex is p12 in RSV, p10 in MLV, and p14 in MMTV (Meric et al., 1984; Bolognesi et al., 1973; Schulein et al., 1978; Nermut et al., 1972; Fleissner and

Tress, 1973; Arthur et al., 1978b; Nusse et al., 1980; and Dickson et al., 1982). These proteins are highly basic and do not seem to have a higher affinity for single-stranded RNA over double-stranded RNA (Dickson et al., 1982).

This RNA-binding protein is the carboxy terminal protein of the precursor polypeptide in the murine systems. The avian retroviruses differ in that there is one more peptide carboxy-proximal to the RNA-binding protein. This protein is known as p15. By a series of *in vivo* and *in vitro* techniques, Vogt et al. (1979) was able to demonstrate that p15 was a protease. They also demonstrated that this protease specifically cleaved Pr76 into the five structural proteins seen in the mature virion. Von Der Helm (1980) also demonstrated that p15 had protease activity by showing that Pr76 could be correctly processed in nonpermissive cell lines (rat TWERC and hampster RBH cells) by microinjecting purified p15. The location of p15 in the virion was determined by Pepinsky et al. (1980). They were able to show by chemical crosslinking that p15 is located between the nucleocapsid and the viral envelope.

No equivalent protease is found in the murine *gag* polypeptide. This activity however is found as part of the *gag-pol* fusion polyprotein and is encoded by the *pol* sequence. This protease has similar specificity for cleavage of the $Pr65^{gag}$ polypeptide in MLV (Levin et al., 1984; Dickson et al., 1984).

Synthesis of Reverse Transcriptase. In RSV virions, active reverse transcriptase is isolated as a two-subunit enzyme: the alpha subunit has a molecular weight of 58 kDa while the beta subunit has a

molecular weight of 92 kDa (Dickson et al., 1982). Both subunits seem to have the RNA-dependent DNA polymerase activity as well as the indispensable RNAse H activity (Verma, 1977). The alpha subunit has been demonstrated by numerous investigators to be derived by proteolytic processing of the beta subunit in the maturing virion (Dickson et al., 1982). It seems that the alpha subunit is derived from the carboxy-terminus of the beta subunit. The endonuclease activity which is required for integration of the cDNA into the host genome, is found at the carboxy terminus of reverse transcriptase. This endonuclease, p32, cleaves the circular double-stranded cDNA prior to the integration step (Varmus and Swanstrom, 1982). It seems reasonable to envision that this endonuclease is also important in the actual process of integration. Dickson et al., (1982) suggest that two beta subunits complex together and that one of these is a precursor to the alpha and p32 subunits in the mature virion. Fractionation studies on purified virions show that the active transcriptase co-purifies with the viral RNA (Stromberg et al., 1974). Panet et al. (1975b) demonstated that reverse transcriptase specifically binds the $tRNA^{trp}$ primer and therefore might possibly be tightly associated with the viral RNA. It has also been shown that the reverse transcriptase selects the correct tRNA and might possibly be responsible, although indirectly, for the correct packaging of viral RNA (Levin and Seidman, 1979; Sawyer and Hanafusa, 1979; Peters and Hu, 1980, Dickson et al., 1082). It is also interesting to note that the sequence required for packaging of the viral RNA (L region) is located directly adjacent to the tRNA binding site (PB-) in RSV.

Reverse transcriptase is first synthesized as part of a *gag-pol* fusion protein (*ie.*, $Pr180^{gag\text{-}pol}$ in RSV) in all retroviruses examined to date. The first direct evidence that Pr180 was the precursor product to reverse transcriptase was obtained by immunoprecipitation experiments using anti-reverse transcriptase antiserum (Oppermann et al., 1977; Hayman, 1978; and Retteniner et al., 1979). These experiments also demonstrated that *gag* specific amino acid domains were present by using antisera raised against purified *gag* proteins. However, the levels of $Pr180^{gag\text{-}pol}$ in infected cells are 20 to 50 fold lower then $Pr76^{gag}$. This is not surprising considering the possible mechanism by which the *gag-pol* fusion protein is made (Stromberg et al., 1974; Panet et al.,1975a). Schwartz et al. (1983) identified, by sequence analysis, two open reading frames, one coding for Pr76 and a second coding for the presumptive reverse transcriptase. The sequence analysis actually generated more questions than it answered. A perplexing question was inspired when it was demonstrated that the two coding regions were actually in different reading frames with *pol* being in the -1 reading frame with respect to *gag*. This data also showed that the two reading frames overlapped by 58 nucleotides with respect to the *gag* amber codon. However, according to the amino acid sequences of the mature reverse transcriptase and Pr76, the two reading frames should be separated by 20 nucleotides on the RNA. The first and most obvious explanation was that the precursor peptide was generated from a spliced RNA that had the intervening sequences removed. Further detailed study of the nucleotide sequence did not reveal the splice donor and splice acceptor consensus sequences (see Fig. 5). Therefore, it was suggested that there must be some minor

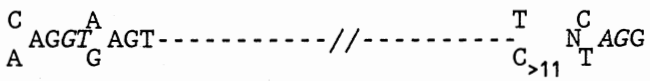

Figure 5. Consensus sequence for known splice donor and splice acceptor sites. The invariant GT and AG are indicated in italics (Mount, 1982).

sequence that is involved in the RNA splicing activites.

Because of the results presented by Jacks and Varmus (1985), the most excepted idea is that there is an occasional ribosomal frameshift during translation that allows the ribosome to fall back into the -1 reading frame and begin translation of reverse transcriptase. They demonstrated with well defined *in vitro* transcription and translation systems that the *gag-pol* fusion protein in RSV was translated via ribosomal frameshifting events. By deletion analysis, they identified a stretch of nucleotides near the 3′ end of the *gag* coding sequence that was important in this event (see Fig. 6).

Jacks et al., (1987) also showed that MMTV translated its *gag-pol* fusion protein by a similar event. This virus is a little bit

-gag
ValAlaMetValArgGlySerIleLeuGlyArgAspCysLeuGlnGlyLeuGlyLeuArgLeu
GUAGCUAUGGUUAGAGGGAGUAUCCUAGGAAGAGAUUGUCUGCAGGGCCUAGGGCUCCGCUUG
***ArgGluTryProArgLysArgLeuSerAlaGlyProArgAlaProLeuA

ThrAsnLeu***
ACAAAUUUAUAGGGAGGGCCA
spLysPheIleGlyArgAla------ *pol*

Figure 6. Diagram of the 58 nucleotide overlap between the two open reading frames for the *gag* and *pol* proteins and the amino acid sequence encoded by the 3′ end of *gag* (upper amino acid sequence) and the 5′ end of *pol* (lower amino acid sequence). The underlined nucleotides indicate were the frameshifting signals are located.

different in that it requires two ribosomal frameshifts to generate the fusion protein. Detailed sequence analysis has identified a potentially important consensus sequence, A AAA AAC, that is found in MMTV, BLV, and HTLV-2. They go on to suggest that the sequence U UUA near the amber codon of *gag* in RSV is the probable frameshifting signal. They also propose that the context in which this sequence lies is very important in that a stem-loop structure must be present downstream in order to stimulate the ribosomal frameshift. The idea of a ribosomal frameshift is further supported by Moore et al., (1987).

Synthesis of the Viral Glycoproteins. The product of the spliced 28S mRNA in RSV is the precursor polypeptide, $Pr95^{env}$ (see Table 1 for analogous proteins in MLV and MMTV). Unlike $Pr76^{gag}$ and $Pr180^{gag\text{-}pol}$, $Pr95^{env}$ is not synthesized on free polyribosomes but rather on the rough endoplasmic reticulum (RER). $Pr95^{env}$ is similar to many other host polypeptides synthesized on the RER in that these peptides are modified by glycosylation and other post-translational modifications. Most of these glycosylated proteins are destined for the cell surface and common among them are the presence of a very hydophobic stretch of amino acids at the amino terminal end of the growing peptide chain. This hydrophobic region is thought to interact with the membrane of the RER.

It has been hypothesized that the amino terminal sequence of Pr95 harbors a recognition signal for a host "signal recognition particle (or SRP). This SRP not only arrests protein synthesis in the cell cytoplasm (Lipp et al., 1987), but it is also recognized by SRP binding proteins on the endoplasmic reticulum. Also present on the

RER are ribosome binding proteins. Through a complicated series of events not fully understood, the ribosome becomes bound to the RER and the leader peptide is translocated across the membrane into the cisterna of the ER. Present in the cisterna are signal peptidases which cleave the signal peptide and recycles the host SRPs allowing protein synthesis to ensue. Glycosylation of the growing peptide results from specific signals on the viral peptide and host glycosylation enzymes (Sefton, 1976; Dickson et al, 1982). For a more intensive review see Hirschberg and Snider (1987).

Both *in vitro* translation and microinjection studies have shown that the 28S mRNA specifically encodes the retroviral *env* product. *In vitro* translation systems using purified 28S mRNA result in a polypeptide of approximately 70 kDa (Pawson et al., 1977). *In vivo* studies using the glycosylation inhibitor tunicamycin also result in an unglycosylated protein of approximately 70 kDa (Shapiro et al., 1976; Moelling et al., 1977; Bishop, 1978). Stacey et al. (1977) also demonstrated that the 28S mRNA encodes the viral gylcoprotein by microinjecting the 28S mRNA into cells infected with an RSV *env* deletion mutant and showing that the RNA could complement the defective virus.

Addition of the high manose oligosaccharide takes place as the nascent polypeptide translocates into the endoplasmic reticulum and translation continues. After the high mannose precursor is transported to the Golgi apparatus where the complex oligosaccharides are added, $Pr95^{env}$ is cleaved by unknown host proteases to generated gp85 and gp37 (Wills et al., 1984). The *env* precursor in MLV $Pr90^{env}$, is similarly processed into gp70 and p15E and $Pr73^{env}$ in MMTV into gp52

and gp36. DNA sequence analysis has predicted the presence of a very hydrophobic region near the carboxy terminus of gp37. This hydrophobic stretch of amino acids serves as a membrane anchoring domain. Thus, a short peptide tail projects into the cytoplasm of the host cell. The function of this tail will be discussed later. The larger processed glycoprotein (gp85) makes up the body of the surface spikes on the mature virion and is covalently linked to the membrane anchoring protein by disulfide bonds (see Fig. 2). In both the avian and murine retroviruses, the smaller peptide can be removed after treatment with reducing agents such as beta-mercaptoethanol (Leamnson and Halpern, 1976; Dickson et al., 1982). The spike complex in its mature form is capable of recognizing and attaching to specific host surface receptors not yet identified (Dickson et al., 1982 and 1984).

RSV Oncogenes and Other Phosphorylated Proteins. The third major message found only in RSV (as described earlier) codes for a phosphoprotein, $pp60^{src}$. This protein, termed v*src* (viral sarcoma), has also been called the transforming protein because of its involvement in transforming infected cells into a neoplastic state. Since this protein is not found in the RSV virion nor involved in retrovirus replication, assembly, or maturation, it will not be discussed in further detail (for a detailed review, see J.M. Bishop and H. Varmus, 1982 and 1984).

Two other viral proteins are also phosphorylated. It is generally believed that this type of post-translational modification is important in biological regulation. Both p19 of RSV and p12 of MLV are phosphorylated (or pp19 and pp12) and are found associated with

the viral membrane. The second viral protein that is similarly modified is reverse transcriptase (Lee et al., 1975; Hizi et al., 1977). The reasons for these modifications are not entirely known. Lee et al. (1975) has suggested that phosphorylation is required for the enzymatic activity of reverse transcriptase. This has not been proven but it seems possible that this modification would be required to create the correct three dimensional stucture needed for the enzymatic activity. The reason for modifying p12 and p19 of MLV and RSV respectively is not known at this time. However, since Erikson et al. (1977) demonstrated that p19 can be modified while still in the precursor form (Pr76). This result suggests that phosphorylation somehow influences the processing of Pr76.

Other post-translational modifications of viral proteins seem to have some effect on the viral life cycle. Most noted of these are the virally induced cell-surface glycoproteins unrelated to *env*. This phenomenon was first described by Tung et al. (1976) while studying leukemic cells from AKR-strain mice infected with MLV. Friend strain murine leukemia virus (FeMLV) infected erythrocytes (Evans et al., 1977) and Moloney murine leukemia virus (MoMLV) infected fibroblasts (Evans and Fan, 1979) also harbor glycosylated cell-surface proteins which are induced by MLV infection.

By using a variety of *in vivo* labeling techniques, these cell surface antigens were identified as glycosylated *gag* proteins (Tung et al., 1976; Ledbetter et al., 1977; Buetti and Diggelmann, 1980b; Dickson et al., 1982). Immunoprecipitation studies have identified that these surface proteins exists as two species with molecular weights of 95 and 85 kDa. The results also suggest that gp85 is derived from

gp95 after proteolytic removal of $p10^{gag}$ (Ledbetter et al., 1978; Tung and Fleisnner, 1980; Dickson et al., 1982). Edwards and Fan (1980) demonstrated that gp95 is synthesized independently of $Pr65^{gag}$ from a precursor protein of 80 kDa, $gPr80^{gag}$.

These glycosylated *gag* proteins do not appear in the viral particles but are present on the cell surface from which the virions were released (Aoki et al., 1972). It is unlikely that these glycoproteins are just an artifact since they are present on virtually all MLV infected cells and exhibit an affinity for extracellular matrices (Fan et al., 1983). This affinity for these matrices may be a result of the extra amino acids found on the amino-terminus of gPr80. These extra residues probably also carry the signals required for glycosylation (Edwards and Fan, 1980; Dickson et al., 1984). Edwards and Fan (1981) went on to further isolate several mutant strains of MLV that are deficient in the production of the surface glycoproteins. Most but not all were also defective for viral production. They hypothesized that these proteins are therefore important in the virus life cycle. Other evidence suggests that the glycoproteins are probably not important. Swartzberg et al. (1983) and Fan et al. (1983 independently isolated mutants of MLV that are incapable of expressing these surface proteins but are capable of virus production. These mutants are also still capable of transforming the infected host. So, it is still not clear what function, if any, these surface glycoproteins play in the retroviral life cycle.

CHAPTER II

VIRION ASSEMBLY AND MATURATION

Since Rous' famous discovery of a transmittable entity responsible for tumor formation in chickens, many virologists of been diligently searching for analogous viruses involved in malignancies in mammals, especially humans. There are countless examples of retroviruses responsible for neoplasms in all mammals studied to date. But it is surprising to note there are only three known retroviruses that are capable of infecting the human population. Two of these are responsible for infectious leukemias, HTLV I and HTLV II, while the third (HIV), causes extensive cell death of the T_4 helper cell population of the immune system.

In spite of the importance of these viruses as human pathogens and the need to understand their replication cycles, it is surprising that very little is known about the process of particle assembly and maturation. What little we do know is based on electron microscopy and gross analysis of replication defective mutants, mostly in MLV. The following discussion will review these mutants as well as describe the current working hypotheses of viral assembly and maturation.

Theories on Assembly and Maturation

The enormous size alone of the two volumes of the "RNA Tumor

Viruses: Molecular Biology of Tumor Viruses" text is evidence enough of the colossal amount of information that has accumulated concerning retrovirology. The two texts combine over 2,500 pages of valuable information. In contrast, what is known about particle assembly and maturation can be summed up in four pages (p 528, and 556-558 of Volume 1).

As mentioned in Chapter 1, the outer membrane of the virion is derived from the host cell surface membrane and is studded with numerous glycoprotein projections or spikes. The proteins are synthesized and processed in the rough endoplasmic reticulum and then are transported to the cell surface where they are incorporated into the viral envelope at the exclusion of all other host surface proteins. The exact mechanism for the specificity of viral glycoproteins is not entirely known but it is generally thought that the cytoplasmic tail of the glycoprotein interacts with *gag* precursor polypeptide or immature nucleocapsid. This proposed interaction does not seem essential for viral budding however because particles can be generated (although noninfectious) in the absence of the viral glycoproteins (Dickson et al., 1982).

Many other investigators have also demonstrated that budding can occur in the absence of reverse transcriptase (Hanafusa et al., 1972; Eisenman et al., 1980; Dickson et al., 1982) and genomic viral RNA (Levin et al., 1974: Linial et al., 1978). However budding can be arrested by deleting the *gag* sequences or mutating them in such a way that Pr76 can not be processed. Thus, Dickson coined the term

"particle-making machine" since it is obvious that $Pr76^{gag}$ is the major and perhaps the only viral peptide required for budding (Dickson et al., 1982).

With the above data in mind, many (Bolognesi et al., 1978; Bishop, 1978; and Dickson et al., 1982) have suggested the following model for type C particle assembly and maturation: (1) The viral *env* precursor polypeptides are synthesized on the endoplasmic reticulum so glycosylation can take place. After this modification, the glycoproteins are transported to the cell surface so they can be incorporated into the budding virion. The kinetics of processing the *env* precursor have shown $Pr95^{env}$ to be overproduced and only about 1% of the total synthesized product is actually incorporated into the virion (Bosch and Schwartz, 1984). The timing of $Pr95^{env}$ processing *in vivo* is not really known. (2) The *gag* precursor polypeptide is synthesized on cytoplasmic ribosomes and then makes its way to the site of budding, by unknown mechanisms. Here the precursor products aggregate with the amino-terminus associated with the cytoplasmic membrane and the carboxy terminus associated via p12 (in RSV), with the viral genomic RNA. Because these proteins are aggregating, the immature particle begins to bud from the cytoplasmic membrane. (3) Also included in the aggregation is $Pr180^{gag\text{-}pol}$ with the associated $tRNA^{trp}$ molecules (Panet et al., 1975b). (4) Once the immature virus buds from the membrane, the precursor polypeptides, $Pr76^{gag}$ and $Pr180^{gag\text{-}pol}$, undergo proteolytic processing. It is believed that the condensing of the nucleocapsid (or viral maturation) is a result of this processing .

This simple model of assembly and maturation is easy to envision but much more difficult to prove. There is strong evidence that $Pr76^{gag}$ is the only peptide required for budding. Te evidence is only indirect since no one as yet has been able to express Pr76 by itsself and demonstrate viral budding. Two important questions have yet to be answered. Are viral maturation and precursor processing actually the same event? And, how is the *gag* precursor targeted to the site of assembly? This site might be the cytoplasmic membrane or some intercytoplasmic location as is the case with type B and type D retroviruses (Yoshinaka and Luftig, 1982).

The Crucial Peptide for Budding.

Bister et al. (1977) reported the first evidence that implicates Pr76 in budding in the avian retroviruses. They demonstrated that a nonproducing cell line transformed with avian myelocytomatosis virus produced a virus specific peptide of 110 kDa. This peptide was shown to be similar to $Pr76^{gag}$ in that it was immunoprecipitated with an anti-p27 antiserum. Further peptide analysis revealed that this cell line did not make Pr76 nor any of the processed *gag* proteins (p19, p10, p27, p12, p15). These observations along with the fact that this defective virus was capable of being rescued by Rous associated virus type 1 suggests that the defect in virus production was due to a lesion in *gag*. Haymon et al. (1979) reported similar data on a nonproducing cell line transformed with avian erythroblastosis.

The idea that $Pr65^{gag}$ in MLV or $Pr76^{gag}$ in RSV is the only peptide required for budding is strengthened by studies using temperature-sensitive (ts) replication mutants of MLV and RSV. Mason et al.

(1979) described the isolation of three ts mutants whose lesions map to the *gag* gene. These mutants were identified by their reduced yields of labeled virions at the non-permissive temperatures. Pulse-chase experiments revealed these mutants were delayed in Pr76 processing.

It is difficult, however, to find in the literature well characterized mutations in which the lesions fall in the *gag* gene since most of the mutations are lethal. Goff and Lobel (1987) describe many spontaneous murine leukemia virus mutants and their mutational effects. However, its dramatic to note the few number of mutants isolated that have an alteration in the *gag* coding region. This is again indirect evidence that the *gag* peptide is solely responsible for budding and virion production. What obviously needs to be done, is to express $Pr76^{gag}$ or $Pr65^{gag}$ by itself and determine if viral particles are released.

Precursor Processing and Viral Maturation.

There is substantial evidence that *gag* precursor processing and viral maturation (see p.3) are closely linked (Vogt et al., 1975; Stephonson et al., 1975; Van de Ven et al., 1978; Lu et al., 1979; Mooren et al., 1980; Yoshinaka et al., 1980; Manly et al., 1981; Durbin et al., 1984; Katoh et al., 1985; and Katsumoto et al., 1987). It is clear that viral particles can not mature without coexistent processing of the *gag* precursor polyprotein. But it is not known whether cleavage of *gag*, *pol*, and *env* polyproteins immediately precedes or accompanies viral budding or maturation (Dickson et al., 1982). If cleavage is required for maturation, and is dependent upon

the viral protease (Dickson et al., 1982), what activates the protease? As will be described later, one of the theories to explain mammalian host restriction of avian retroviruses is that the mammalian cells lack the required host protease that activates the viral protease (Dickson et al., 1982). Others have speculated that the *gag* precursors must associate in some way at the site of assembly to stimulate budding and maturation (Pepinsky et al., 1980; Dickson et al., 1982).

Targeting of the *gag* Precursor Polypeptide.

Although retroviruses require processing of the *gag* precursor for maturation, viral assembly can still occur in the absence of processing (Gazdar et al., 1971; Pinter and deHarven, 1979; and Edbauer and Naso, 1984). Thus, the mature forms of the viral core proteins are not required for assembly. This is in line with the idea that the *gag* precursor polypeptide must be targeted to the site of assembly were it interacts with other precursors to stimulate budding. With this in mind, many investigators have attempted to find the mechanism of targeting.

Edbauer and Naso (1983) reported that $Pr65^{gag}$ of MuMLV binds rapidly and specifically to cytoskeletal elements in infected cells. They went on to demonstrate that a temperature sensitive (*ts*) mutant of MLV had increased amounts of $Pr65^{gag}$ associated with the cytoskeletal elements. They suggested that, "the majority of $Pr65^{gag}$ molecules in a cell exist in the membrane-bound form via p15 and these peptides

are direct precursors to the mature viral proteins." The cytoskeletal-associated proteins are then probably required for correct targeting of $Pr65^{gag}$ to the cell surface.

Other evidence has accumulated which shows MLV and probably all mammalian retroviruses to be myristylated at the amino terminal glycine of $Pr65^{gag}$ (Henderson et al., 1983). Rein et al. (1986) demonstrated by using site directed mutagenesis that this acylation of MuMLV is required for the association of the *gag* protein with the plasma membrane and that this association is required for viral assembly. On the other hand, all avian C-type retroviruses but one lack this myristic acid modification but are acylated with an acetyl group (Palmiter et al., 1979). Avian reticuloendotheliosis virus has been shown to be myristylated and it is known that this particular avian retrovirus can replicate in mammalian cells (Schultz and Oroszlan, 1983; Weiss, 1982). Therefore it is easy to speculate that myristylation must be important in targeting $Pr76^{gag}$ to the plasma membrane since other lipid-associated proteins can be similarly acetylated (Dickson et al., 1985).

Another interesting experiment to note was performed by Bosch and Schwartz (1984). They used an inhibitor of glycoprotein transport (monensin) to study the processing of $Pr95^{env}$. A startling result was reported with monensin treated infected cells. In these cells with arrested glycoprotein processing and targeting, viral particles were seen budding into intercellular vesicles. They suggest that targeting of the core precursors to the plasma membrane might result from some unidentified host protein.

Phosphorylated *gag* Polyproteins.

As mentioned in Chapter I, two types of post-translational modifications of the *gag* precursor have been observed but the functions of the modified proteins remain obscure. The glycoproteins do not appear in the viral particle but are present on the cell surface of the infected host. The observed phenomenon of intracellular budding mentioned above, might be explained by glycosylated *gag* precursors being required for transporting the direct core precursor polyproteins to the plasma membrane.

In addition, Schultz et al. (1979) suggested that the direct core precursor polypeptides were phosphorylated prior to cleavage. Since they have been able to identify a kinase activity in the viral particles, they speculate that the phosphorylation is required to activate the cleavage events needed for viral maturation. This idea has been strengthened by the observations that avian myeloblastosis virus also harbors two protein kinases distinct from the viral oncogene products (Rosok et al., 1979). It has also been shown by *in vitro* phosphorylation that the *gag* precursor is modified at an amino acid residue within p12 (Yoshinaka and Luftig, 1982; Sen at al., 1977). These results are similar to the observations of Naso et al. (1979). They too suggest that p12, while still part of $Pr65^{gag}$, is phosphorylated and this apparently is related to rapid cleavage of $Pr65^{gag}$ and viral maturation.

Host Restrictions on Replication

An approach in elucidating viral assembly is to determine in detail the restrictions imposed by nonproducing cell lines such as

mammalian cells transformed with RSV. Svet-Moldavsky (1958) first demonstrated that RSV could infect mammalian cells and transform them. This observation was soon confirmed by a number of other investigators (Schmidt-Ruppin, 1959; Jenson et al., 1964; Munroe and Windle, 1963). These cells were determined to be true nonproducer cells since no virus particles were released when assessed by infectivity assays (Svoboda and Hlozanek, 1970; Weiss, 1982). The block in particle formation was determined not to be a result of altered provirus structure since it was determined that virions could be rescued by membrane fusion with uninfected avian cells (Svoboda et al., 1967; Shevliaghyn et al., 1969; Machala et al., 1970). Therefore, the avian cells must harbor the correct factors to induce virus production (Svoboda et al., 1971).

Von der Helm et al. (1980) and Vogt et al. (1982) did most of the pioneering work in pursuing the nature of the block in mammalian cells. They were able to show that the levels of $Pr76^{gag}$ in transformed mammalian cells were dramatically reduced and Pr76 was not processed into the major core proteins. Therefore, they suggest two possibilites that could explain the block in replication. The first possibility is that the concentration of $Pr76^{gag}$ in these cells is too low to allow the correct intermolecular interactions required for particle formation. Therefore, the block must be at the level of transcription or translation. This is a plausible explanation since it is known that enhancer elements can be species or tissue specific (Banerji et al., 1983; Gillies et al., 1983; Varmus and Swanstrom, 1985; Varmus and Swanstrom, 1985). The second possibility is that some avian host product is required for targeting the precursor to the

site of assembly and for processing.

SV40 Expression Vector

The use of the SV40 virus as a eukaryotic expression vector in mammalian cells has been well documented (Gething and Sambrook, 1981 and 1982; Wills et al., 1983 and 1984). The advantages for using this viral system to express a cloned gene are numerous and diverse; 1) The most obvious is, of course, the normal host of SV40 are mammals in particular primates. In tissue culture, investigators routinely use an African green monkey (*Cercopithecus aethiops*) kidney cell line, CV-1. Coincidentally, this cell line was established in 1964 to study RSV transformations (Jensen et al., 1964). 2) The naked DNA of SV40 is infectious. This is important since the recombinant SV40 DNAs can be directly transfected into cell cultures. 3) The methods used for transfecting recombinant SV40 DNA have been worked out in great detail (McCutchan and Pango, 1968; Sompayrac and Danna, 1981; Luthman and Magnusson, 1983; Perez et al., 1986). 4) The late promoter (P_L) used to drive expression of the subcloned genes is very strong (Gething and Sambrock, 1981). By driving expression of *gag* from this promoter in a recombinant SV40 vector, the levels of Pr76 in mammalian cells should be sufficient to address the question whether or not the block in replication in mammalian cells is because of poor expression. 5) The nucleic acid sequence has been determined (Reddy et al., 1978) which will greatly expedite the subcloning of the RSV *gag* sequences into the vector. 6) The SV40 genome replicates independently of the host chromosome.

Two important matters must be considered when using this

virus as an expression vector. First in order to propagate the recombinant viruses, the total size of the circular DNA must be between 70% and 100% of the wild type SV40 genome although the actual size limitation have not been worked out. This is important for DNA packaging of the recombinant DNA. The other point is that the recombinant virus will be replication defective since the late genes needed for virion assembly will be replaced with the foreign DNA. In order to rescue the recombinant virus, the late viral products must be supplied by cotransfecting DNA from a helper virus. When the vector DNA and the helper DNA are cotransfected into CV-1 cells, complementation between the early region of the vector molecule and the late region of the helper molecule allows for correct replication and packaging of both DNA molecules. The viruses can then be harvested and used to infect virtually 100% of a fresh monolayer. This is also important in analyzing the foreign product since at best only about 5% of the monolayer is actually cotransfected.

SV40 Replication Cycle: A Mini Review.

The SV40 replication cycle is perhaps the best understood of the eukaryotic viruses to date. Therefore, this minireveiw will only discuss the important details pertaining to the use of SV40 as a eukaryotic expression vector.

SV40 and the closely related polyoma virus belong to the papova viral family. They are small DNA tumor viruses that replicate in monkey and mouse cells, respectively. SV40 was first identified in 1960 in a batch of contaminated polio vaccines prepared from Rhesus monkey cells. It was described as a noncytopathic virus present in

Rhesus monkey cells that produced strong cytopathic effects when cultured in African green monkey kidney cells (Sweet and Hilleman, 1960; Fried and Prives, 1986).

As reviewed by Freid and Prives (1986), the nucleic acid of SV40 is a double-stranded circular DNA molecule consisting of 5,297 base-pairs. The genomic organization is divided into three distinct regions. The two largest regions, comprising nearly half the genome each, encode the products required for viral replication. These two regions are bidirectionally and differentially expressed, during the course of an infection, from the opposite strands and are termed the early and late regions. The third region is a relatively small region located between the early and late regions. Most of the regulatory sequences needed for viral gene expression (enhancers and promoters) and DNA replication (origin of replication) are found in this region.

The early viral products found in an SV40 infection are the large "T" and small "t" antigens (so named for their tumor inducing properties). The coding regions for these two proteins were mapped and determined to originate from the same early promoter, P_E. They were also found to share portions of the same reading frame and thus must originate from a spliced mRNA. In fact, the first documented examples of such splicing originated with this virus (Shenk et al., 1976; Berk and Sharp, 1978).

Early transcription of the "T" antigens not only relies on the upstream "TATA" box, but also the 21 bp repeats and the 72 bp enhancer elements. It is not known but it is suspected that host Spl, along with other factors not yet identified, interacts with the enhancer and the three 21 bp repeats to activate early transcription (Dynan and

Tjian, 1983).

The major product from the transcripts is the large "T" antigen. This early gene product begins to accumulate soon after viral penetration and their cellular concentration increases for about 8-10 hours until DNA replication begins (Schirmbeck and Deppert, 1987). This particular protein is perhaps the best understood eukaryotic regulator protein. It has been shown to be involved in SV40 DNA replication, late gene expression, cell transformation, host range restriction, and induction of host DNA replication factors that are required for viral DNA replication (Fried and Prives, 1986). The small t antigen is not as well characterized and will not be discussed in futher detail.

Elucidating the strategy for regulating late gene expression remains a difficult task. It is known though that expression of the late region relies on DNA replication and the activity of the large T antigen. The origin of replication (*ori*) has been mapped to a region between nucleotides 5208 and 30. DNA sequencing has revealed a 27 bp perfect inverted repeat adjacent to a long (17 bp) stretch of A/T nucleotides. This region (nucleotides 5208 to 30) also contains one of the T antigen binding sites (site II). This is not surprising since it has been demonstrated numerous times that the only viral product needed for DNA replication is the T antigen. It has been suggested that this protein acts in concert with host alpha-primase to initiate replication.

Once DNA replication has begun, T antigen is no longer needed since it is only required for initiation of DNA replication and not DNA synthesis. Some have suggested that the 21 bp repeat as well as the 72 bp enhancer region are required for replication but this has

not been fully worked out (Murakami et al 1986; Fried and Prives, 1986).

Late gene expression initiates after DNA replication. It is not known if expression is stimulated, by the act of DNA replication melting the supercoiled DNA complex, by the T antigen activating the late promoter maybe at the enhancer region, by the T antigen stimulating the expression of a host product which is required to act *in trans*, or by some type of transcriptional attenuation (Hay et al., 1982; Brady and Khoury, 1985; Fried and Prives, 1986).

The late products of SV40 infection, VP1, VP2, and VP3, are required for particle formation and DNA packaging. VP1 has been reported as being responsible for SV40 chromatin spacing, a possilbe requirement for packaging (Blasquez et al., 1986). But, the roles of the other products are unclear. Like most other viruses, the mechanisms of particle formation remain obscure.

In recent years a new late viral product has been discovered. This protein, called the "agnoprotein," was first suspected after the nucleic acid sequence was deduced. It was later found in in the perinuclear cell fraction and seems to bind to DNA cellulose. The exact role this product plays in the viral life cycle is not known. But, it has been suggested that this protein plays an important role in late gene expression and possibly viral assembly (Fried and Prives, 1986).

I have decided to use a molecular genetics approach to begin to elucidate the problem of particle formation in mammalian cells. This is a very important project because elucidating the block in particle formation in mammalian cells will lead to a better understanding of

the normal process of particle formation in the natural host and possibly lead to new novel approaches for combating retroviral infections.

CHAPTER III

Materials and Methods

Organisms

All plasmids were propagated in *Escherichia coli* strain DH-1 (F^-, *recA1, endA1, gyrA96, thi-1, hsdR17*(r_k^-, m_k^+), *supE44, relA1?, lambda*$^-$) (Hanahon, 1983) on L.B. agar plates + 35ug/ml. ampicillin (see appendix). *E. coli* transformants were stored in L.B. broth + 25% glycerol at -80°C.

The expression and characterization of the recombinant SV40 vectors were studied in CV-1 cells (Jenson et al., 1964), a continuous line of African green monkey kidney cells that are permissive for SV40 replication (Acheson, 1981), and Cos-1 cells, an SV40 transformed CV-1 cell line that is constitutive for T-antigen expression (Gluzman, 1981). Both cell lines were purchased from ATCC (Rockville, MA) and passaged not more that ten times in these experiments. Both cell types were grown in Dulbecco's modification of minimal essential medium (MEM) (Flow laboratories, Inc., Mclean, Va). The medium was supplemented (per liter) with 1.68 g $NaHCO_3$, 1.2 x 10^{-5} g penicillin G (20 Units), and 1.0 x 10^{-5} g streptomycin. After the medium was sterilized by filtration through a 0.45 um nitrocellulose membrane, heat-inactivated calf and fetal bovine sera (Hyclone Laboratories, Inc., Logan Utah) were added to 6% and 3% respectively (growth medium). All mammalian cell cultures were incubated at 37°C in 5% CO_2.

Long Term Storage and Thawing of
Eukaryotic Cell Cultures

To store CV-1 and Cos-1 cells, the cell monolayers were detached with 0.05% trypsin. As soon as the cells were released, the trypsin was diluted in nine volumes of growth medium (as described above). The cells were pelleted by low speed centrifugation at 800 rpms for 15 minutes in an IEC model CL clinical centrifuge at room temperature. The cells were then washed twice in 10 ml of growth medium. The cells were again pelleted and resuspended in 0.25 ml of growth medium per 1.0×10^7 cells and colled on ice. While the cells were pelleting, the freezing medium was made as follows: 11 parts MEM: 5 parts Dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, Mo): 4 parts fetal bovine serum. The freezing medium was then added to the cell suspension one drop at a time to a final concentration of 50%. After the cells were resuspended in the freezing medium, 0.5 ml aliquots were added to each cryotube and placed at -137°C as quickly as possible.

To thaw the cells, the frozen cultures were removed from the freezer and brought to room temperature as quickly as possible by warming in a 50°C water bath only until the ice had disappeared. Growth medium (0.3 ml) was added one drop at a time, then all the cells were added to a tissue culture flask + 30 ml growth medium. The medium was changed after 24 hr to remove any residual traces of DMSO.

DNAs and Viruses

The Prague C *gag* gene used in this investigation was derived from a recombinant plasmid pATV-8, which contains the entire genome of Rous sarcoma virus (Schwartz et al., 1983). The SV40 late region replacement vector, d12005 (Sleigh et al., 1978), used for the expression of RSV *gag* and all other subsequent mutations in the *gag* sequences, was subcloned into a derivative of pBR322 for propagation in *E. coli* (pQD). SV40 late functions were provided by a early deletion mutant, d11055 (Pipas et al., 1982 and 1983). Both SV40 DNAs were generously provided by Dr. Mary-Jane Gething and Dr. Joe Sambrook (University of Texas Health Science Center, Dallas Tx.). A recombinant SV40 vector carrying the RSV *env* gene was used as a positive control in all experiments unless otherwise noted. This vector, designated SV.KX (see Fig. 7), is essentially the same as SV.KB (Wills et al., 1984). The only difference between the two vectors is that SV.KX retains the original RSV Xba I site (downstream of the *env* coding sequence) adjacent to the Bam HI site whereas SV.KB does not (Wills, pers. comm).

DNA Manipulations

Restriction Endonuclease Digestions

Restriction endonucleases were prepared and used according to manufacturers published protocols. DNA was purified away from contaminating proteins by extracting once in buffer equilibrated phenol:chloroform (1:1) (see, Phenol Chloroform Extraction of Nucleic Acids). The DNA concentrations were estimated by comparing the band

intensities with known standards (Lambda HindIII standards, 20 ug/ml, New England Biolabs, Beverly, Ma.) after agarose gel electrophoresis and staining with ethidium bromide (see below) (Sigma Chemical Co., St. Louis, Mo)

Agarose Gel Electrophoresis

DNA Fragments were analyzed by agarose gel electrophoresis (Maniatis et al., 1982). The standard gel used in this investigation contained 0.8% agarose (International Biotechnologies, Inc. (IBI), New Haven, Ct.) in Tris-Acetate Buffer (see Appendix) with 0.5 ug/ml ethidium bromide (Sigma Chemical Co. St. Louis, Mo.). The same Tris-Acetate buffer was used for the electrophoresis buffer and also contained 0.5 ug/ml ethidium bromide.' Gels were run at a constant 13.3 volts/cm.

DNA fragment sizes were estimated by visually comparing and curve fitting their electrophoretic mobilities to DNA size standards (Lambda DNA digested with Hind III, New England Biolabs, Beverly, Ma).

Phenol-Chloroform Extractions
of Nucleic Acids

DNA was always extracted as follows. The approximate volume of DNA was estimated and the final concentration of NaCl was adjusted to 0.1 M using a stock solution of 5M NaCl. The nucleic acid concentration was also adjusted to 30 ug/ml using a stock solution of 30mg/ml tRNA (Sigma Chemical Co. St. Louis, Mo.). The nucleic acids were then extracted once by adding an equal volume of a 1:1 ratio of a Tris-HCl, pH 8.0, buffer saturated phenol:chloroform (Maniatis et al., 1982).

The mixture was thoroughly vortexed and the two organic and aqueous phases were separated by centrifugation for 2 minutes at 16,500 x g. The upper aqueous phase was transferred to a clean tube and extracted twice in a like manner with chloroform to remove any residual traces of phenol. The DNA was then precipitated by adding two volumes of 100% ethanol. The DNA was pelleted by centrifugation at 16,500 x g after the suspension was allowed to set for at least 15 minutes at -20°C. The ethanol was removed by drying the pellet in a vacuum chamber. The DNA was dissolved in an appropriate volume of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0) + 20 ug/ml RNAse A.

Plasmid Isolations

Many protocols have been developed for the isolation of plasmid DNA. I chose to use the method of lysis by alkali as described by Maniatis et al. (1982). When screening for transformants, mini-lysates were prepared as follows. Four ml of L.B. broth plus the antibiotic of choice (see Appendix A) was inoculated with a single colony. The cultures were incubated overnight at 37°C with vigorous shaking.

Bacterial cells were harvested by centrifugation at 16,500g for 2 minutes. The pellets were completely resuspended in 100 ul of Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, and 5 mg/ml lysozyme). The cultures were incubated at room temperature for 5 minutes to allow the cell wall to be digested by the lysozyme. The integrity of teh remaining spheroplast was retained by the osmolarity of 50 mM glucose. Two hundred microliters of freshly prepared Solution II (0.2 N NaOH, and 1% SDS), was then added, mixed, and allowed

to set on ice for 5 minutes. The SDS solubilizes the cell membrane which is accessible due to the weakened cell wall resulting. Both the chromosomal DNA and plasmid DNA were then denatured by the alkaline conditions. One hundred and fifty microliters of Solution III (3 M KOAc, pH about 6.0, was prepared as follows; to 60 ml of 5 M KOAc, add 11.5 ml of glacial acetic acid and 28.5 ml H_2O) were added to the lysates to allow reannealing of the plasmid DNA. The chromosomal DNA which was nicked and denatured by the protocol does not reanneal under these conditions and is removed along with the cell debris by centrifugation at 16,500g for 5 minutes. The plasmid DNA was then extracted as described above and resuspended in 50 ul of TE buffer with 20ug/ml RNAse A.

"Midi-Prep" DNA was prepared from 50 ml cultures as described above except with 1 ml of Solution I, 2 ml Solution II, and 1.5 ml Solution III. After the DNA was extracted and pelleted, it was dissolved in 100 ul TE buffer plus 20 ug/ml RNAse A.

For large preparations of plasmid DNA (or "Maxi-Preps"), 25 ml of L.B. medium was inoculated with 0.1 ml of an overnight culture and incubated at 37°C with vigorous shaking until the $O.D._{600}$ reached 0.6. This culture was added to 500 ml L.B. medium with the appropriate antibiotic. The culture was incubated at 37°C with vigorous shaking for 2.5 hr. Plasmid DNA was selectively amplified by adding chloramphenicol to 170 ug/ml. Chloroamphenicol amplifies the number of copies of plasmid by inhibiting host protein synthesis. In the absences of protein synthesis, replication of the plasmid carrying the

Col EI replicon continues whereas chromosomal DNA replication is inhibited. The cultures were incubated for another 12 to 16 hr at 37°C with vigorous shaking.

Lysis of the cultures were carried out as described above except with 10 ml of Solution I, 20 ml Solution II, and 15 ml Solution III. Plasmid DNA was precipitated with an equal volume of isopropanol and pelleted at 16,500g for 15 minutes. The pellet was resuspended in 3.2 ml TE buffer and plasmid DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide density gradients.

Purification of Plasmid DNA.

Supercoiled plasmid DNA can be purified due to its buoyant density in ethidium bromide-cesium chloride gradients. The gradients were prepared by adding 1 g of cesium chloride and 100 ul of ethidium bromide (10 mg/ml) to every ml of DNA followed by centrifugation for 16 to 18 hr at 60,000 rpm in a Beckman vTi-65.2 rotor at 20°C (Maniatis at al., 1982).

After centrifugation, two DNA bands could be seen with ultraviolet irradiation. The upper band contains linear and open circular plasmid DNA and the lower band contains closed circular plasmid DNA. The lower band was removed with a 20 gauge needle and the ethidium bromide was extracted with water-saturated N-butanol (Maniatis et al., 1982). The DNA was precipitated by adding two volumes of H_2O and then two more volumes of 100% ethanol (final concentration of 70% ethanol). After centrifugation to pellet the DNA, it was resuspended in 1 ml. TE buffer. The concentration was estimated by comparisons to DNA standards after agarose gel electrophoresis.

Subcloning and In Vitro Mutagenesis

The RSV *gag* coding sequence (nucleotides 380 to 2485) is contained within a 2485 bp Sac I-Hind III fragment (nucleotides 255 to 2740 respectively). This fragment was modified by adding one dephosphorylated Cla I linker (d[CATCGATG]) to the Sac I site with T4 DNA ligase after blunt-ending the fragment with the Klenow fragment of *E. coli* DNA Polymerase I. The fragment was subcloned into pJC-2, a derivative of pAT153 missing the Eco RI site, in place of the Cla I-Hind III fragment (Twigg and Sheratt, 1980). The resulting plasmid was further modified by the addition of one Xba I linker (d[CTCTAGAG]) to the Hpa I site in RSV to facilitate further manipulations. This plasmid was designated pGAG.

SV40 DNA lacking the late region was obtained from a derivative of SVEHA3 (Gething and Sambrook, 1982) in which the Hpa II site was modified by the addition of a Cla I linker (see above). The vector was further modified by the addition of an Xba I linker (see above) adjacent to the Bam HI site following Klenow treatments. The 3056 base-pair SV40 Cla I-Cla I fragment was gel purified and subcloned into the unique Cla I site of pGAG. The resulting plasmid was designated pSV.GAG. Correct orientation of the SV40 DNA was confirmed by restriction analysis. This construction leaves a functional SV40 late promoter to drive transcription of the inserted *gag* sequences (Brady et al., 1982).

Because pSV.GAG is approximately 105% of the size of wild type SV40 DNA and borders, on the size limit for SV40 packaging (Elder et al., 1981), further manipulations were carried out to reduce the size

of pSV.GAG. For this, a 1729 base-pair Nde I-Kpn I fragment from d12005, a viable early deletion mutant, was ligated in place of the wild-type fragment in pSV.GAG. This mutant has a 230 base-pair deletion that removes the intron (nucleotides 4639 to 4875) in the early region of the SV40 genome (Pipas et al., 1980 and 1983). The resulting construction was designated pΔSV.GAG and is approximately 100.5% the size of the wild-type SV40 genome (see Fig. 7).

To introduce deletions into the 3' end of *gag*, pΔSV.GAG was cut with Eco RV at the unique site in pJC-2 which is adjacent to *gag*. The linear DNA was digested for various amounts of time with the double strand specific Bal31 exonuclease. The digestions were quenched by the addition of EGTA to 1 mM; extracted with phenol-chloroform, and the DNA blunt ended with Klenow. The DNA was circularized by ligation at low DNA concentrations (4ug/ml.) in the presence of Xba I linkers and used to transform *E. coli* DH-1 cells (see below). Clones were screened for the presence of Xba I sites in the plasmids and the approximate size of the deletions was estimated by restriction analysis. These deletion mutants were constructed and generously provided by Dr. J.W. Wills (Louisiana State University Medical Center Shreveport, Shreveport LA)

It was also of interest in this investigation to determine whether or not p27 could induce viral budding if it was expressed by itself in the SV40 vector. In order to facilitate the subcloning into the vector, the p27 coding region was first subcloned into the plasmid pAT153 as follows. The plasmid pATV-8 which contains an infectious clone of Rous sarcoma virus was cut to completion with Rsa I and the 1900 bp fragment which contains the p27 coding domain plus an extra

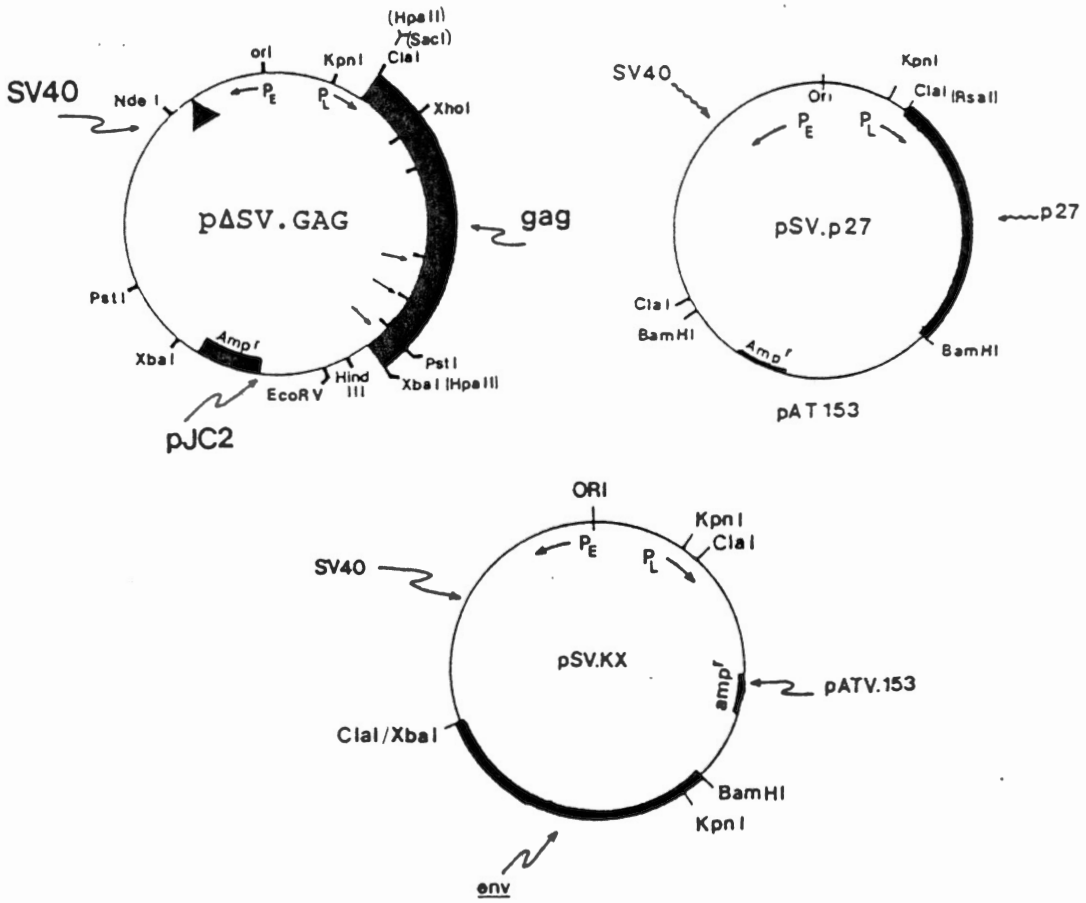

Figure 7. Generalized structure of three SV40 late region replacement vectors used for the expression of RSV specific poylpeptides in CV-1 cells. The region of *gag* which codes for the amino terminus of Pr76 is located near the Cla I site of the vector pΔSV.GAG. Three *in vitro* mutations were created in the *gag* coding sequence with the Bal 31 exonuclease starting at the unique Eco RV site. The approximate end points of the deletions are indicated by the black arrows. The region of the coding sequence which codes for the amino terminus of p27 is located near the Cla I site of pSV.p27. The plasmid sequences of both these vectors are between the early and late regions of the SV40 genome and must be removed with the appropriate enzyme prior to transfections. The region of *env* which codes for the amino terminus is located near the KpnI site of the vector pSV.KX. The plasid sequences are located between the two Kpn I sites.

1172 bp was purified by electroelution. The blunt ends were modified by the addition of Cla I linkers (see above) with T_4 DNA ligase. The DNA was then digested again with Cla I and Bam HI (see Fig. 24). This fragment was again purifed by electroelution and ligated at 4 ug/ml overnight at 15°C into the corresponding sites in pAT153. This plasmid pAT.p27 was used not only as an intermediate to facilitate the subcloning into the SV40 vector but also as a probe to detect p27 sequences in Southern hybridizations. This plasmid was constructed and generously provided by Chris Mhor. The SV40 sequences were introduced into this plasmid by isolating the 3000 bp Cla I-Cla I fragment of pSV.KX and ligating the frament into the unique Cla I site of pAT.p27 with T_4 DNA ligase. The resulting plasmid, pSV.p27, was digested with Bam HI to remove the plasmid sequences prior to transfection. After purifing the vector sequences (SV40 and p27), the linear fragment was circularized with t_4 DNA Ligase and the used to transfect CV-1 cells.

Transforming Competent *Escherichia coli* DH-1

Preparation of Competent *E. coli* DH-1 cells. *E. coli* DH-1 cells (Hanahan, 1983) were grown overnight in 3 ml of L.B. medium. One tenth ml of this culture was used to inoculate 10 ml of L.B. broth and incubated at 37°C until the $O.D._{600}$ reached 0.2. Five ml of this culture was subcultured into 400 ml of prewarmed L.B. broth and again incubated at 37°C until the $O.D._{600}$ reached 0.2. The cultures were chilled on ice for 10 minutes and the cells harvested by centrifugation at 5000 rpm in a GSA type rotor for 10 minutes at 4°C. The cells were washed in 200 ml of Transformation Buffer X (50 mM $CaCl_2$, 15% glycerol, and 10 mM MOPS, pH. 6.6) at 4°C. The cells were pelleted

again and resuspended in 20 ml of Transformation Buffer X and were split into 200 ul aliquots. The cells were then quickly frozen on dry ice and methanol and stored at -70°C.

Transformation of Competent *E. coli* DH-1 Cells. The mechanism of transformation in *E. coli* is not well understood. But the protocol for transformation is widely used. It is believed the calcium along with a heat shock are the major components of *E. coli* transformations.

Briefly, 200 ul of competent *E. coli* DH-1 cells (see above) were thawed at room temperature for 10 minutes. After the cells were then chilled in ice water for 10 minutes, up to 25 ng of DNA was added and further incubated on ice for 10 minutes. Heat shock was done by placing the cells at 37°C for 5 minutes then adding 0.8 ml prewarmed L.B. broth. The culture was incubated at 37°C for 30 minutes then plated onto L.B. agar plates containing the appropriate antibiotic and incubated inverted overnight at 37°C.

Southern Hybridizations

Southern hybridizations were performed as described by Southern (1975). After agarose gel electrophoresis, the DNA was fragmented by soaking the gel in several volumes of 0.25 M HCl for 15 minutes at room temperature for minigels (2' x 3' x 1/4'), or 30 minutes for preparative gels (4' x 5.5' x 1/2'). The DNA was then denatured to single stranded DNA by soaking the gel in 0.5 M NaOH, 1 M NaCl for the same time. The gel was neutralized in 0.5 M Tris-HCl, pH 7.7, 3 M NaCl for 15 or 30 minutes.

Southern Transfer of DNA to Nitrocellulose. The DNA was blotted onto nitrocellulose paper, type BA85 (Schleicher & Schuell Inc., Keene N.H.) as follows. One blotting pad (Gel Blot Paper GB004, Schleicher & Schuell) was saturated with 20 x SSC buffer (see appendix). One piece of Whatman 3 MM paper was cut just smaller than the blotting pad and placed on top of the pad followed by the treated gel. Water saturated nitrocellulose paper (cut just slightly larger that the gel) was carefully placed on top of the gel. Air bubbles were worked out with a gloved hand. One piece of 3 MM paper and 3-4 cm of paper towels were then placed on top followed by a 200 g weight.

The capillary action provided by the paper towels pulls the SSC buffer up through the nitrocellulose. The single-stranded DNA is carried along with the buffer up through the gel and is bound by the nitrocellulose. After 3-4 hours, to overnight for larger gels, the nitrocellulose was marked for orientation and peeled away from the gel. It was then soaked in 6 x SSC buffer for 5 minutes prior to baking for 2 hr at 80°C under vacuum. The filters can know be stored until needed in an air tight bag at room temperature.

Before hybridizing the ^{32}P-labeled probe DNA with the filters, the filters were prehybridized overnight at 42°C in a sealed bag with 5 ml of prehybridization buffer (see Appendix D) to block nonspecific binding of the probe DNA.

The ^{32}P nick-translated probe DNA (see below) was boiled for 5 minutes then quickly chilled in ice water. The probe (500 ng of DNA) was injected into the sealed bag using a 27 gauge needle. After the bag was resealed, the probe was hybridized to the bound DNA for 24 to 36 hr at 42°C. After sufficient hybridization, the filters were

removed from the bag and any unbound probe DNA was washed off with three 5 minute washes with 2x SSC buffer, 0.5% SDS at room temperature. Any weakly bound probe DNA was washed off under high stringency conditions. This consisted of two thirty minute washes in 0.1x SSC, 0.5% SDS at 68°C (in the presence of formamide) (Maniatis et al., 1982).

The filters were placed next to a preflashed X-ray film (see Fluorography) and two intensifying screens (Dupont). The autoradiography was carried out from 15 minutes to 3-4 days, depending on the strength of the probe, at -80°C. The exposed film was developed according to the manufacturer's protocols.

^{32}P Nick-Translation. Five tenths ug of probe DNA were nicked with a dilute sample of DNAse I that was previously titrated to a concentration that would nick plasmid DNA to linear form in 1 hr at 15°c (approximately 0.1 ug/ml). This dilution resulted in plasmid DNA that had only a few nicks per molecule. The nicked probe DNA was simultaneously incubated with 1 unit of *E. coli* DNA Polymerase I in the presence of 50 uCi ^{32}P-dCTP (specific activity of 619 Ci/mmol, New England Nuclear, Boston, Ma.),2 mM dATP, dTTP, and dCTP, and Nick-translation buffer (see Appendix D) for 2.5 hr at 15°C.

The percent incorporation of radiolabeled nucleotides into nucleic acid was calculated by adsorption to DE-81 filters as described by Maniatis et al., (1982). Briefly, 0.5 ul of the nick-translation reaction was spotted onto two DE-81 Filters (Whatman) and dried. One of the filters was washed three times for 5 minutes each in 0.5 M Na_2HPO_4. The salt was removed by washing once for 1 minute in

H_2O. Finally the filters were washed once for 1 minute in 95% ethanol and dried. Both the unwashed filter (corresponding to total radioactivity) and the washed filter (total radioactivity incorporated into nucleic acid) were counted in 3 ml of liquid scintillation fluid in a liquid scintillation counter.

The unincorporated nucleotides were removed from the DNA by precipitating the DNA with 1/10 volume of 3 M sodium acetate and one volume of isopropanol. After the pelleted DNA had been dried, the DNA was resuspended in 1 ml of TE buffer and stored at -20°C.

VIRAL INFECTIONS AND DNA TRANSFECTIONS

SV40 Infections

SV40 virus stocks were made by freezing (-20°C) and thawing transfected or infected plates three times. Once a fresh monolayer reached a confluency of 80%-90%, the medium was removed and 0.5 ml of the virus lysate was added. The virions were allowed to infect the monolayer for 30 minutes at 37°C. The lysate was then removed and fresh medium was added and the cells were incubated at 37°C.

SV40 DNA Transfections

Before transfecting the recombinant SV40 DNAs, the bacterial sequences (pJC-2) were removed by Xba I digestion of pΔSV.GAG or Kpn-I digestion of pSV.KX. The SV40-*gag* or SV40-*env* sequences were agarose gel purified (Maniatis et al., 1982) and circularized by ligation at low DNA concentrations (4 ug/ml).

Virions carrying the recombinant SV40 DNAs were produced in CV-1 cells by complementation with a co-transfected SV40 helper DNA (dl1055) using a modification of the method of Sambrook and Gething (1982). Tissue culture plates (60mm) were seeded with 2.5 x 10^5 cells and incubated at 37°C in 5% CO_2. When the monolayer became approximately 80% confluent, the cells were washed twice in phosphate buffered saline (PBS) and twice in tris buffered saline (TBS) (see Appendix A). Three tenths ml of TBS containing 120 ng circularized vector DNA, 36 ng helper DNA, and 300 ug DEAE-DEXTRAN (McCutchan and Pangano, 1968) were added to the monolayers. The cells were then incubated at 37°C, 5% CO_2. The DNA was removed after 60 minutes and the plates were washed once with TBS, once with PBS and then growth medium containing 100 uM chloroquine (Luthman and Magnusson, 1983) was added for 4 hr at 37°C. The plates were incubated in medium without chloroquine for 4-7 days or until cytopathic effects were observed.

Extraction of SV40 DNA

SV40 DNA was extracted by the method of Hirt (1967). The monolayers (60 mm plate) were washed twice with cold PBS and drained. 400 ul of lysis buffer (0.6% SDS, 10 mM EDTA, pH 7.4) were added to the plates and allowed to incubate for 20 minutes at room temperature. The lysates were scraped with a rubber policeman into a microfuge tube and the final NaCl concentration was adjusted to 1.56 M with 5 M NaCl. The tubes were inverted 10 times gently to mix the salt and lysate without shearing or detaching the DNA from the nuclear membrane. The DNA was allowed to precipitate overnight, then the chromosomal DNA was pelleted with the cellular debris by microcentrifuging at 16,500g for

8 minutes at room temperature. The supernatant was removed and RNAse A was added to 10 ug/ml and incubated at room temperature for 30 to 60 minutes. The DNA was extracted as described earlier and precipitated with one tenth volume of 20% potassium acetate and 2 volumes of 100% ethanol. The DNA was pelleted by centrifugation and dried under vacuum. The DNA was then resuspended in 50 ul TE containing 20 ug/ml RNAse A.

DNase I Protection

In order to test whether or not both the vector and helper DNAs were packaged into DNase I resistant virus particles, 70-80% confluent monolayers (100 mm plates) were cotransfected for 1 hour followed by a 4 hr chloroquine treatment. The monolayers were then digested for 1 hr with DNase I as described in the DNA replication experiments to degrade any DNA not ingested by the cells. SV40 DNA (vector or helper), either in released particles or still within the monolayers was harvested at 89 hr post-transfection as follows. The medium was removed and stored at -20°C overnight. The monolayer was gently scraped off the plates in 1 ml of RSB buffer (100 mM tris-Cl, pH 7.4, 10 mM KCl, and 1.5 mM $MgCl_2$) using a rubber policeman. The cells were then frozen at -20°C overnight (Stow et al., 1983). The cells were then thawed at room-temperature and lysed by five 2 second bursts of sonication. One tenth volume of 13% deoxycholate and 0.1 ml of 0.25% trypsin were added to both the cell lysate and the culture medium and allowed to incubate at room-temperature for 20 minutes to disrupt any membrane bound vesicles that might have formed. SV40 particles have previously been shown to be resistant to this concentration of trypsin

(Khoury and Lai, 1979). The cell lysates were each split into four 0.5 ml aliquots. To the first was added 1 ug of plasmid (pUC19) DNA (to check for DNase I activity) and 50 ug/ml of DNAse I. The second alquot received 1 ug plasmid DNA only. The third sample received 50 ug/ml DNAse I and the fourth was left untreated. Any unprotected DNA was degraded by incubating these samples for one hour at 37°C. The DNase I was then inactivated by adding EDTA pH 8.0 and SDS to 1 mM and 0.6% respectively. SV40 particles were then disrupted by adding Proteinase K to 500 ug/ml and incubating at 37°C for two hours. The DNA was purified by two phenol-chloroform and two chloroform extraction and concentrated by precipitation with two volumes of 100% ethanol. The DNA was pelleted by centrifugation at 16,000 x g dried and resuspended in 20 ul of TE buffer.

Meanwhile, the medium was clarified by centrifugation at 10,000 x g for 10 minutes at 4°C. SV40 particles were concentrated by centrifugation in an SW41 rotor for 3 hr at 122,000 x g. The pellets were gently resuspended overnight in 0.5 ml of RSB buffer at 4°C by gentle agitation. The samples were split into two 0.25 ml aliquots and 1 ug of plasmid DNA was added to each . DNAse I was added to one of the samples as before and incubated at 37°C for one hour. The DNAse I was inactivated and the DNAs were purified as described above.

Fifteen percent of each extract were digested with Kpn I and Xba I to distinguish between helper and vector DNA molecules. The DNAs were transferred to nitrocellulose after agarose gel electrophoresis and hybridized with ^{32}P-nick translated pSV.KX.

Expression Assays

Intracellular Immunofluorescence

Viral specific proteins and their locations were identified by indirect immunofluorescence. CV-1 or Cos-1 cells were grown in 35 mm plates and stained after 60 hr or until cytopathic effects (CPE) were observed (usually 140 hr post-transfection). The monolayers were washed twice with PBS and fixed with 95% ethanol: 5% acetic acid for 0.5 to 2 hr at -20°C. The fixative was removed and the monolayers were washed once with PBS + 0.1% bovine serum albumin (BSA). Fifty ul of primary antibody (rabbit anti-p27, rabbit anti-gp85, or goat anti-RSV) diluted 1:20 in PBS + 0.1% BSA was added to the monolayers and allowed to adsorb for 45 minutes at room temperature. The rabbit anti-p27 and anti-gp85 were generously provided by Dr. E. Hunter (University of Alabama Birmingham, Birmingham Al) and the goat anti-RSV was purchased from Microbiological Associates, Bethesda Ma. The unbound antibody was removed by two one-minute washes in PBS + BSA and one 5 minute wash in the same buffer. Fifty ul of fluorescein-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG (Antibodies Inc., Davis, Ca.) diluted 1:10 in PBS + BSA was added and allowed to adsorb for 30 minutes at room temperature. The secondary antibody was removed by the same series of washed as described above. A cover slip was mounted on the monolayer with 50% glycerol in PBS + BSA. The staining was then visualized with an ultraviolet irradiation using a fluorescence microscope.

Metabolic Labeling and Immunoprecipitation

Viral specific products could be followed through the course of infection by radiolabeling newly synthesized proteins and then selectively precipitating (Staph A) viral specific proteins with antisera raised against the individual proteins or the whole virus (Cullen and Schwartz, 1976). To follow the processing or degradation of the RSV proteins, transfected or infected monolayers were starved in medium lacking leucine and serum for one hour. The cells were pulse labeled with 200 uCi ^{3}H-leucine (1 mCi/ml of ^{3}H-leucine, New England Nuclear, Boston, Ma, in MEM without serum) for 15 minutes. Chases with unlabeled leucine were performed in MEM + 3% fetal bovine and 6% calf bovine serum.

After radiolabeling the cells (60 mm plate), the medium was removed and the cells immediately lysed in 1 ml of lysis buffer without SDS (1.0% Triton X-100, 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% deoxycholate). The monolayer was gently disrupted with a pastuer pipet and transferred to a microcentrifuge tube. The nuclei were pelleted by centrifugation in a microcentrifuge (16,500g) for 5 minutes. The lysate was removed and SDS was added to a final concentration of 0.1%. The lysates were then stored at -20°C. To assay for labeled proteins released into the cell culture fluid, the medium (0.8 ml) was removed and 1/5 volume (200 ul) of 5x lysis buffer containing SDS (1 x recipe is the same as above but with 0.1% SDS). The samples were then processed the same as the above lysates.

Twenty ul of a 1:10 dilution of primary antisera were added to 0.5 ml of radiolabeled lysate and allowed to adsorb overnight at 4°C. One

tenth ml of prepared Staph A (see below) which had previously been determined to adsorb 95-99% of the IgG in 2 ul of the undiluted rabbit anti-p27 and anti-gp85 antisera preparations, were added to the lysates to precipitate the antibody-antigen complexes. After 30 minutes, the Staph A-antibody-antigen complexes were pelleted for 10 seconds in a microcentrifuge and washed twice in 500 ul of 1 x Lysis Buffer with SDS. The pellet was washed a third time in 20 mM Tris-HCl pH 7.5 to remove the salt and then suspended in 20 ul of dissociation buffer (see Appendix B). The sample was boiled for 1 minute to dissociate the antigen and antibody from the Staph A. Immunoprecipitated proteins were then analyzed by SDS-PAGE (see below).

To determine whether or not the *gag* precursor polypeptides were being processed in mammalian cells, it was of interest to increase the amount of protein being synthesized in the transfected monolayers. Since expression of Pr76 could only be analyzed in transfected cells, the easiest way to increase the yield of Pr76 was to increase the number of cells being transfected. Also, since only transient expression was being analyzed, there was no need to cotransfect helper DNA. Therefore, 1 ug of vector DNA (ΔSV.GAG or SV.KX) was transfected into nearly confluent CV-1 cell monolayers (60 mm plate). After 48 hr. when maximal expression of the SV40 late region occurs, the medium was removed and the cells washed twice with prewarmed serum-free Dulbeccos (MEM) lacking L-methionine (Gibco, Grand Island, N.Y.). The monolayers were then labeled with 70 uCi of TRAN^{35}*S*-LABEL (ICN Biomedicals Inc., Costa Mesa, Ca.) in 0.8 ml. of the same serum-free medium as above. After 30 minutes, L-methionine was added to one tenth the concentration of methionine in normal Dulbeccos MEM (final

concentration of 1.2 x 10^{-4}g/ml). The cells were then labeled for an additional 2 hr at 37°C, 5% CO_2 to insure steady state levels of labeled protein and precursor processing. The labeling was then terminated by placing the cells on ice.

To determine if the *gag* antigens were released, the culture medium form the labeled cells was collected and any RSV *gag* specific antigens were concentrated by immunoprecipitation with an equal mixture of goat anti-RSV (Prague strain): goat anti-RAV-0: goat anti-AMV p27 (Microbiological Associates, Bethesda, Md.) rabbit anti-goat IgG (Organon Teknika Co., West Chester, Pa.) and Staph A. For a negative control, the culture medium from SV.KX transfected monolayers was similarly collected and assayed for *env* proteins using the rabbit anti-gp85 antibody (Wills et al., 1984).

To characterize the intracellular *gag* antigens, the same monolayers were gently lysed in the presence of phenylmethylsulfonyl floride and Pepstatin (1 x 10^{-3}g/ml and 1 x 10^{-5}g/ml respectively). The *gag* and *env* polypeptides were collected from half the lysates by immunoprecipitation using the same antibodies as used on the culture fluid. The precipitated proteins from both the lysates and culture fluid were separated on a 10% SDS polyacrylamide gel and analyzed by fluorography.

Preparation and Freezing of Staph. A. Ten ml of Brain Heart Infusion (BHI) medium (Difco Laboratories, Detroit, Mi.) was inoculated with *Staphylococcus aureus* Cowan 1 strain (ATCC #12598) and grown overnight at 37°C with vigorous shaking. One liter of BHI broth was then inoculated with the 10 ml culture and incubated for 24 hr at 37°C. The

cells were harvested by centrifugation at 5000 rpm in a GSA type rotor for 10 minutes at 4°C; washed in 200 ml of PBS + 0.2% sodium azide pH 7.2; pelleted again; and resuspended in 10% w/v PBS + 1.5% formaldehyde. The cell suspension was then stirred at room temperature for 90 minutes. After pelleting the cells again, they were resuspended in the same volume of PBS + 0.2% sodium azide and incubated at 80°C for 2.5 to 5 minutes. The suspension was cooled immediately in ice and washed again in the same volume of PBS-sodium azide. The prepared Staph A was then stored at -80°C in 1 ml aliquots.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Immunoprecipitated proteins were released from the Staph A-antibody complex by boiling for 1 minute in dissociation buffer (see Appendix D for SDS-PAGE recipes). These proteins were then separated on thin (1.5 mm) sodium dodecyl sulfate (SDS) acrylamide slab gels using the discontinuous buffer system described by Laemmli (1970) and Ames (1974). The standard resolving gel contained 10% acrylamide and NN'-methylene-bis-acrylamide at a ratio of 30:0.8 in 0.4 M Tris-HCl pH 8.8 and 0.1% SDS. The polymerization of the acrylamide was initiated by the addition of ammonium persulfate and tetramethylethylenediamine (TEMED) to final concentration of 0.1 and 0.067% respectively. A 7mm stacking gel (1.5 mm thick) containing 3% acrylamide, 0.1% SDS, and 60 mM Tris-HCl pH 6.8 was also polymerized with ammonium persulfate and TEMED. Twenty five ul of sample were loaded in each lane of a 14 x 16 cm gel and electrophoresed for 5 to 6 hr at a constant 0.134 mA\cm^2 (30 mA). Gels were fixed and stained for 30 minutes at room temperature with Coomassie Brilliant Blue R250 (Sigma, St. Louis, Mo.) in 95%

ethanol; 5% acetic acid. The gels were destained overnight in 5% methanol:7% acetic acid. After destaining, the gels were prepared for fluorography according to the manufacturer's protocols.

Fluorography. Destained gels were soaked for one hour in the autoradiography enhancer, "ENhance" (New England Nuclear, Boston Ma.). The organic enhancer was impregnated into the gels by soaking the gels for 30 minutes in water plus 5% glycerol. The gels were the dried onto Whatman 3 MM paper under vacuum using a slab gel drier (Hoeffer Scientific Instruments, San Francisco, Ca). The gels were usually dried for 1.5 hr at 60°C. The protein standards (see below) were marked with a small 5 ul drop of ^{14}C ink. The dried gels were then placed on Kodak X-Omat AR film which was preflashed (Laskey and Mills, 1975). The films were exposed at -80°C for 2 days to 2 weeks depending on the amount of labeled protein in the gels. Exposed films were developed according to manufacturer's protocols.

Protein Molecular Weight Determination. The molecular weight of the ^{3}H-labeled protein bands were estimated by comparisons with the known electrophoretic mobilities of the protein standards (Low molecular weight standards, Bio-rad, Richmond, Ca.). The following proteins were used as standards: Phosphorylase B (97.4 kDa), Bovine Serum Albumin (66.2 kDa), Carbonic Anhydrase (31 kDa), Soybean Trypsin Inhibitor (21.5 kDa), and Lysozyme (14.4 kDa). The purchased low molecular weight standards were diluted to 100 ug/ml in Dissociation Buffer. Ten to twenty five ul was run as standards.

PART I:

EXPRESSION OF THE ROUS SARCOMA VIRUS *GAG* GENE IN MAMMALIAN CELLS

CHAPTER IV

RESULTS

Expression of $Pr76^{gag}$ in Mammalian Cells By the Vector pΔSV.GAG

The SV40 late region replacement vector used in this study is capable of autonomously replicating its DNA in permissive cells since it contains an intact copy of the gene encoding the T Ag. This vector is not only capable of expressing the early genes, but the late genes as well. As mentioned earlier, the RSV *gag* gene has been subcloned into the late region of the SV40 genome in place of the SV40 coat proteins VP1, VP2, and VP3. This vector therefore can only be propagated by complementation by a helper virus providing the late proteins *in trans* (see Fig. 8). The helper virus, d11055, is an early deletion mutant the requires T Ag *in trans* for DNA replication. Complementation between the two cotransfected DNAs in the same cell should result in the production of infectious virus; half of which should contain the vector DNA. The virus produced by these cells could then be used to infect virtually 100% of a fresh monolayer and induce cytopathic effects in 2-3 days.

Intracellular Immunofluorescence

To determine if the vectors, ΔSV.GAG and SV.KX, were expressing either RSV *gag* or *env* proteins, transfected as well as infected

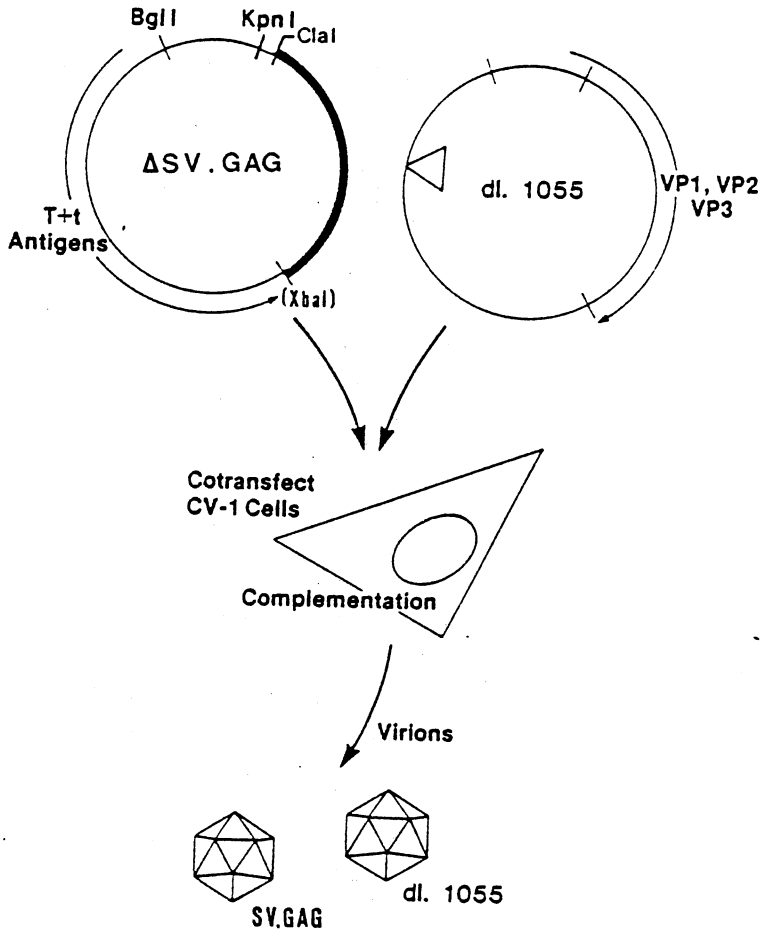

Figure 8. Schematic representation of cotransfecting CV-1 cells with vector (ΔSV.GAG) and helper (dl1055) DNA. Complementation *in trans* between the vector and helper allows the production of infectious recombinant SV40 virions. This illustration of the cotransfection procedure was adopted from a figure provided by Dr. E. Hunter (University of Alabama Birmingham, Birmingham Al).

monolayers were fixed with 95% ethanol, 5% acetic acid (vol/vol) at various times post-transfection or post-infection. The permeable cells were then assayed for the respective RSV proteins by immunofluorescence using rabbit anti-viral antisera (either anti-p27 or anti-gp85) and fluorescein-conjugated goat anti-rabbit IgG (Antibodies Inc., Davis Ca).

Photographs of typically stained cells at 45 hr post-transfection are shown in Fig. 9. Both RSV *gag* and *env* products were detected and both display a staining pattern that is expected for each protein. The ΔSV.GAG transfected cells show the *gag* product throughout the cytoplasm as a result of synthesis form cytoplasmic ribosomes. The SV.KX transfected cells show the *env* gene product localized around the nucleus resulting form synthesis on the RER No attempts were made to quantitate the fluorescence intensities. Unlike that seen in SV.KX infected cells, intracellular fluorescence in ΔSV.GAG infected cells was rarely observed after the first passage of the recombinant viruses and never observed after the second passage. Elucidation of the inability to propagate the vector was studied in detail and will be addressed in Chapter VI. However, since *gag* expression could not be analyzed in infected cells, the characterization of the *gag* antigens could only be studied in transfected cells.

Metabolic Labeling and Immunoprecipitations

To characterize the *gag* antigens expressed in CV-1 cells and to determine the optimal time of expression, nearly confluent monolayers (60 mm plates) were cotransfected with vector DNA (either SV.KX or

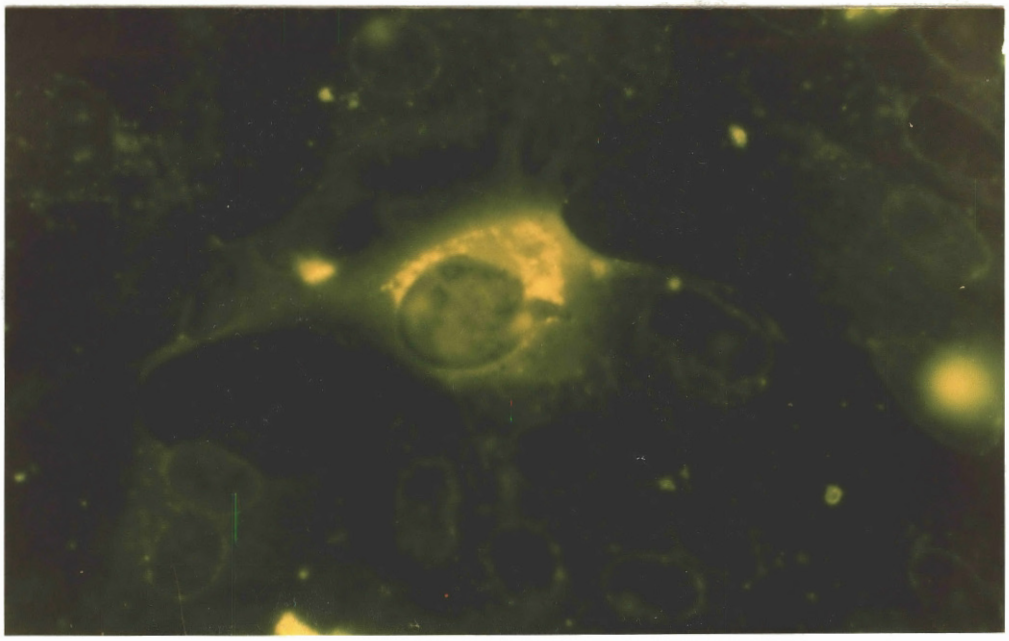

Figure 9.A

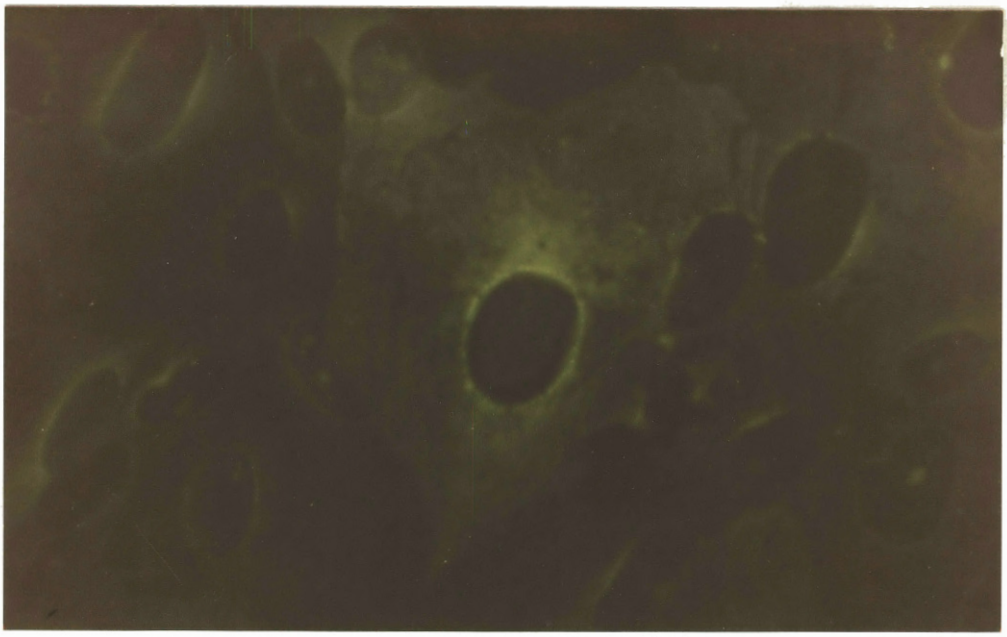

Figure. 9.B

Figure 9. Intracellular immunofluorescence of CV-1 cells cotransfected with helper and: 9.A, △SV.GAG at 45 hr post-transfection, and 9.B, SV.KX at 45 hr post-transfection. The antibodies used to detect the antigens were a rabbit anti-p27 and rabbit anti-gp85 respectively.

△SV.GAG) and helper DNA. At various times post-transfection (15, 36, 53, and 77 hr) the monolayers were labeled with 200 uCi [^{3}H] leucine for 15 minutes and lysed. The *gag* specific antigens were collected by immunoprecipitation using the same antisera as above and separated by electrophoresis in a 10% SDS polyacrylamide gel. The labeled products were analyzed by fluorography. Figure 10 shows Pr76 to be the only *gag* protein immunoprecipitated with the anti-p27 antiserum (lanes 1-4). The *env* precursor polypeptide, Pr95, was also the only peptide detected after the 15 min pulse (lanes 5-8). This figure also shows that maximum expression of both Pr76 and Pr95 occurs around 50 hr post-transfection. The dark bands at the bottom ant top of the gel are cellular proteins nonspecifically precipitated by the Staph A.

To determine whether or not the *gag* precursor polypeptides were processed in mammalian cells, CV-1 cell monolayers were labeled, 48 hr after transfection, with TRAN^{35}*S*-LABEL for 2.5 hr then lysed. Also, the cell culture fluid from the same monolayers were collected and both the cell lystes and culture fluids were assyaed for the presence of *gag* or *env* antigens by immunoprecipitations and 10% SDS-polyacrylamide gel electrophoresis. One such fluorogram is shown in Figure 11.

After 2.5 hr, labeled Pr95^{env} could easily be detected in the cell lysate and the larger of the two processed forms of Pr95, gp85, was barely detectable (Fig. 11.A, lane 1). The slight appearance of gp85 at 2.5 hr is consistent with observation that 50% of Pr95 is processed 2 to 3 hr after pulse-labeling CV-1 cells (Wills et al., 1984). Also consistent with Wills et al. (1984) is that nether Pr97 nor gp85 is réleased into the culture fluid (Fig. 11.B, lane 1). The

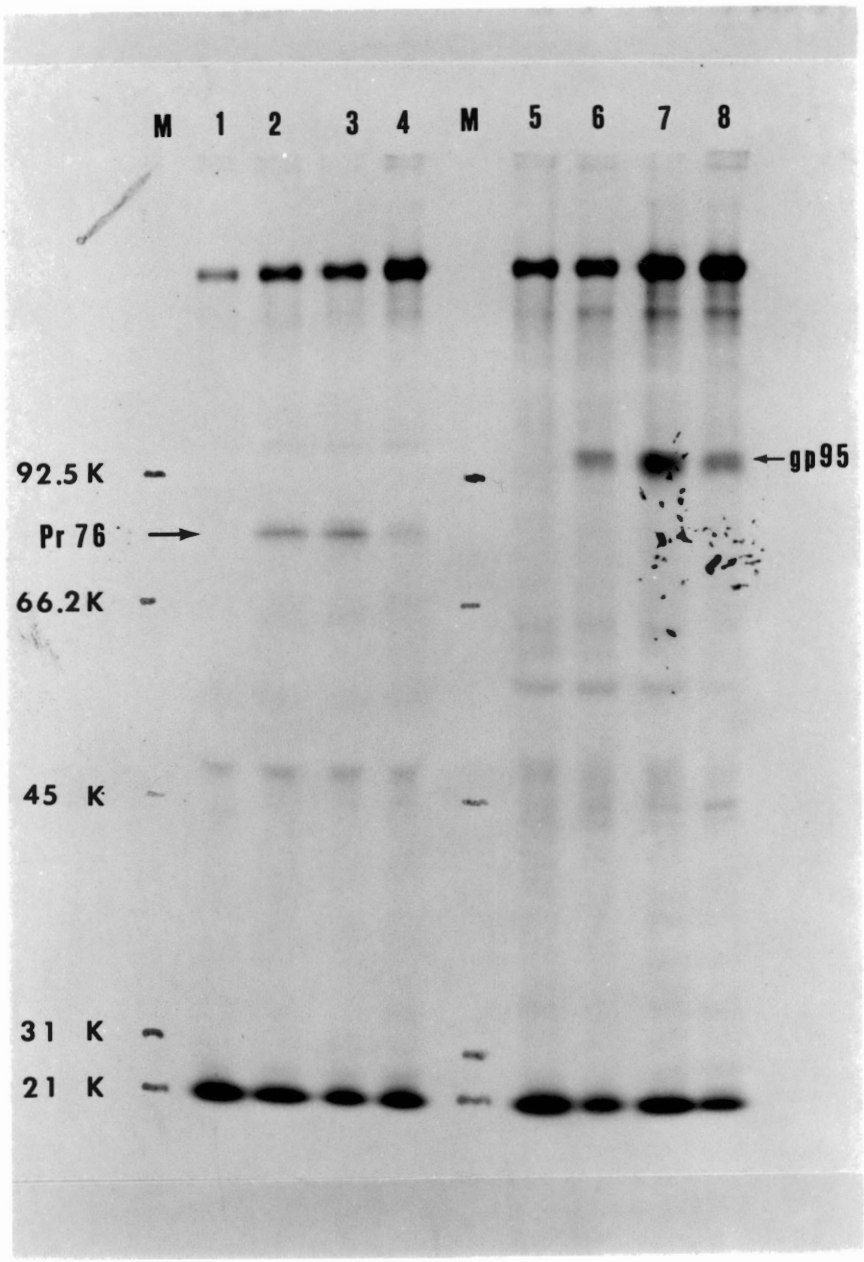

Figure 10. Characterization of the polypeptides immunoprecipitated from CV-1 cells transfected with the recombinant *gag* and *env* vectors. Cotransfected cells were pulse-labeled at various times after transfection and lysed. Samples of the lysates were separated by 10% SDS-PAGE and visualized by fluorography. The lanes are defined as follows: M, low molecular weight standards; (lanes 1-4, ΔSV.GAG cotransfected cell; lanes 5-8, SV.KX cotransfected cells) lanes 1 and 5, 15 hr; lanes 2 and 6, 36 hr; lanes 3 and 7, 53 hr; and lanes 4 and 8, 77 hr post-transfection.

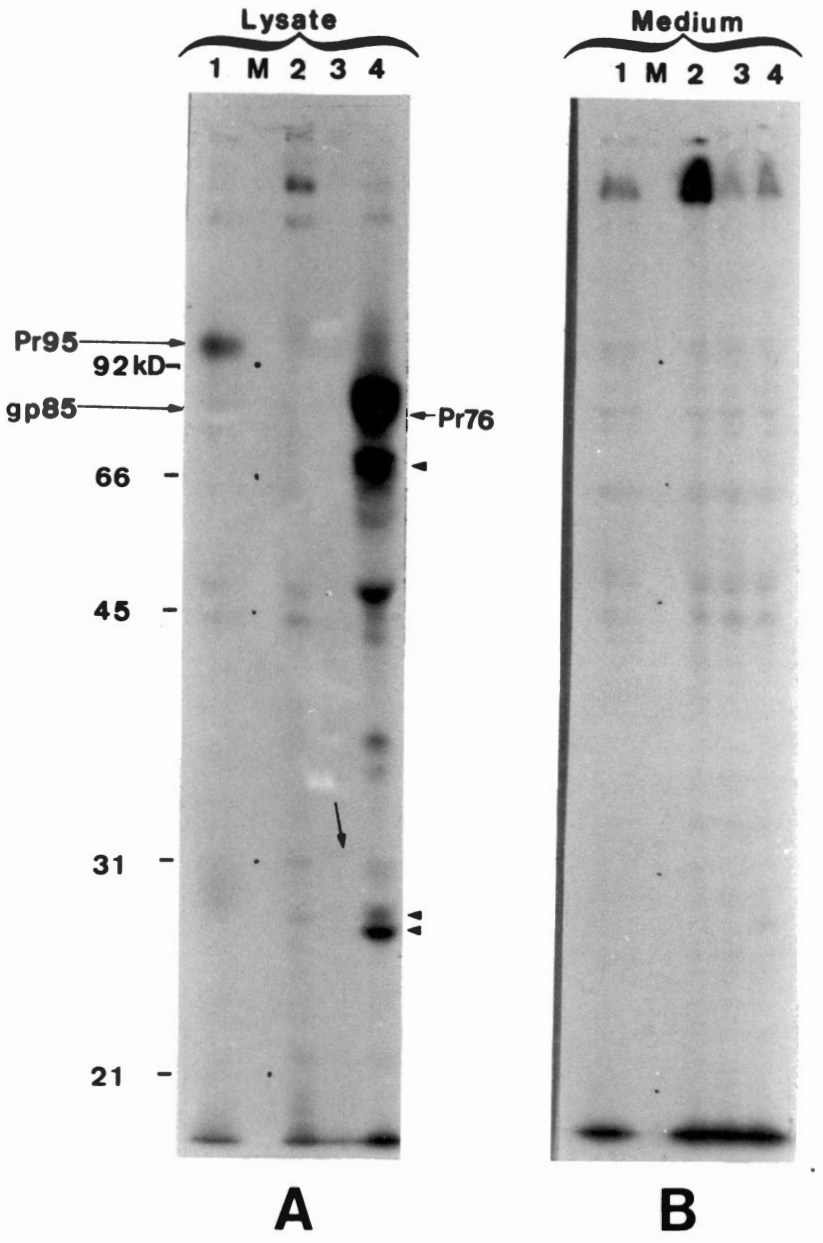

Figure 11. Steady state labeling of transfected cells with TRAN^{35}S-LABEL. CV-1 cells transfected with 1 ug vector DNA only were labeled with [^{35}S] methionine at 48 hr post-transfection and lysed. Immunoprecipitates were analyzed by 10% SDS-PAGE and fluorography. Samples from the lysates are shown in Panel A, medium in Panel B. The lanes are defined as follows: lane 1, SV.KX, M low molecular weight standards; lane 2, uninfected; lane 3, SV.p27, and lane 4, ΔSV.GAG transfected CV-1 cells.

band at the top of all the lanes represents fibronectin or some other host protein nonspecifically precipitated by Staph A. Lane 2 in both panels depicts the protein profile from the lysates and cell culture fliuds from uninfected cells processed in the same manner as all the transfected monolayers and immunoprecipitated with the goat anti-RSV antibody mixture. Likewise, lane 3 in both panels depicts the profile of lysstes and culture fluids of CV-1 cells transfected with 120 ng of an SV40 vector carrying the RSV p27 coding sequence only and 36 ng of helper DNA and immunoprecipitated with the same goat antibody mixture. These lanes help to identify those proteins specific to the transfected cells. The construction of the SV.p27 vector was described earlier. There is a faint band present in panel A, lane 3 (arrow) that has an electrophoretic mobility of approximately 32-33 kDa. This band was visible after longer exposure and may represent a protein initiating at the SV40 agno AUG and terminating dounstream of the p27 coding sequence since it is recognized by the anti-p27 antisera. If this product is a modified p27 product, it has a very short half life when expressed form this vector in CV-1 cells since the amount of protein present is very low.

Lane 4 in both panels depicts those proteins immunoprecipitated with the goat anti-RSV mixture from ΔSV.GAG transfected cells. A diffuse band which migrated with a mobility characteristic of a 76kDa polypeptide was detected. There were also a number of smaller proteins detected that ranged in size form 27kDa to 69 kDa. Comparison of this profile to those of uninfected, transfections (using different vectors *ie.* SV.KX and SV.p27), or ΔSV.GAG transfected cells pulse labeled for 15 minutes (see Figure 10), demonstrated the 76, 68,

28, and 27 kDa bands (arrow heads) to be unique to CV-1 cells transfected with 1 ug of ΔSV.GAG DNA. Lane 4, panel B, clearly shows that no RSV *gag* specific proteins were released into the cell culture fluid.

Inability to Propagate ΔSV.GAG

Although we were able to successfully express Pr76 in transfected cells, repeated attempts to detect the *gag* antigens in infected monolayers failed. This is in contrast to SV.KX which continued to express *env* antigens after three serial passages. (Wills et al., 1984). Because the total size of ΔSV.GAG is slightly larger than the wild-type SV40 genome (100.5%), we initially attributed the inability to propagate ΔSV.GAG to a size packaging limitation. To test this hypothesis, we introduced 3'-end deletions into the *gag* coding region with the double-stranded specific Bal 31 exonuclease. Three clones were chosen for further analysis and the extent of each deletion was estimated by restriction analysis. The end points of these deletions are shown in Figure 7 (black arrows). These clones are approximately 82%, 88%, and 96% of the size of the wild-type SV40 genome.

To determine the effect of these deletions on the ability to propagate the mutant vectors, the vector DNAs were cotransfected with helper DNA into nearly confluent monolayers as previously described. The amount and type of SV40 DNA present in the transfected monolayers 7-days post-transfection was determined by extracting the circular DNA according to the method of Hirt (1967). As shown in Figure 12, SV.KX was the only vector capable of propagating in the transfected monolayers (lane 7). The major 3 kb band was determined to consist of

both SV.KX and helper (d11055) DNA by restriction analysis (data not shown). The inability to propagate ΔSV.GAG or any of the BAl 31 deletion mutants was repeatedly observed after numerous transfections (over 60).

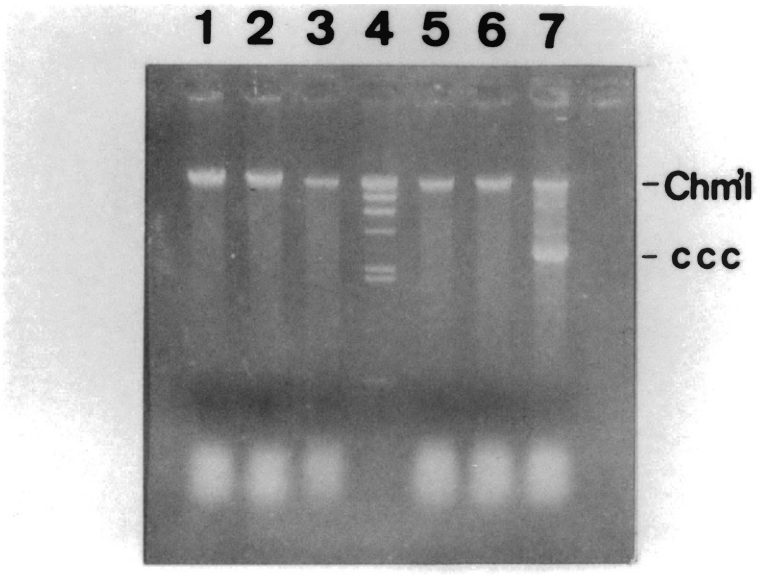

Figure 12. Samples of Hirt extracts from cells cotransfected with 3'-end deletions in *gag* which were created with the exonuclease Bal 31. Samples were taken seven days post-transfection. Lane 1) Smallest *gag* deletion (96% of the size of SV40; lane 2) medium *gag* deletion (88%); lane 3) smallest *gag* deletion (82%); lane 4) Marker DNA; lane 5) untransfected cells; lane 6) ΔSV.GAG tranfected cells; 7) SV.KX transfected cells. Chromosomal (Chm'l) and covalently closed circular (ccc) DNA are indicated.

To evaluate the possibility that Pr76 might be toxic to the transfected cells and therefore inhibit propagation, a +1 frameshift

was introduced at a unique Xho I (nucleotide position 630 in RSV) in the p19 coding region (see Fig. 7). The pΔSV.GAG DNA was linearized with Xho I and blunt ended with the Klenow fragment of *E. coli* polymerase and dNTPs. The DNA was circularized by blunt-end ligation (which creates a new Pvu I site) and transformed in to *E. coli* DH-1 cells (see Material and Methods). Transformants were picked and the plasmids were screened for the loss of the Xho I site and the creation of a new Pvu I site (data not shown). The 4 bp insertion should eliminate expression of all Pr76 except for about 50% of p19.

We tested the stability of this mutant in the SV40 vector by cotransfection and subsequent DNA extraction 7 days post-transfection. Figure 13 shows that mutants defective for Pr76 synthesis are also unstable in CV-1 cells and gave rise to wild-type SV40 after 8 to 10 days (data not shown).

The possibility that the "toxicity" might be due to a truncated p19 product in the frameshift mutants was examined. Also, the RSV splice donor site in p19 might allow aberrantly spliced messages that could code for products toxic to the CV-1 cells or inhibit SV40 replication. The 5′ region of *gag* which contains the Pr76 AUG start codon was therefore deleted by removing the Kpn I-Xho I fragment of pΔSV.GAG. This 427 bp fragment was removed by digesting with Kpn I and Xho I. The remaining 8600 bp fragment was gel purified and circularized by blunt-end ligations with the *E.coli* Klenow fragment, dNTPs, Polymerase I and ligase and ATP (see Material and Methods). Three positive clones were choosen and tested for their ability to be propagated previously described.

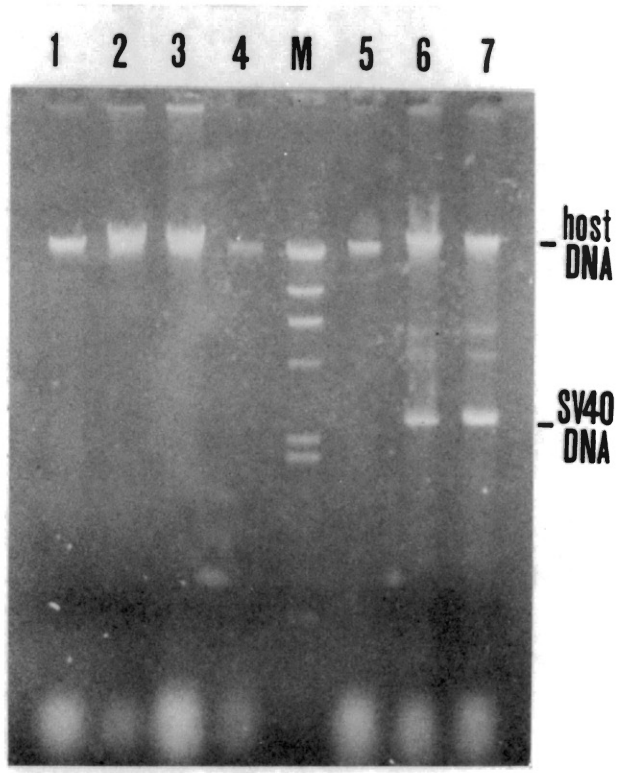

Figure 13. Recombinant SV40 vector DNA carrying a +1 frameshift mutation near the 5′ terminus of the *gag* gene were cotransfected along with helper DNA into CV-1 cells and seven days later, Hirt extracts were obtained. Samples of these DNAs were analyzed by agarose gel electrophoresis to determine if the vectors had replicated. The lanes are defined as: 1) untransfected cells; 2) Frameshift mutation of ΔSV.GAG; 3) Frameshift mutation of the largest *gag* Bal 31 mutant clone #1; 4) frameshift mutation of large Bal 31 mutant clone #2; M) Lambda DNA digested with Hind III (standards); 5) frameshift mutation of smallest Bal 31 mutant; 6) SV.KX; 7) Frameshift mutation at 5′ Xho I site of SV.KX.

Hirt extractions were performed 9 days post-transfection. Since the efficiency of transfections is low and varies with the confluency of the monolayer and other not well defined parameters, the time at which the monolayers are sacrificed was based on SV.KX's ability to induce major cytopathic effects. Figure 14 shows that the mutant vectors were unstable in CV-1 cells (lanes 5-7) and only SV.KX (lane 2) was able to replicate. The faint 3 kb band in lanes 1, 5, 6, and 7 was determined by restriction analysis to be wild-type SV40 DNA that probably arose from homologous recombination between the vector and helper DNA (data not shown).

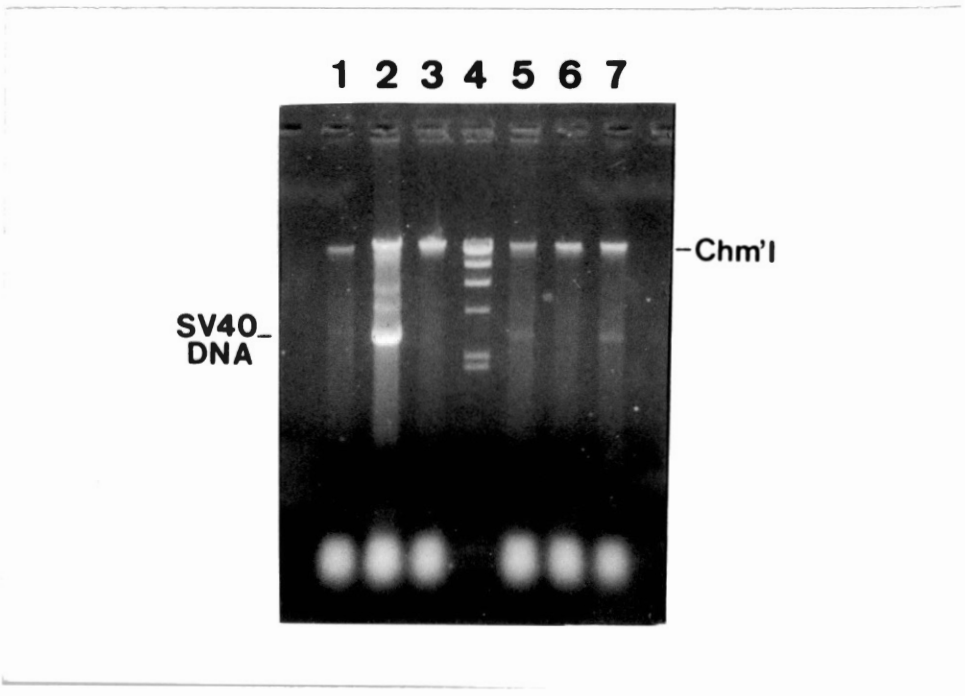

Figure 14. Hirt extracts of CV-1 cells cotransfected with the 5'-end deletions. Samples were taken nine days post-transfection. Lanes are as follows: 1) ΔSV.GAG; 2) SV.KX; 3) untransfected cells 4) marker DNA; 5) 5'-end deletion #1; 6) 5'-end deletion #2; 7) 5'-end deletion #3.

CHAPTER V

DISCUSSION

Von der Helm et al. (1980) and Vogt et al. (1982) demonstrated that RSV infected mammalian cells expressed very low levels of the RSV "particle making machinery," $Pr76^{gag}$, and failed to process the precursor molecules into the mature structural proteins. To determine whether or not the block to replication of RSV in mammalian cells is actually due to insufficient levels of transcription or translation of Pr76, the SV40 late region replacement vector was chosen to express the RSV *gag* gene in a transient expression system in order to increase the levels of Pr76 in mammalian cells. If increased expression of Pr76 results in precursor processing, it would suggest that specific avian products or avian protein sorting signals are not required for correct targeting of the precursor molecules to the site of assembly. It would also suggest that elevated concentrations of Pr76 are essential for the intermolecular interactions needed for processing. The results in Chapter IV demonstrated the usefulness of the SV40 late region replacement vector in expressing elevated levels of $Pr76^{gag}$ in mammalian cells.

Protein Characterization

It has been demonstrated that CV-1 cells, cotransfected with either ΔSV.GAG or SV.KX and helper DNA, were capable of expressing

high levels of the respective *gag* or *env* antigens when assessed by intracellular immunofluorescence. The staining pattern in ΔSV.GAG-helper cotransfected cells, shown in Figure 9.A, is consistent with the observations that $Pr76^{gag}$ is synthesized on cytoplasmic ribosomes (Lee et al., 1979 and Purichio et al., 1980). Similarly, the pattern in the SV.KX-helper cotransfected cells shows $Pr95^{env}$ (or gp85) localized around the nucleus. Wills et al. (1984) determined that this "halo" was actually *env* polypeptides in the rough endoplasmic reticulum and the continuous nuclear membrane.

The immunofluorescence also showed numerous cells which were not transfected. Those transfected cells which were labeled with the fluorescein conjugated antisera, are much larger than the uninfected cells and have an enlarged nucleus typical of SV40 infected cells (Martin, 1981).

The antigens recognized in the transfected cells by the antiviral antibodies were determined by immunoprecipitation of the cell lysates which had been pulse-labeled for 15 minutes. It was demonstrated that the major antigens selectively precipitated were either $Pr76^{gag}$ or $Pr95^{env}$. The appearance of only the precursor products was expected since processing is known to occur post-translationally. Since it was repeatedly observed that the *gag* antigens could not be detected in <u>infected</u> monolayers, precursor processing could only be determined by transient expression in the transfected monolayers. To achieve steady state protein labeling needed for the characterizations, monolayers, labeled for 2.5 hr were transfected with eight times as much vector DNA as normally used and no helper DNA was added. Figure 11 showed that $Pr95^{env}$ was easily

detected in SV.KX transfected monolayers whereas gp85 was only slightly visible on the autoradiogram. These results are consistent with Wills et al. (1984) who demonstrated that Pr95 processing begins around 2.5 to 3 hr post-infection. It can be concluded then that the expression system was working correctly

The protein profile observed after ΔSV.GAG transfection demonstrated that the majority of the antigens present in the ΔSV.GAG transfected monolayers was in the precursor form. It was observed however that a few bands were present in these profiles which had not been previously reported in mammalian cells expressing the RSV *gag* products when protease inhibitors were added to the lysis buffer. These three bands with estimated molecular weights of 27, 28, and 68 kDa are unique to the ΔSV.GAG transfected cells and may represent authentic precursor processing (albeit very inefficient).

Even though Pr76 processing was observed in mammalian cells, it is not clear why it was such an inefficent processing it would be expected that all the precursor would act in a simialar manner. The results presented here suggest that only a small portion of the molecules are capable of being processed. A possible explanation for these results might be that the 76 kDa band observed in the immunoprecipitates of the pulse labeled cells (Figure 10) was not homogeneous population. In fact, the predicted nucleotide sequence of the vector reveals two start codons upstream of the *gag* start codon. The first AUG, which is used to initiate translation of the SV40 *agno* protein, lies in a good initiation context according to Kozak (1986) and lies 142 bp upstream of and out of frame with the *gag* start codon. Initiation at this codon would cause the ribosomes to bypass the *gag*

AUG codon and probably terminate just down stream of the Pr76 AUG codon at any of the stop codons in frame with the agno AUG. This however does not explain the possible heterogenity. If this proposed event did occur and the ribosomes were able to reinitiate, ribosomes might be able to reinitiate translation at the next AUG codon. This second start codon lies 28 codons downstream of and in frame with authentic Pr76 AUG codon. Reinitiation at this codon would result in a polypeptide 28 amino acid shorter than the full length Pr76 molecule. This truncated precursor might possibly share similar antigenic determinants of Pr76 thus its presence in the immunoprecipitates. If the important control signals for targeting or processing are located at or near the amino terminus of the precursor molecule, the truncated protein would not be expected to be processed or released from the transfected cells.

Assuming an average of 100 daltons per amino acid, the truncated product should be approximately 2.5 to 3 kDa smaller than the full length precursor molecule. Figure 10 in fact revealed a very faint band just slightly higher than the labeled 76 Kda band. This larger product may represent the full length precursor molecule and the smaller may represent the truncated form. The reason the smaller band is more pronounced may be explained by Kozak's rules which, based on the context of the sequences surrounding the different AUG codons, suggest the *agno* start codon would be used more often than the Pr76 start codon.

Initiation at the second codon upstream of the *gag* AUG would not be expected to result in a truncated precursor molecule since there is

an in frame stop codon upstream of the *gag* AUG. However, initiation at this upstream AUG would further results in diminished tranlsation initiation at the authentic *gag* AUG codon.

The results shown in Figures 10 and 11 are inconsistent with those reported by Vogt et al. (1982). They reported that only the precursor form of the *gag* proteins was present in several mammalian cell lines transformed with RSV. They suggested that in those mammalian cell lines where the *gag* precursor seemed to be autocatalytically processed (Fleissner, 1970 and Ghysdael et al., 1977), processing was actually due to nonspecific protein degradation that occurred immediately upon cell lysis. They demonstrated that they could inhibit this nonspecific or host regulated processing by the addition of the sulfhydryl-blocking reagent *N*-ethylmaleimide (NEM). It is unlikely that the smaller molecular weight proteins observed resulted from nonspecific protein degradation since the cells were lysed in the presence of the protease inhibitors phenylmethylsulfonyl fluoride and pepstatin. Also, if the processing observed in Figure 11 was due to nonspecific degradation, the same break down products should have been observed in Figure 10. However, nonspecific degradation can not be ruled out since NEM was not included.

There are a couple of explanations as to why processing has never been reported in most mammalian cells since Chapter IV established that processing can occur (albeit very inefficiently) in mammalian cells upon increased expression of $Pr76^{gag}$. In all of the reports to date which suggest that processing does not occur in mammalian cells, the investigators were studying RSV transformed cells which had been shown to express very low levels of Pr76. Therefore,

the only way to even detect Pr76 in mammalian cells would be to increase the actual number of transformed cells being assayed and use more sensitive detection methods like western blotting. Since processing is such an inefficient event in mammalian cells, merely increasing the amount of Pr76 to just detectable levels would not reveal the processed intermediates or the final products. Another explaination is that some inherent intracellular concentration of Pr76 is required for processing and is not obtained in RSV infected mammalian due to inefficient expression.

Since processing is a rare event in RSV transformed mammalian cells but can be induced upon increase expression, there must be some type of processing signal or pathway specific for avian cells that differs from that in mammalian cells. One such possible signal present in only the mammalian retroviruses may be the addition of a myristic acid to a glycine residue at amino acid position number two. In fact this addition is essential for Moloney murine leukemia virus precursor processing and particle formation (Rein et al., 1986).

Even though it has been shown that Pr76 can be processed (albeit inefficently), it can not be determined from these experiments whether or not *gag* specific products were released from transfected mammalian cells. The *gag* products (full length Pr76 or processed forms) might have been release but have escaped detection by metabolic labeling and immunoprecipitation. Likewise, previous reports (Volker et al., 1982) which showed that neither RSV *gag* specific antigens nor virus particles are capable of being released from RSV transformed mammalian

cells should be reconsidered since low levels of Pr76 expression in transformed cells would not give rise to detectable levels of antigens or reverse transcriptase activity in the cell culture fluids.

According to one model, the apparent inability of Pr76 to be correctly processed in mammalian cells is a result of an inability to correctly assemble virus particles (a prerequisite to processing). Another model suggest that processing is a prerequisite to budding. Therefore the block to particle formation in mammalian cells is due to an inhibition of precursor processing (Dickson et al.,1982). A third model suggests that cleavage of Pr76 can only occur in the final stages of, or subsequent to, the budding of virus particles from the plasma membrane. Thus, if Pr76 were unable to interact with the plasma membrane correctly, then it might not be able to achieve the right conformation required for processing and subsequently budding (Vogt et al., 1982). The results presented here suggest that the later model is probably more correct but not entirely true. Our results suggest that a subpopulation of Pr76 molecules (ones that are full length) are capable of being processed in mammalian cells. These results however can not distinguish between either processing or budding being a prerequisite to the other. One plausible explanation is that myristic acid addition is required to achieve the correct conformation required for budding or processing since mammalian retroviruses which are not modified act just like Pr76 in mammalian cells (Schultz and Oroszlan, 1983 and Rein et al., 1986). Although these results are highly suggestive of precursor processing, more detailed experiments are required to demontrate that the lower molecular weight polypeptides are authentic processed *gag* proteins.

Inability to Propagate ΔSV.GAG

Although we were able to successfully express Pr76 at high levels in mammalian cells upon transfection, we were unable to propagate the vector on fresh monolayers. Our first hypothesis was, since the size of the vector genomes was 100.5% of that of the wild-type SV40 genome, the vector was incapable of being packaged to due a size constraint. We therefore created a series of end-point deletion mutations that ranged from 82% to 96% of the SV40 genome. These deletion mutants were also unstable in the transfected monolayers.

Another possibility was that the RSV *gag* products were either lethal to the mammalian cells or to the SV40 replicative strategy. To test this hypothesis, two different mutations were created that abolish Pr76 synthesis. The first mutation was created by introducing a +1 frameshift at a unique Xho I site located in the middle of the p19 coding region. This construction, confirmed by restriction analysis, was shown to be just as unstable as ΔSV.GAG in the transfected monolayers.

This altered vector might be making a detrimental product such as a truncated p19 peptide or some other protein arising from an spliced message since the splice donor site is still intact. We therefore constructed another vector in which the Kpn I-Xho I fragment was removed. This vector should be unable to synthesize any *gag* related product since the SV40 *agno* and Pr76 start codons were removed. This vector should also be unable to transcribe any mRNAs from this region since the SV40 late promoter ha been altered. This construction however was also unstable in the SV40 vector. We therefore con-

clude that the inability to propagate the ΔSV.GAG vector lies at either the DNA or RNA level since other constructions (SV.KX) which do not contain the *gag* sequences are fully capable of being propagated in CV-1 cells. This inability to propagate the ΔSV.GAG vector was studied in detail and is addressed in Part II of this thesis.

PART II.

ELUCIDATING THE CAUSE OF THE BLOCK TO ΔSV.GAG REPLICATION

CHAPTER VI

RESULTS

Elucidating the block to ΔSV.GAG Propagation

Time Course Hirt Extraction

As shown in Part I, neither ΔSV.GAG nor helper (dl1055) DNA could be detected in CV-1 cells 5 to 7 days post-transfections when Hirt extracts were analyzed on ethidium-bromide stained agarose gels. This could be accounted for by a number of explanations. If the efficiency of packaging was low, the DNA would be lost after a couple of rounds of virus replication in the transfected monolayers. As a consequence, the helper DNA would also be lost due to a lack of T Ag. To test this hypothesis, cotransfected monolayers were sacrificed at various times post-transfection (25, 62, 104, and 177 hr) and the SV40 DNA was purified by Hirt extractions in an attempt to find the time at which the recombinant SV40 DNA was lost in the monolayers.

Figure 15 shows that neither ΔSV.GAG nor dl1055 DNA could be detected on ethidium-bromide stained agarose gels at any time post-transfection (gel A, lanes 1-4). In contrast, gel B shows that both SV.KX and dl1055 DNAs replicated in the monolayers and was detectable by 62 hr (gel B, lane 2). The amount of DNA in the monolayers greatly increased by 104 hr. By 177 hr, the time at which the greatest cytopathic effects were observed, the amount of DNA present in the monolayers reached its maximum. Hirt extracts at longer times

after transfection generally yield lower amounts of DNA since the monolayers were completely destroyed by the infection. The time of vector DNA replication (after 28 hr) in transfected cells is consistent with that observed with infected cells (gel A lanes 5-7).

COS-1 Cell Cotransfections

The above experiments suggest that the inability to propagate the *gag* vectors is due to an inability to assemble recombinant SV40 virus since no SV40 DNA (vector or helper) was detected even after one or two rounds of viral replication unlike SV.KX which was detectable by 62 hr. Another possible explanation for this inability to propagate SV40-gag vectors might be that the insertion of the *gag* coding region, which contains a directional enhancer (Arrigo et al., 1987) into the SV40 genome alters T Ag expression and subsequently alters DNA replication and late gene expression. In order to eliminate this possibility, vector DNAs were cotransfected into COS-1 cells, a cell line constitutive for T Ag expression. T Ag expression in these cells was confirmed by intracellular immunofluorescence using a monoclonal antibody against the T Ag expression and subsequently alters DNA replication and late gene expression. In order to eliminate this possiblity, vector DNAs were cotransfected into COS-1 cells, a cell line constitutive for T Ag (data not shown).

Transfected COS-1 cell monolayers were sacrificed and assayed for SV40 DNA after 4 and 7 days. Figure 16.A, which represents 5% of the extract, shows a prominent band of about equal intensity in both the ΔSV.GAG and SV.KX cotransfected samples at both 100 and 170 hr (lanes 1, 2, 4, and 5).

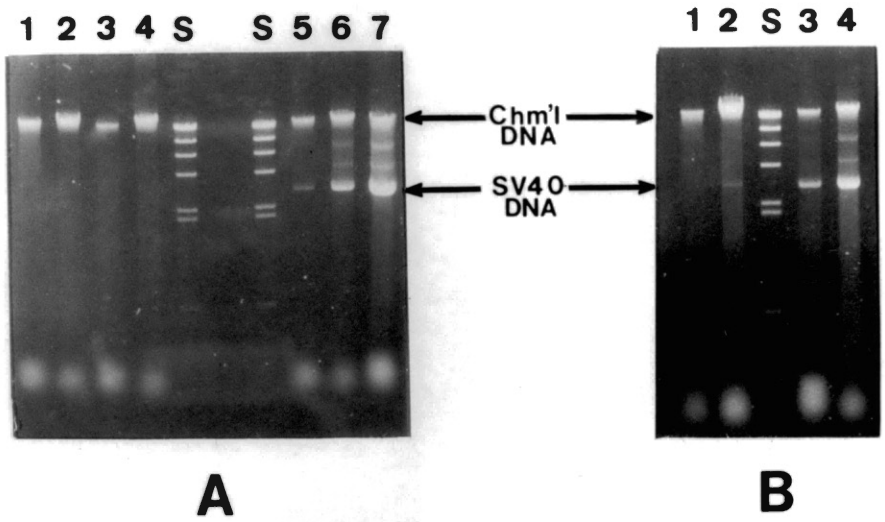

Figure 15. Time course Hirt extracts. CV-1 cells were either cotransfected with ΔSV.GAG or SV.KX and helper DNA or infected with SV.KX. Cells were lysed and SV40 DNA was extracted. Panel A, lanes 1-4, ΔSV.GAG transfected cells, lanes 5-7, SV.KX infected cells, Pabel B lanes 1-4, SV.KX transfected cells. Lanes 1 (both Panel A and B), cells lysed after 25 hr; lanes 2, cells lysed after 62 hr; lanes 3, cells lysed after 104 hr;, lanes 4, cells lysed after 177 hr. Lanes 5-7 (Panel B) are extracts from cells infected with SV.KX and lysed at 28, 62, and 104 hr post-transfection.

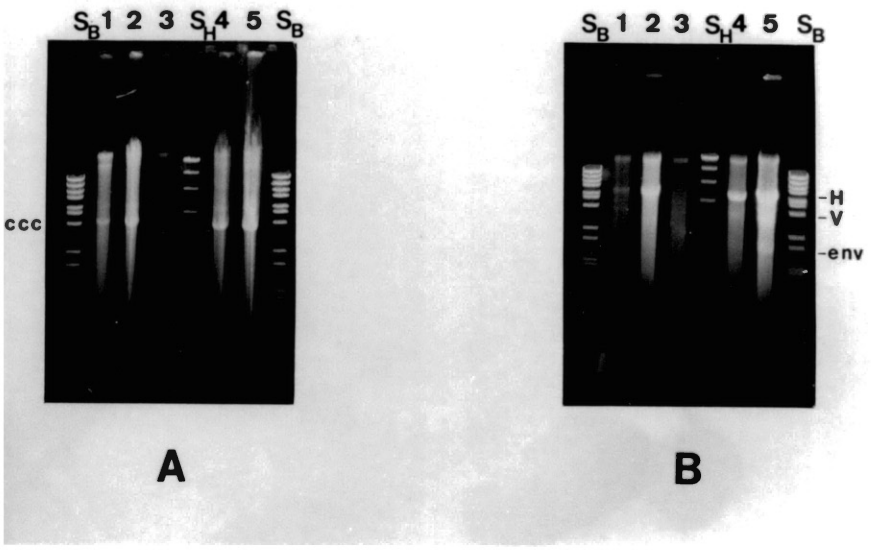

Figure 16. Hirt extracts from cotransfected COS-1 cells. To test the ability of the recombiant vectors to replicate with constitutive T-Ag expression, COS-1 cells were cotransfected and lysed at various times. Panel A depicts samples from Hirt extracts of ΔSV.GAG cotransfected cells after 4 days (lane 1) and 7 days (lane 2) and SV.KX cotransfected cells after 4 days (lane 4) and 7 days (lane 5). Panel B demonstrates the type of DNA present in the samples in Panel A. The same amount of sample was subjected to restriction enzyme digestion (see text for specific enzymes used) to separate the helper DNA from the vector and insert DNA. Again lanes 1 and 2 are samples from ΔSV.GAG tranfected cells at 4 and 7 days post-transfection and lanes 45 are samples from SV.KX transfected cells at 4 and 7 days post-transfection respectively. "H" is helper (dl1055) DNA. "V" is vector sequences and likewise, "env" is RSV *env* sequences

The type of DNA present in these Hirt extracts was determined by digesting 5% of the extract with Kpn I and Xba I. This double digest only linearizes the helper DNA (5000 bp) and releases the RSV *gag* sequences (2528 bp) or *env* sequences (1800 bp) from the vector (3000 bp). Figure 16.B shows that only one 5000 bp band was present in the ΔSV.GAG-helper extracts (lanes 1 and 2). In contrast, lanes 4 and 5 show that all three sequences (helper, vector and *env*) are present. Lane 3 (uninfected) again shows that these bands are unique to the transfected cell extracts. The possibility remains that the linear band present in lanes 1 and 2 might be wild type SV40 DNA that arose from homologous recombinantion between the vector and helper DNA molecules. However, it is unlikely since wild type SV40 was never detected in this system after only four days especially at these intensities.

Characterization of the Vector's
Replication Cycle

In order to determine whether or not the recently described directional enhancer element located in the p10 coding region of the RSV *gag* gene or some other element in ΔSV.GAG might be interfering with the SV40 replicative strategy and thus inhibiting vector replication, the replicative cycle (DNA replication and late gene expression) of ΔSV.GAG was characterized. One way to assess early gene expression was to determine the timing of vector DNA replication since SV40 DNA replication is dependent upon T Ag (Freid and Prives, 1986).

Vector DNA Replication. Since it has already been established that the vector ΔSV.GAG is not capable of producing infectious progeny virions, it was of interest to determine whether or not the vector DNA was replicating in those cells which were actually transfected. If so, it was important to characterize the timing of DNA replication to assess any alterations in the timing of SV40 DNA replication invoked by the vector sequences. However, since only very few of the cells in a monolayer are capable of being transfected, DNA replication could only be addressed by Southern hybridizations. This technique also allows differentiation of helper DNA from vector DNA by hybridizations to specific probes. The probe used to differentiate between the helper genome and the ΔSV.GAG genome was the plasmid pAT.p27 which contains the plasmid pJC-2 and the RSV p27 coding region. The construction of this plasmid was described earlier. The helper sequences as well as the vector and *env* sequences were detected by hybridizations to the [^{32}P]-labeled plasmid pSV.KX.

In order to discern whether or not the transfected DNAs were replicating, a series of nearly confluent CV-1 cell monolayers (60 mm plates) were cotransfected with either ΔSV.GAG or SV.KX and dl1005 DNA. Any extracellular DNA remaining after the 4 hr chloroquine treatment was degraded by washing the monolayers twice in PBS and then incubating the cells in serum-free Dulbecco's MEM containing 50 ug/ml DNase I for 1 hr at 37°C. The DNAse I was removed by washing the monolayers twice in PBS. The monolayers were then further incubated in growth medium for 15, 27, 40, or 50.5 hr. At the time of sampling, the monolayers were sacrificed and SV40 DNA was purified by Hirt extractions. Eight percent of each extract was run on two 0.8%

Agarose gels containing 0.5 ug/ml ethidium-bromide. The DNA was transferred to nitrocellulose filters by the method of Southern (1975). One filter was hybridized to ^{32}P-nick translated pAT.p27 (approx. 22 uCi). The second filter was hybridized to the ^{32}P-nick translated pSV.KX (approx. 33 uCi). The DNA was allowed to hybridize for 24 hr at 42°C in the presence of 45% fromamide and then washed under high stringency conditions.

Figure 17 shows the autoradiogram of both hybridizations. Gel A was probed with pAT.p27 and Gel B was probed with pSV.KX. The intensities of the bands on both autoradiograms were measured by densitometric scanning at 633 nm. The results of the scans are shown in Figure 18. These figures demonstrate both ΔSV.GAG and SV.KX DNAs begin to replicate around 30 hr post-transfection. The amount of DNA dramatically increases by 40 hr. SV.KX DNA (and helper DNA) continued to exponentially increase by 50.5 hr due to its ability to infect the whole monolayer. The amount of ΔSV.GAG and helper DNA however began to decrease after 40 hr due to degradation and an inability to produce infectious particles. The two bands in Gel A (Fig. 17) lanes 7 and 8 are either the pSV.KX plasmid DNA replicating in the CV-1 cells due to T Ag and the SV40 origin of replication or dimers of the circular vector DNA.

The type of DNA present in these Hirt extracts was determined by digesting the extracts with Kpn I and Xba I. This double digestion linearizes the helper DNA (5200 bp) and releases the SV40 vector sequences (3000 bp) from the *gag* (2528 bp) or *env* sequences (1800 bp). The DNAs were separated on 0.8% Agarose gels and hybridized to the same two probes. Figure 19 shows that all three sequences (helper,

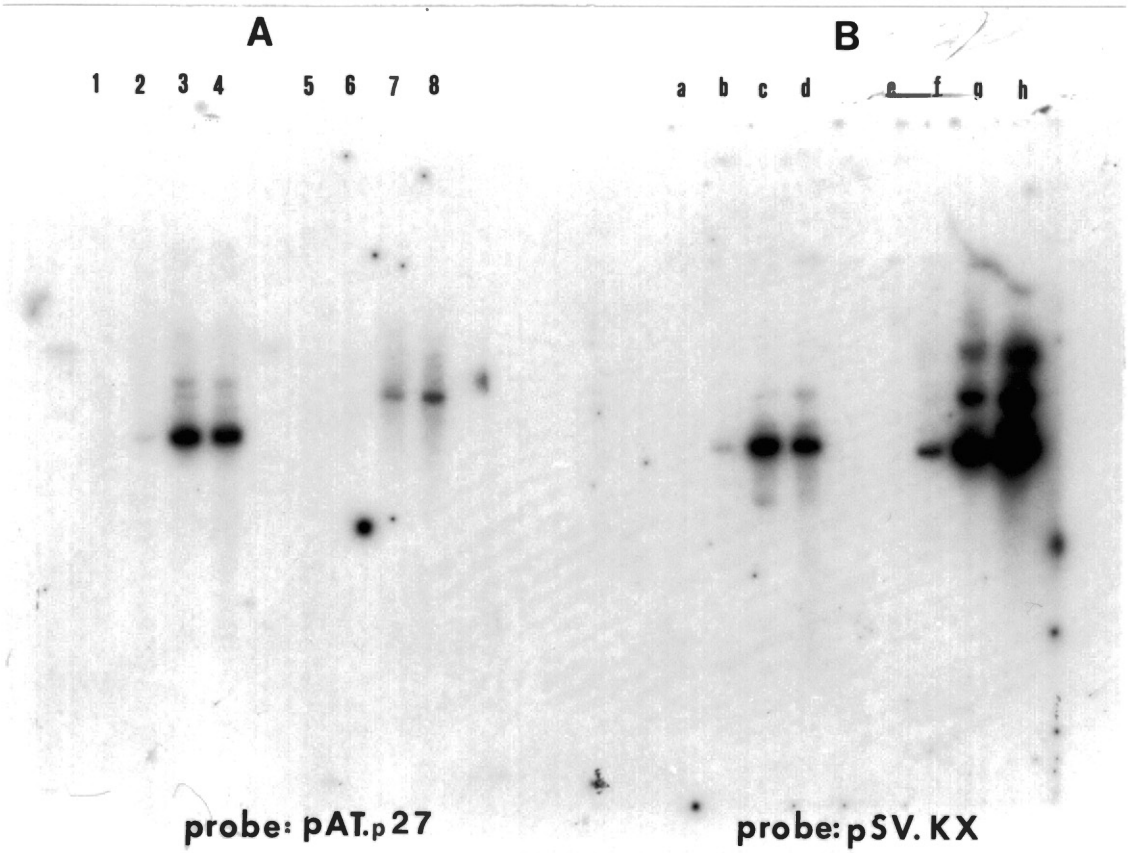

Figure 17. Vector DNA replication assessed by Southern hybridization of Hirt extracts. Vector DNA replication was determined by extracting SV40 from CV-1 cells that had been cotransfected with vector and helper DNA 15, 27, 40 and 50.5 hr before. The DNA was separated by agarose gel electrophoresis and Southern blotted to nitrocellulose filters. ^{32}P-Nick translated probes (either pAT.p27 or pSV.KX) were then allowed to hybridize for 24 hr at 42°C in the presence of formamide. The filters were then washed under high stringency conitions before autoradiography. Panel A depicts samples probed with pAT.p27 which detects p27 sequences only. Lanes 1-4 are samples from ΔSV.GAG cotransfected cell at 15, 27, 40, and 50.5 hr post-transfection. Lanes 5-8 are samples from SV.KX transfected cells taken at the same time (15, 27, 40, and 50.5 hr respectively) post-transfection. Panel B demonstrates SV40 and *env* sequences present in the same samples as those in panel A. Lanes a-d are from the same extracts as lanes 1-4. Lanes e-h are indentical samples as those in lanes 5-8.

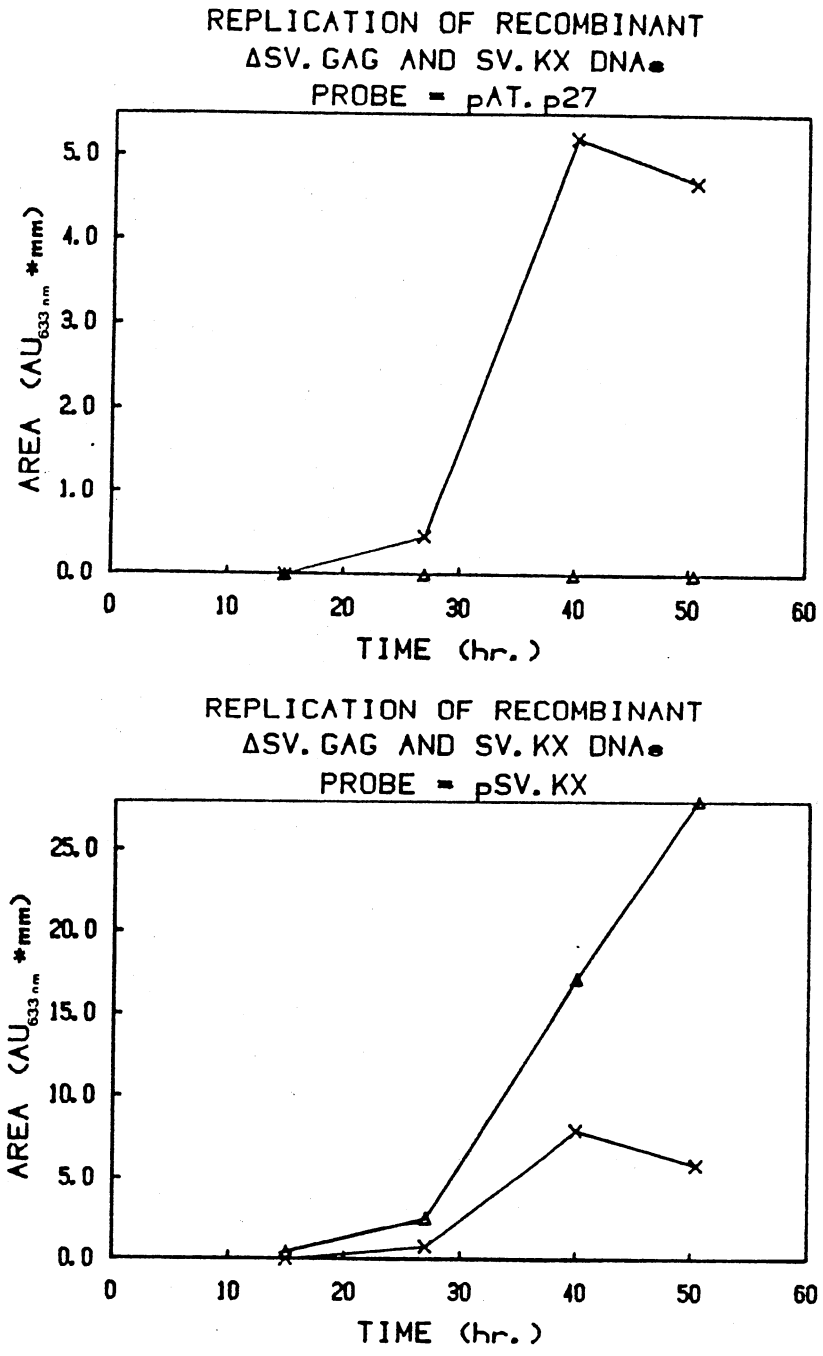

Figure 18. Results from a densitometric scan of the autoradiogram in Figure 17. The plots represent the band intensities of the autoradiogram (aborabance at 633 nm x width of the band) -vs- time after transfection The top scan represents ths scan of Fiter A. The time of ΔSV.GAG DNA replication is shown by (-x-). SV.KX replication (-Δ-). The bottom graph represents the scan from filter B.

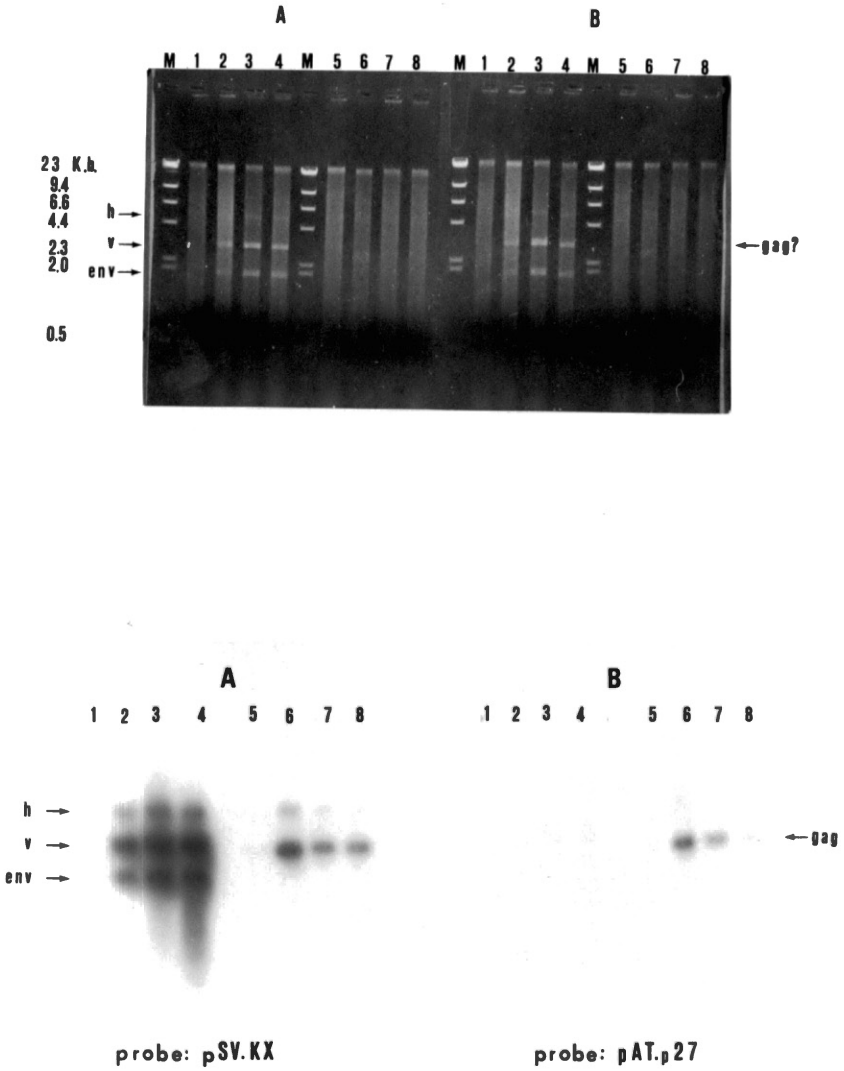

Figure 19. Determination of the type of DNA present in the extracts from Fig 17, The same samples were digested with restriction enzymes to linearize the helper genome and release the vector sequences from either the *gag* or *env* sequences. After agarose gel electrophoresis, the DNAs were transfered to nitrocellulose and probed with the same probes in Figure 17. The top gel is the ethidium stained gel of the autoradiograms below. Lanes 1-4 are samples from SV.KX cotransfected cells after 15, 27, 40, and 50.5 hr. Lanes 5-6 are samples from ΔSV.GAG cotransfected cells after 15, 27, 40, and 50.5 hr respectively. "h" designates helper sequnces whereas "v" designates the vector sequences.

vector, and *env*) from the SV.KX transfections were detected at 27 hr when probed with pSV.KX (Gel A, lane 2). Both helper and vector sequences were also detected at 27 hr in ΔSV.GAG extracts. The *gag* sequences were also detected at 27 hr when probed with pAT.p27 (Gel B). The 3:1 ratio of the amount of vector DNA to helper DNA initially cotransfected was still observed after densitometric scanning of the autoradiogram in Fig. 19, confirming that both vector and helper DNAs were equally replicated (data not shown). These result suggest that, at the DNA level, neither the vector nor the helper DNA had a selectable advantage to replicate in CV-1 cells

Time-Course Immunofluorescent Assays. Since it has been demonstrated that the ΔSV.GAG DNA replicates in the transfected cells, the possibility existed that the timing of late gene expression is altered. To determine this, cotransfected cells were fixed for intracellular staining as previously described at various times post-transfection (23, 35, and 45 hr). Figure 20 demonstrates that Pr76 synthesis begins between 23 and 35 hr post-transfection. SV.KX cotransfected cells showed similar times of expression of $Pr95^{env}$ (data not shown).

Time-Course Pulse Labeling. To further demonstrate the timing of Pr76 and Pr95 synthesis, cotransfected monolayers were pulsed labeled for 15 minutes with [^{3}H] leucine at 15, 36, 53, and 77 hr post-transfection and then lysed. Immunoprecipitates (using the same antisera as above) were separated on 10% SDS polyacrylamide gels and the radiolabeled products were visualized by fluorography. The fluorogram (see Fig. 10) was then analyzed by densitometric scanning

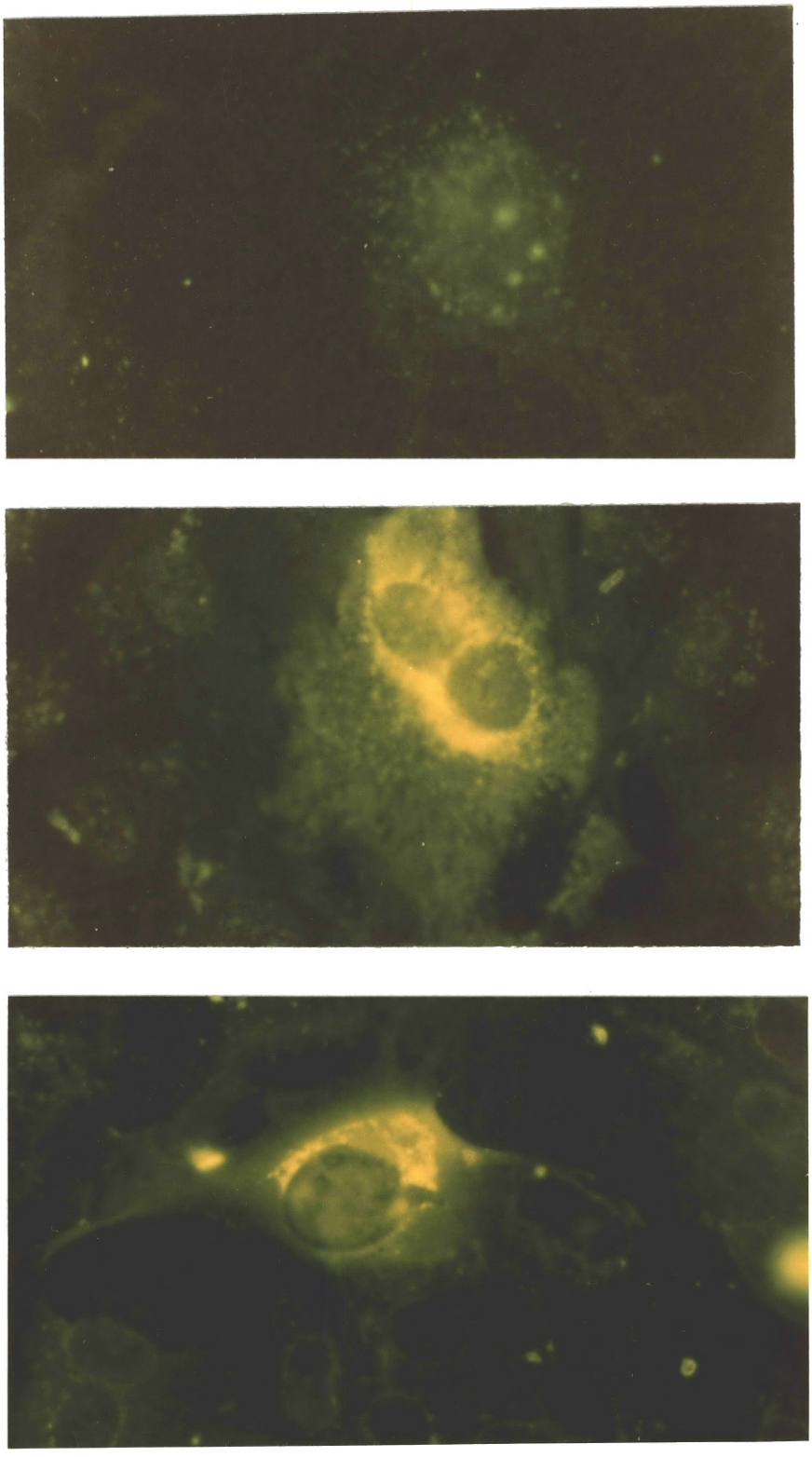

Figure 20. Intracellular immunofluorescence of CV-1 cells cotransfected with ΔSV.GAG after 23 hr (top), 35 hr (middle), and 45 hr (bottom).

at 633 nm using the LKB Ultrascan XL Laser Densitometer. Figure 21 shows SV40 late gene expression of both Pr76 and Pr95 begins prior to 36 hr post-transfection. Maximal expression for both polyproteins occurred around 50 hr and by 77 hr, the levels of expression had decrease probably marking the end of viral replication. The difference in the amount of protein between SV.KX and ΔSV.GAG can be explained by possible differences in translational efficiencies and protein half-lives (see Part I).

DNAse I Protection. Since the inability to propagate ΔSV.GAG did not lie at the protein level nor was it due to insufficient DNA replication or altered gene expression, we tested the ability of the ΔSV.GAG DNA to be packaged into DNase I resistant SV40 particles. It the vector DNA was incapable of being packaged into virions, but the helper was, only the helper DNA should be protected from DNase I.

Figure 22 shows the autoradiogram of a Southern hybridization in which the ability of the vector and helper DNA to be packaged into DNase I resistant virions was tested (see Materials and Methods). Lanes 1 through 4 are samples from a monolayer infected with SV.KX and dl1055 virions at 84 hr post-infection. Lane 1 shows the untreated extract. THe helper DNA (5000 bp) was detected as well as the vector (3000 bp) and *env* (1800 bp) sequences. Lane 2 which only had plasmid DNA added shows the same bands as in lane 1 except for new band (linearized pUC19) between the SV40 and *env* sequences. Lane 3 which had both plasmid DNA and DNase I indicates that the plasmid DNA was susceptible to DNase I while the helper, vector, and *env* sequences were resistant. Also the smear of chromosomal DNA that was present in

lanes 1 and 2 disappears after the DNase I treatment. Lane 4 had only DNase I added and again demonstrates that helper, vector, and *env* were encapsidated into DNase I resistant virions.

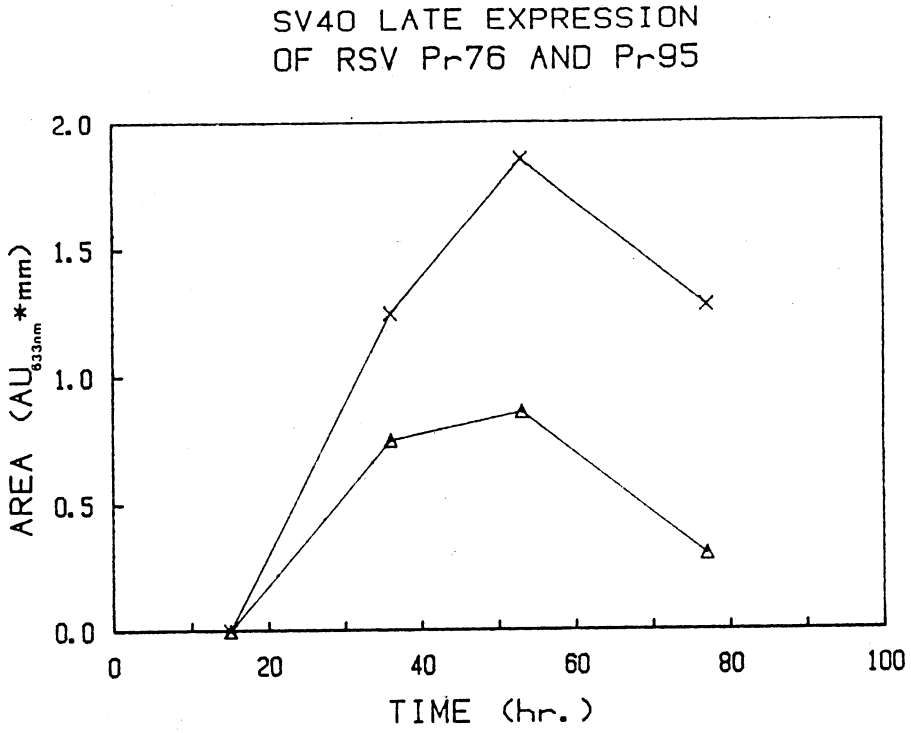

Figure 21. Plots of the densitometric scans of time course pulse-labeling (see Fluorogram in Figure 10), (-x-) represents Pr95 and (-Δ-) represents Pr76.

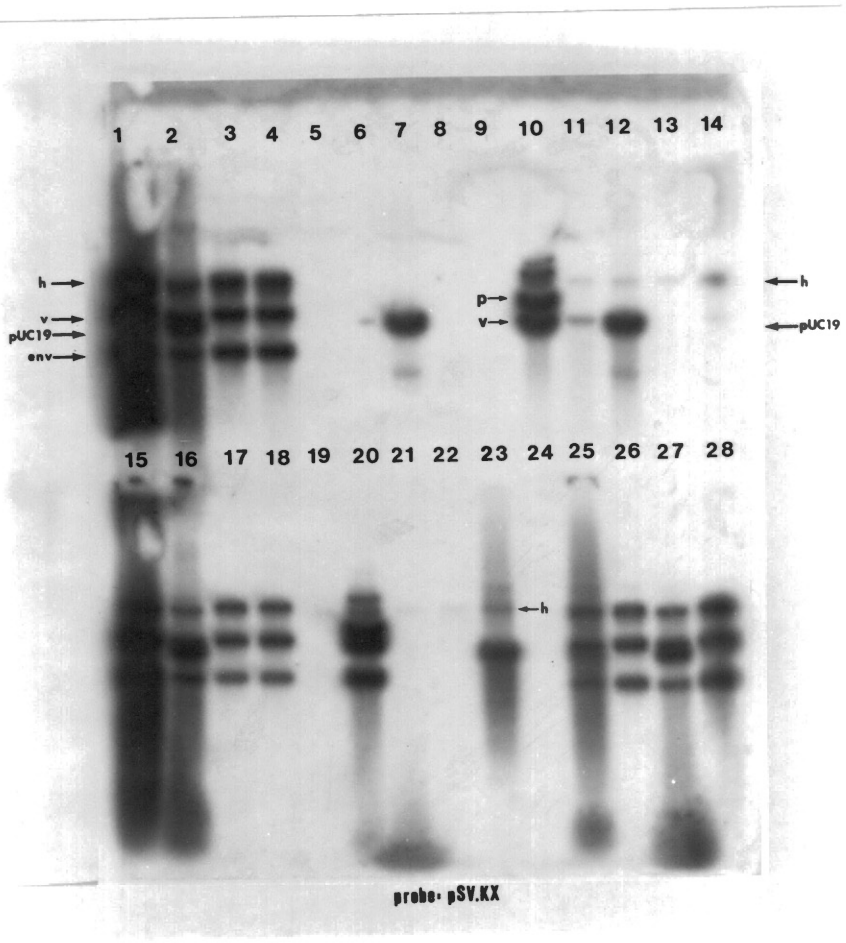

Figure 22. Southern hybridization using ^{32}P-labeled pSV.KX to detect any SV40, *env* or *E.coli* sequences that were resistant to DNase I after the lysates and culture fluid were digested with trypsin/deoxycholate. Lanes 1-4 demonstrate the DNA from SV.KX infected cells. Lane 1 was untreated, lane 2 had pUC19 DNA added, lane 3 had pUC19 DNA and DNase I added, lane 4 had DNase I only. Lanes 6-9 show DNA from untransfected cells. The order of processing was the same as lanes 1-4. Lane 10 was pΔSV.GAG digested with Kpn I and Xba I. Lanes 11-14 are samples from ΔSV.GAG cotransfected cells. Lane 11 untreated samples, lane 12, samples with pUC19 DNA added, lanes 13 and 14 had pUC19 + DNase I and DNase I added respectively. Lanes 15-18 are samples from SV.KX cotransfected cells. The order of processing for lanes 15-18 was the same as before. Lane 19 were DNA markers. Lane 20 was pSV.KX digested with Kpn I and Xba I. Lanes 21-28 are samples collected from the cell culture fluid. Lanes 21 and 22 are from ΔSV.GAG cotransfected cells. Lanes 23-24 are samples from uninfecteced cells lane 23 had pUC19 DNA added only were as lane 24 had both pUC19 and DNase I added. Lanes 25 and 26 are from SV.KX infected cells and processed in the same order as lanes 23 and 24. Likewise, lanes 27 and 28 are from SV.KX cotransfected cells.

all resistant to DNase I. These results are consistant with the observation that the vector, SV.KX, and helper DNA are packaged into infectious SV40 particles as assessed by antigen and DNA detection in infected monolayers. Lanes 6-9 were samples from uninfected CV-1 cells processed and run on the gel in the same order as before. Thus, the major band in lane 7 is linear plasmid DNA. Lane 10 contained pΔSV.GAG DNA digested with Cla I and Xba I to release the vector, plasmid, and *gag* sequences from one another. The figure shows the pJC-2 sequences (3484 bp) and the SV40-vector sequences (3017 bp). The *gag* fragments were present on the ethidium-bromide stained gel but not seen on this autoradiogram because of the probe used. The upper bands are residual open circular DNA resulting from incomplete restriction enzyme digestion. Lanes 11 through 14 show extracts of Δ SV.GAG cotransfected cells at 89 hr. Lane 11, samples which did not receive DNase I or plasmid DNA, show both the helper DNA and the SV40 vector sequence of ΔSV.GAG. The ratio of vector to helper that was originally used for the transfections (3:1) was observed on the densitometric scan. The vector sequence disappears after the DNase I treatment (lanes 13 and 14) where as the helper DNA remains DNase I resistant. These results suggest that ΔSV.GAG DNA was not packaged into virions. This is consistant with the observations that ΔSV.GAG could not be passaged onto fresh monolayers. Lanes 15-18 are extracts from SV.KX cotransfected cells at 89 hr. The order of processing was the same as above. The extracts from the cotransfected cells look the same as the extracts from the SV.KX infected cells. Briefly, all the expected DNase I resistant DNA molecules (helper, vector, and *env*) are present after the DNase I treatment. These results futher strengthen

the results from lanes 1 through 4 in which the SV.KX vector is packaged into SV40 virions. Lane 20 was pSV.KX plasmid DNA digested with Kpn I and Xba I and included on the gel as a positive hybridization control. Three major bands are seen corresponding to the plasmid (upper band of the doublet), vector (lower band of the doublet) and *env* (lower band) sequences. As in lane 10, the minor bands represent partially digested plasmid DNA.

It indeed the vector ΔSV.GAG failed to be packaged into DNase I resistant virions, then the vector DNA should not be detected in the cell culture fluids whereas the helper DNA should. The remaining lanes (21 through 28) show DNAs purified from the pellet obtained from the cell culture fluids. Lanes 21 and 22 show DNA purified from the pellet produced from ΔSV.GAG cotransfected monolayers. The sample in lane 22 was digested with DNase I whereas lane 21 was not. Inadvertently, plasmid DNA was omitted from both samples. As shown by the autoradiogram, only the helper DNA was present in the trypsin resistant particles isolated from these culture fluids again demonstrating that ΔSV.GAG was not packaged into SV40 virions. Longer exposures of the autoradiogram failed to show any vector sequences (data not shown). Lanes 23 and 24 are uninfected controls. Both samples received plasmid DNA but only the sample in lane 24 was digested with DNase I. The upper band in lane 23 probably consists of open circular plasmid molecules. Lanes 25 and 26 contain extracts purified from trypsin resistant particles produced in the SV.KX infected cells. Lane 27 and 28 contain DNAs purified from the medium of SV.KX cotransfected monolayers. All four of these lanes received plasmid DNA whereas only lanes 26 and 28 were digested with DNase I prior to

viral DNA extraction. All four lanes reveal helper, vector and *env* sequences to be present. Lanes 26 and 28 demonstrate that all of these sequences are resistant to DNAse (prior to the proteinase K digestion) but the plasmid DNA was sensitive. It can be included from all the results described that the block to ΔSV.GAG propagation is due to an inability to package the vector DNA into SV40 virions.

Localization of the *Cis*-Acting Sequence
Interfering With SV.GAG Encapsidation

In an attempt to localize the *cis*-acting element which interferes with the encapsidation of ΔSV.GAG DNA, many deletion mutants were made. Figure 23 shows a summary of these deletions and the effect they had on the propagation of their respective vectors. As shown in the table, the deletions span the entire length of the *gag* coding region. Three of these mutants also contain deletions which extend into the 5′ proximal SV40 sequence. Two of these, named ΔSph I-Sph I 0.5 and ΔSphI-Sph I 1.5, were constructed by Eric Howard and were included in to table for completeness of the deletion analysis. The 0.5 and 1.5 indicate the number of SV40 enhancers deleted from each vector. The actual construction however will not be discussed but the effect they have on the propagation of the vector will be discussed later. Likewise, the propagation of SV.envDE and SV.envCE which were constructed by Lynette Bangs and Sue Denman will be ad dressed later.

All of the constructions in this figurure have been previously described in this text except the Xho-Sph I deletion mutant. The original deletion of the Xho I-Sph I fragment from the *gag* coding

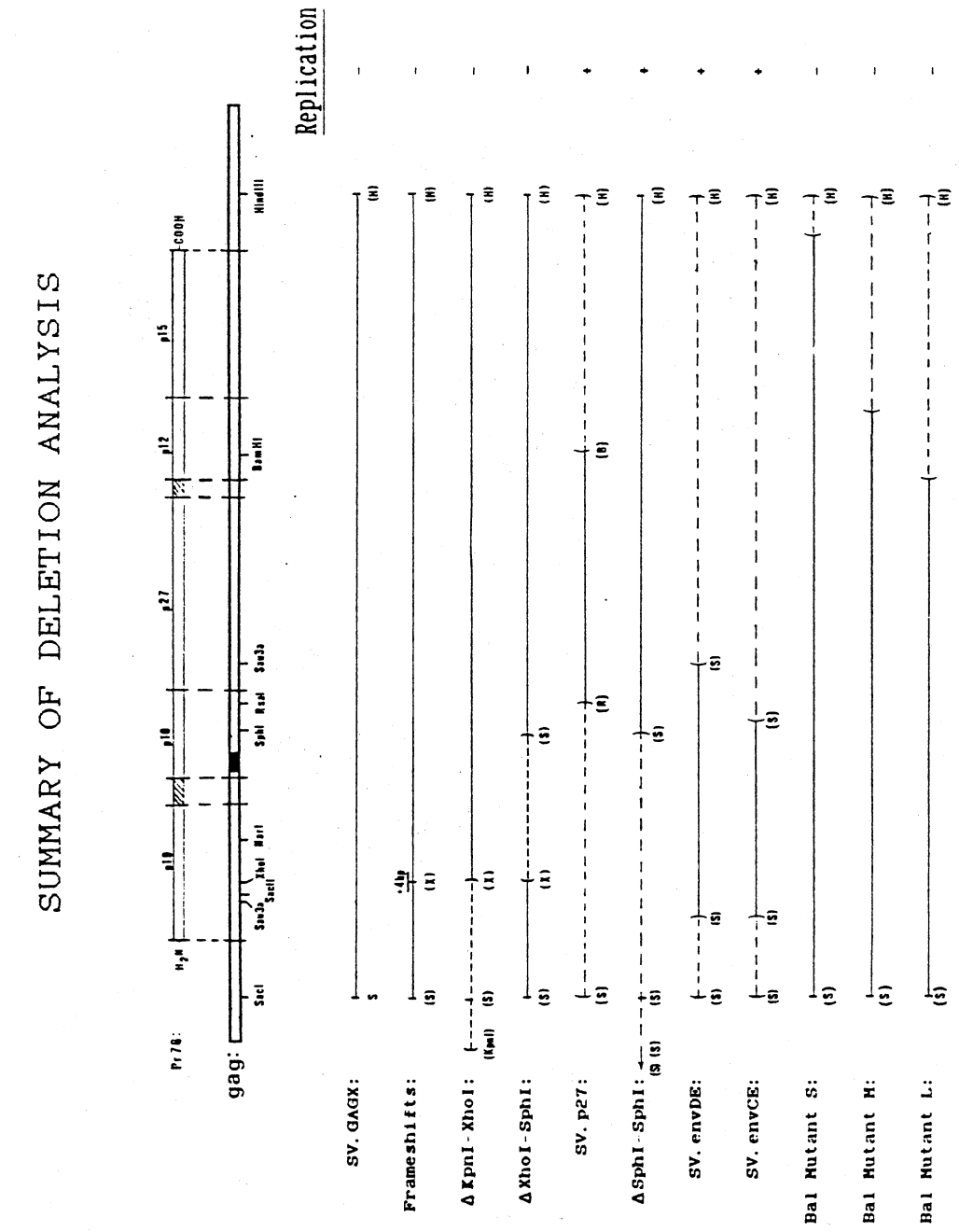

Figure 23. Summary of the different deletion mutants constructed and their effects on the propagation of the respective vectors. Replication was assayed by detection of vector and helper DNA in Hirt extracts of the cotransfected monolayers by ethidium bromide-agarose gel electrophoresis as previously described.

region was made by Arrigo et al. (1987). This deletion mutation was introduced into the ΔSV.GAG genome by replacing the Sac II fragment of ΔSV.GAG with the Sac II fragment, containing the deletion mutation, from the proviral clone of Pr-CRSV. This clone was generously provided by Dr. K. Beeman (Johns Hopkins University, Baltimore, Ma.). Again this vector was unable to be propagated in CV-1 cells upon cotransfected (data not shown).

The vector, SV.p27, was made by subcloning the p27 coding region of RSV (from the Rsa I to Bam HI sites) into the SV40 late region (see Materials and Methods). This vector was cotransfected into CV-1 cells and 5 days post transfection the monolayers were lysed and the SV40 DNA was purified as described previously. Figure 24 shows that this vector was capable of replicating (lane 1).

The results listed in Fig. 23 suggest that the inhibitory sequence is not located between the Sau 3a site in p19 and the Bam HI in p12 since these fragments alone did not inhibit propagation of the respective vectors. The other mutations listed which were constructed by deleting the remaining sequences of ΔSV.GAG. These deletions failed to demonstrate the location of the inhibitory sequences since the respective vectors were still unable to be propagated in CV-1 cells.

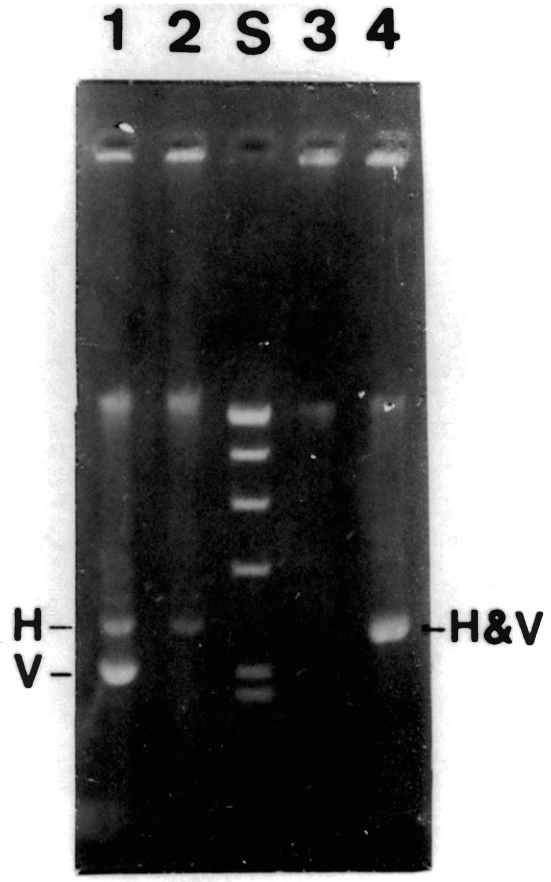

Figure 24. Samples of Hirt extracts from CV-1 cells cotransfected with helper and SV.p27 (lane 1), ΔSV.GAG (lane 2), or SV.KX (lane 4). Samples from untransfected cells are shown in lane 3. "H" represents helper DNA and "V" represents vector DNA.

CHAPTER VII

DISCUSSION

Part I initially described the phenomenon of the inability to propagate the vector ΔSV.GAG. It was further demonstrated in Chapter IV that this inability to propagate the vector was not due to a size packaging limitation or some other detrimental effect(s) imposed on the system by polypeptides synthesized by the vector. This phenomenon was examined in detail in hopes of understanding how some *cis*-acting element could inhibit the normal replicative strategy of simian virus 40 and was reported in Chapter VI.

Elucidating the Cause of the Block of ΔSV.GAG Propagation

It was demonstrated in Figure 15 that neither ΔSV.GAG nor helper DNA could be detected in Hirt extracts from monolayers cotransfected with the DNAs at any time post-transfection when assayed by ethidium bromide-agarose gel electrophoresis. This phenomenon was also observed with the 3′ end deletion mutants and those mutants defective for Pr76 expression. These results are in contrast to those observed from extracts from both SV.KX-helper infected and cotransfected monolayers in which both the helper and the SV.KX DNAs were detected after 28 hr and 62 hr respectively.

To determine whether or not the possible *cis*-acting element in the ΔSV.GAG vector was interfering with or altering the regulation of T Ag expression via the early promoter, the vector was cotransfected into an SV40 permissive cell line, COS-1, which is constitutive for T Ag expression. Hirt extracts from these cotransfected COS-1 cells revealed that only the helper DNA was capable of being propagated. However, the possibility remains that the DNA observed in these extracts in not helper DNA but wild-type SV40 DNA arising from homologous recombination between the helper and vector genomes. It seems unlikely though that such an event would occur at a frequency high enough to allow wild-type SV40 DNA to replicate and subsequently be detected by 90 hr.

These results suggest, although indirectly, that the regulation of SV40 gene expression is not altered in the ΔSV.GAG vector. So to further define the different stages of the SV40 replicative cycle, the timing of the first round of DNA replication and late gene expression for both the SV.KX and ΔSV.GAG expression vectors was determined.

Vector DNA Replication
and Late Gene Expression

Since it has already been shown that the vector, ΔSV.GAG, was not proficient at producing infectious progeny virus, it was of interest to determine whether or not the vector was even capable of replicating in the transfected cells. Since only a few cells are competent to take in exogenous DNA, DNA replication could only be detected by Southern hybridizations.

Figure 17 demonstrated that both vectors, ΔSV.GAG and SV.KX, were capable of DNA replication in the cotransfected monolayers and both had similar replication times. The time of replication is consistent with that seen in SV.KX infected COS-1 cells as well as that reported by Schirmbeck and Deppert (1987). It was also shown in Figure 19 that neither the vector nor helper DNAs had a selective advantage of being replicated since the ratio of vector to helper (3:1) was maintained after DNA replication.

SV40 late gene expression was also determined for both vectors by both time course intracellular immunofluorescence and immunoprecipitations using antiviral antibodies specific for the different antigens. Both experiments demonstrated that late gene expression beings prior to 36 hr but after 23 hr post-transfection. This result is consistent with the regulation of SV40 since the late phase of the viral replication cycle is marked by the onset of viral DNA replication.

Since correct DNA replication and late gene expression have been confirmed, the ability of the ΔSV.GAG DNA to become DNase I resistant by being packaged into particles was tested. The results in Figure 22 imply that both the helper and SV.KX vector DNAs, both in the cell lysate and released into the culture fluid, are resistant to DNase I. Only the helper DNA, on the other hand, remains DNase I resistant in the ΔSV.GAG-helper cotransfected cells. It was also shown in this figure that only the helper DNA was released from the cells into particles that could be collected by centrifugation. The centrifugal force at which these "particles" were collected is the same force required to pellet SV40 virus particles. The fact that only the helper

virus was capable of being packaged in SV40 particles is consistent with the results described earlier in which only the helper virus was capable of replicating in COS-1 cells. These observations, along with the following proposed requirement of SV40 chromatin structure for SV40 DNA encapsidation, raises some very interesting questions regarding the chromatin structure of the ΔSV.GAG "minichromosome."

All eukaryotic organisms have approximately 99% of their genome highly condensed by histone proteins into transcriptionally inert heterochromatin (Steitz, 1987). Active chromatin (euchromatin) on the other hand is generally found in more open or DNase I hypersensitive regions (Weintraub, 1985; Ryoji and Worcel, 1984; and Rose and Garrrard, 1984). It has been established that these hypersensitive sites often reside within *cis*-acting regions of transcriptionally active chromatin (Cockerill and Garrard, 1986; and Igo-Kemenes et al., 1982). It has also been suggested that some of these hypersensitive sites might arise from *trans*-acting factors recognizing enhancer elements in transcriptionally poised regions (Weintraub. 1985; and Wang and Calame, 1986). Other hypersensitive regions of the chromosome possibly represent promoter regions where RNA Polymerase can gain entry into the double helix and DNA origins of replication where the DNA replication machinery can gain access (Gross and Garrad, 1987). The SV40 genome is no exception.

The SV40 chromosome seems to always be packaged into 20 to 24 nucleosomes with a nucleosome periodicity of approximately 198 bp (Shelton et al., 1980). Even though it is not clear what the structure of the transcriptionally poised nucleosome is, it is known that transriptionally active genes can be associated with nucleosomes

(Igo-Kemenes et al., 1982). These nucleosomes however are not evenly spaced about the viral chromosome (Scott and Wigmore, 1978). There is a major DNase I hypersensitive region located around the SV40 origin of replication. This region not only contains the SV40 *ori* but also contains both the early and late promoters as well as the two 72 bp repeats (also known as the SV40 enhancer elements) (Saragosti et al., 1982). Blasquez et al. (1986) suggest that during SV40 replication but prior to viral assembly, the 24 nucleosomes are placed nonrandomly on the SV40 DNA so that the hypersensitive region is exposed to *trans*-acting factors like DNA polymerase and the T Ag. After late gene expression, viral capsid proteins may redirect nucleosomes toward the "open" region thereby increasing the nucleosome periodicity to 211 bp. This periodicity they suggest is a prerequisite for SV40 packaging. They also presented evidence that a temperature-sensitive SV40 late protein, VP1, was incapable of directing the change in nucleosome periodicity at the nonpermissive-temperature and this *ts* mutant was incapable of virion assembly. This *ts* mutant was however capable of directing the nucleosome rearrangement and and packaging its DNA into virus particles at permissive temperatures (Blasquez et al., 1983).

Given the data presented in Chapters IV and VI along with the observations of Blasquez et al (1983 and 1986), I propose that ΔSV.GAG contains some *cis*-acting element(s), with or without *trans*-acting factor(s), which may alter the nucleosome spacing or rearrangement such that correct nucleosome periodicity, required for packaging, is not achieved. Such elements might include the directional enhancer described by Arrigo et al. (1987) or some other unidentified element(s) located in the *gag* coding region.

Localization of the Responsible

Cis-acting Sequence

In attempts to localize the *cis*-acting sequence in the *gag* coding region responsible for inhibiting the encapsidation of the vector, a series of different deletion mutations spanning the entire length of the *gag* coding region was made (see Figure 23). The possibility that Pr76 or some other product initiating translation at the 5′ end of the *gag* coding region was eliminated by the creation of the +1 frameshift mutant (at the unique Xho I site) and the Kpn I-Xho I deletion mutant. These two mutations along with the fact that only the vector and not the helper DNA was inhibited from being packaged established that a *cis*-acting element unique to the *gag* coding region was probably responsible for inhibiting the production of ΔSV.GAG virions. We have not ruled out though the possibility that an RNA molecule might be exerting some effect(s) on the SV40 replication cycle. This possibility though seems unlikely since the RNA would have to act in *cis* or selectively act *in trans*.

The results presented in figure 23 suggest that the elements(s) in the *gag* coding region which are responsible for the block to virion production are not located between the Sau 3a site in the p19 coding region and the Bam HI site in the p12 coding region.

It has also been shown that the responsible sequence is not located 3′ of the p27 coding domain since the Bal 31 deletion mutants were also unstable (see Part I, Chapter V). The only sequences

remaining are those 5′ of the Sau 3a site. However, when these sequences were removed form the ΔSV.GAG vector, they did not alleviate the block to virion production.

Of all the ΔSV.GAG deletion mutants made, only two were stably maintained in CV-1 monolayers. Interestingly these two deletion mutants (ΔSph I-Sph I 0.5 and ΔSph I-Sph I 1.5) were either missing 1/2 or 1 1/2 of the SV40 enhancer elements. As shown in Figure 23, removal of the SV40 *cis*-acting elements alleviated the block in propagation of the ΔSV.GAG vector.

This is not too surprising since its known that enhancer elements can alter the nucleosome periodicity leading to the creation of DNase I hypersensitive sites (Weintraub, 1985 and Wang and Calame. 1986). Removal of these elements might have allowed random nucleosome positioning in the DNase I hypersensitive region of the SV40 minichromosome regardless of any inhibitory elements located in the *gag* gene and thus allow encapsidation of the vector genomes.

Even though every sequence in the Pr76 coding domain of the vector, ΔSV.GAG, has been systematically omitted, localizing a *cis*-acting element which interfered with the packaging of the vector DNA into virus particles failed. This does not mean however that such an element does not exists. It may be that the element along with other flanking or nonflanking sequences work in tandem to inhibit genome packaging, possibly by interfering with nucleosome rearrangements. The possibility that some host factor(s) may preferentially bind to the *gag* sequences thus altering a required DNA packaging structure has not been eliminated. All of the results presented in Part I and Part II strongly suggest that there are probably more than one element

residing in the *gag* gene which inhibit encapsidation of the vector genome. These elements which seem to exert a negative effect on the normal process of SV40 packaging may be very important in regulating retroviral gene expression in the avian system by possibly altering nucleosome periodicity thus creating a DNase I hypersensitive domain at the site of provirus integration. This however is purely speculative.

The work presented in Part I suggests that the block to Rous sarcoma virus replication in mammalian cells lies solely at the transcriptional or translational level and not at the level of precursor processing. The work presented in Part II possibly suggests that important sequences which govern RSV expression, other than the LTRs, may lie in the Pr76 coding domain and possibly influence expression by altering the nucleosome positioning around the viral enhancers and promoters.

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APPENDICES

APPENDIX A

MEDIA, ANTIBIOTICS, AND BIOLOGICAL BUFFERS

L.B.(Luria-Bertani) Medium

Per liter;

Bacto-typtone	10g
Bacto-yeast extract	5g
NaCl	10g

Adjust pH to 7.5 with 5N NaOH

Ampicillin

Disslolve 25 mg per milliliter of water.

Sterilize by filtration.

Chloramphenicol

Prepare stock solution at 34 mg/ml in 100% ethanol

Penicillin-Streptomycin

Per 100 ml;

Penicillin G	1.2g
Streptomycin	1.0g

Use 0.5 ml per 500 ml of medium

Tris Buffered Saline (TBS)

Per liter;

NaCl	8.0g
KCl	0.39g

Na_2HPO_4	0.1g
glucose	1.0g
trizma base	6.0g
H_2O	750ml

pH to 7.2 with HCl and filter sterilize

Phosphate Buffered Saline (PBS)

Per liter;

$CaCl_2$	0.13g
KCl	0.2g
KH_2PO_4	0.2g
$MgCl_2$-$6H_2O$	250ul 2 M $MgCl_2$ stock
NaCl	8.0g
Na_2HPO_4 (anhydrous)	1.14g
H_2O	750ml

pH to 7.4 with HCl and filter sterilize

Standard Buffer

Per liter;

NaCl	5.85g
trizma base	1.21g
Na_2EDTA	0.42g
Penicillin G	0.067g
Streptomycin	0.057g
H_2O	750ml

pH to 7.2 with HCl and filter sterilize

Concentrated Trypsin (0.25%)

Per 500 ml;

Trypsin	1.25g
TBS	50ml

Stir overnight at 4°C

Pellet particulate matter by centrifugation at 9,000 x g in a type SS35 rotor.

Pour supernatant into 450 ml sterile TBS

APPENDIX B

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SOLUTION	AMOUNT	CONCENTRATIION

Stock A. Acrylamide:Bis (30:0.8)

Per liter;

Acrylamide	300 g	30%
Methylene-bis-acrylamide	8.0 g	0.8%

H_2O to make 1 liter.

Store at 4°C in the dark.

Stock B. 2.0 M Tris-Cl, pH 8.8 (Resolving Gel Buffer)

Per liter;

tris base	484.6 g	2.0 M

Adjust pH to 8.8 with HCL

Add H_2O to 1 liter

Stock C. 0.5 M Tris-Cl, pH 6.8 (Stacking Gel Buffer)

Per liter;

tris base	60.6 g	0.5 M

Adjust pH to 6.8 with HCl

Add H_2O to 1 liter

Stock D. Electrophoresis Buffer (10x)

Per liter;

tris base	30.34 g	0.45 M
Glycine	144.0 g	3.84 M

Sodium dodecylsulfate	10.0 g	1.0%

Dissociation Buffer (1x)

Per 20 ml;

0.5 M tris-Cl pH. 6.8	2.5 ml	65 mM
Glycerol	2.0 ml	10%
Bromphenol Blue	0.02 g	1.4 mM
10% SDS	4.0 ml	2%
2-Mercaptoethanol	0.4 ml	0.3 M
H_2O	10.9 ml	

Preparation of Resolving Gel (10% acrylamide)

Per 30 ml (1 gel);

Acrylamide:BIS (30:0.8)	10 ml
2 M tris-Cl pH. 8.8	6 ml
H_2O	13.7 ml
10% SDS	0.3 ml
10% ammunium persulfate	0.3 ml
TEMED	0.020 ml

Preparation of Stacking Gel (3% acrylamide)

Per 15 ml (for 1-2 gels);

Acrylamide:BIS (30:0.8)	1.5 ml
0.5 M tris-Cl pH. 6.8	1.8 ml
H_2O	11.5 ml
10% SDS	0.150 ml
10% ammunium persulfate	0.150 ml
TEMED	0.010 ml

Coomassie Blue Stain for Protein Gels

Per 500 ml;

Coomassie Blue R250	1.375 g	0.275%
95% ethanol	250 ml	47.5%
Glacial acetic acid	50 ml	10%
H_2O	200 ml	

Destain for Protein Gels

Per 500 ml;

Methanol	25 ml	5%
Glacial acetic acid	35 ml	7%

APPENDIX C

AGAROSE GEL ELECTROPHORESIS

Tris-acetate Buffer (50x)

Per 500 ml;		[Final 1x]
trizma base	2.5 g	0.04 M tris-acetate
Glacial acetic acid	29 ml	
0.5 M EDTA, pH. 8.0	50 ml	1 mM EDTA, pH. 8.0

Sample Dye

Per 10 ml;

Ficoll	2.5 g
10% Bromphenol Blue R250	1.0 ml

Make up in TE Buffer
(10 mM tris-Cl, pH. 8.0, 1 mM EDTA, pH. 8.0)

APPENDIX D

SOUTHERN HYBRIDIZATION SOLUTIONS AND BUFFERS

20x SSC Buffer

Per liter;

NaCl	173.9 g
NaCitrate	88.2 g
H_2O	800 ml

Adjust pH. to 7.0 with HCl and add H_2O to one liter

Sterilize by autoclaving, store at room temperature

0.25 M HCl

Per liter;

Concentrated HCl	20.8 ml
H_2O	979.2 ml

0.5 M NaOH, 1.0 M NaCl

Per liter;

5 M NaOH	100 ml
NaCL	58.44 g

0.5 M tris-Cl, pH. 7.7, 3.0 M NaCl

Per liter;

trizma base	60.54 g
NaCl	175.32 g

Adjust pH to 7.7 with HCl

Prehybridization Buffer

Per 20 ml;

20x SSC buffer	5 ml
10% SDS	1 ml
50x Denhart's solution	2 ml
0.5 M EDTA, pH. 8.0	40 ul
10 mg/ml Salmon Sperm DNA (denatured)	200 ul
deionized formamide	9 ml
H_2O	2.79 ml

50x Denhart's Solution

Per 500 ml;

Ficol	5 g
Polyvinylpyrrolidine	5 g
Bovine serum albumin (BSA) (Pentax fraction V)	5 g

Sterilize by filtration and store at -20°C

10x Nick Translation Buffer

Per 1 ml;

1 M tris-Cl	500 ul	0.5 M
2 M $MgSO_4$	50 ul	0.1 M
0.5 M dithiothreitol	2 ul	1 mM

VITA

Robert Allen Weldon, Jr.

Candidate for the degree of

Master of Science

Thesis: EXPRESSION AND CHARACTERIZATION OF THE ROUS SARCOMA VIRUS GAG GENE PRODUCT IN MAMMALIAN CELLS USING AN SV40 LATE REGION REPLACEMENT VECTOR

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