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# VIRUS-SPECIFIC MESSENGER RNAS IN PERMISSIVE CELLS INFECTED WITH AVIAN SARCOMA VIRUS

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I also wish to acknowledge many people in the laboratory who helped me in the course of my training.

Finally, I thank my parents for their past patience and their present pride.

All yet seems well, and if it end so meet,
The bitter past, more welcome is the sweet.

"All's Well That Ends Well"

- William Shakespeare

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ABSTRACT

The cytoplasm of permissive cells infected with avian sarcoma virus (ASV) contains three species of virus-specific RNA with sedimentation coefficients of 38S, 28S, and 21S (Weiss, et al, 1977; Hayward, 1977). Each of these RNAs is presumed to be a messenger for the synthesis of viral proteins. I have substantiated this presumption by characterizing the virus-specific RNA contained in polyribosomes isolated from chicken cells infected with either wild type ASV or deletion mutants of the virus. The 38S and 21S RNAs, which are presumed to be the messengers for the viral genes gag - pol, and src respectively, were found largely or entirely in free polyribosomes. By contrast, the 28S RNA was contained predominantly in membrane-bound polyribosomes, where it apparently serves as messenger for the viral gene env. Although I utilized recently developed techniques to improve the resolution and sensitivity of my analysis, I failed to identify any additional virus-specific RNAs in either polyribosomes or cytoplasmic extracts. In particular, I could not confirm previous reports of a viral RNA smaller that the 21S species, and I found no viral RNA that might contain the gene pol at its 5' terminus.

I conclude that permissive cells infected with ASV contain three major classes of viral mRNA. The glycoprotein products of env are synthesized on membrane-bound polyribosomes and are presumably integral membrane proteins, as required by current models of virion assembly. The products of the other viral genes (gag, pol, and src) are synthesized on free polyribosomes and, hence, are neither integral membrane proteins nor secretory proteins.

I further studied the synthesis of ASV gene products by immunoprecipitating polyribosomes with antisera specific for products of each gene. My data support the contentions that the mRNA for gag and pol gene products is 38S in size, the mRNA for env is 28S in size, and the mRNA for src is 21S in size. Finally, my results suggest, but do not prove, that $Pr76^{gag}$ and $Pr180^{gag/pol}$ are synthesized from identical 38S mRNAs and I propose that a mechanism other than classical tRNA suppression is utilized to accomplish the readthrough of termination codons at the end of the coding sequences for $Pr76^{gag}$.

# INTRODUCTION

## RETROVIRUSES

Retroviruses are a class of animal viruses which possess diploid or poly-ploid RNA genomes complexed with structural proteins to form a dense core, and an outer envelope, similar to plasma membranes. The envelope contains specialized glycoproteins which mediate attachement to target cells. The outstanding characteristic of these viruses is that they contain, associated with the virion particles, an RNA-dependent DNA polymerase (Temin and Mizutani, 1970; Baltimore, 1970). This enzyme is critical in the establishment of infection by the virus, because it transcribes the single-stranded RNA genome to produce double-stranded DNA, and this DNA subsequently integrates into the host DNA (Varmus,et al., 1976, Weinberg, 1977) where it can persist in the progeny of the originally infected cell. Many types of retroviruses exist; they infect many different animals including representatives of all classes of vertebrates. The most well studied examples include avian leukosis-sarcoma virus (ALSV), murine leukemia virus (MuLV), feline leukemia virus (FeLV), and mouse mammary tumor virus (MMTV), among several others. The molecular biology and genetics of retroviruses have recently been reviewed (Bishop, 1978; Vogt, 1977). In this section, I will attempt to define the problems which are pertinent to my work.

### ALSV - Disease, structure, and genetics

Avian sarcoma virus (ASV) is a retrovirus recognized in 1911 by Peyton Rous as a causative agent for solid tumors of chickens. The disease as well as avian leukosis, which began to be studied in 1908, could be transmitted by cell-free filtrates, although only very small quantities of infectious viruses were produced. The original isolates of ASV probably

differed significantly from the virus studied today, in that they were apparently defective and required infection by another virus, a helper virus, in order to establish infection and disease. By serial transplantation of the tumors and filtrates in the laboratory studies, several strains became independent of helper viruses. The viruses which resulted were highly oncogenic. Despite the probable dissimilarities between the presently studied viruses and the original isolates, the laboratory evolution has greatly facilitated the biological and biochemical characterization of the viruses.

An important event in the study of ALSV was the demonstration that the virus could "transform" fibroblasts in vitro (Manaker and Groupe', 1956). Upon infection by the virus, the appropriate cell undergoes significant morphological and metabolic changes - transformation. It was shown that the virus could infect and transform the cells with single-hit kinetics and therefore, that all the information for these events was presumably contained in one virion particle. Only avian cells are permissive for both transformation and replication of ALSV. However, the virus has also been shown to transform many types of mammalian cells, albeit at much lower frequencies.

The ALSV virion contains several basic components, all of which are essential in the establishment of infection. Figure 1 illustrates our present conception of the virion structure. First, its single-stranded RNA genome, approximately $3.3 \times 10^6$ or 38S in size, serves as the template for the initial replication step of the virus life cycle. Two identical 38S molecules (except in mixed infections when two homologous but not necessarily identical subunits may form heterozygotes) are linked to form a 70S complex (Beemon, et al., 1976; Bender & Davidson, 1976). Base-paired near the 5' end of the genome RNA is a molecule of transfer RNA for

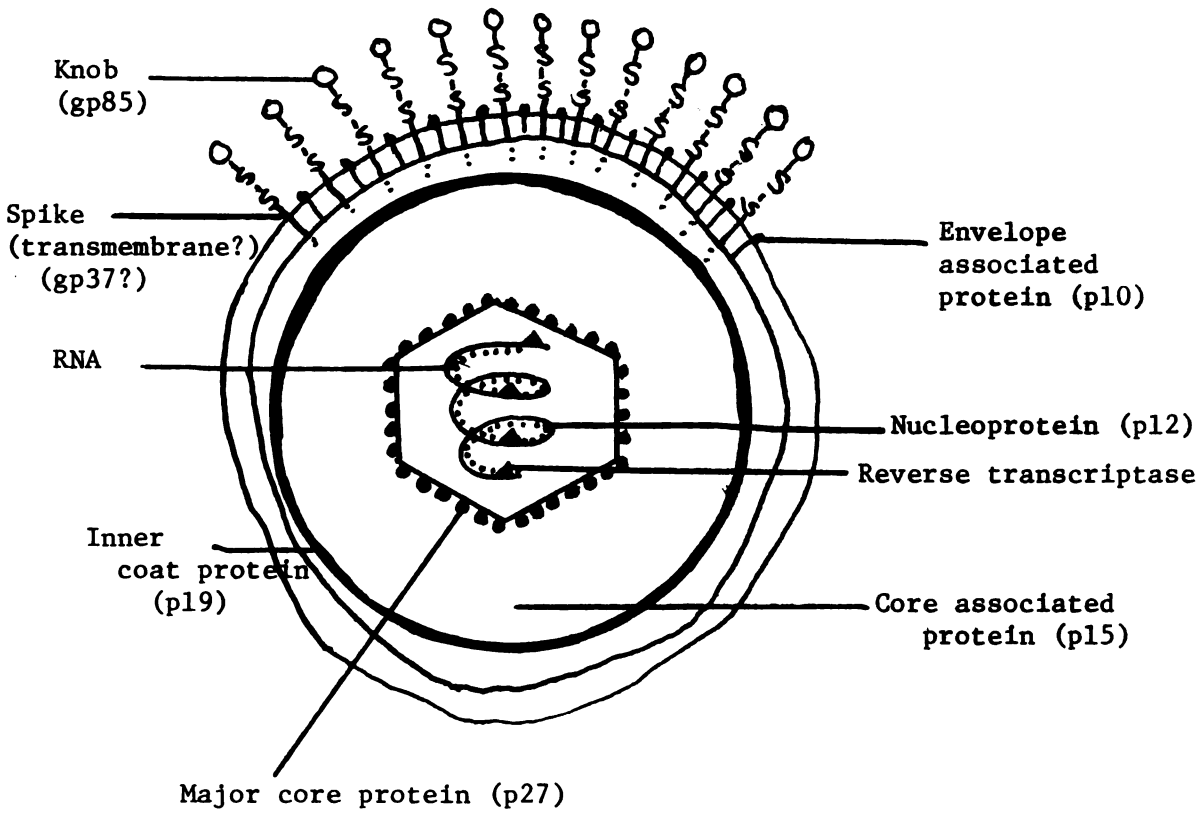


Figure 1. Structure of avian sarcoma virus (from Bolognesi, et al, 1978).

tryptophan, $tRNA^{trp}$ (Taylor & Illmensee, 1976). The 3' end of the $tRNA^{trp}$ serves as a primer for *in vitro* and presumably *in vivo* transcription by the RNA-dependent DNA polymerase (Dahlberg, et al., 1974; Harada, et al., 1975). Two or more copies of the genome RNA, 10-12 copies of $tRNA^{trp}$, and several other 4S and 5S RNAs appear to be present in the virion core. The function of these latter 4S and 5S RNAs are unknown.

Another important structural feature of the virus is its dense nucleo-protein core. The proteins that make up the core are known as the group specific antigens (gag). The major gag protein in the ALSV virion on the capsid surface is p27. p 15 and p10 are also probably located there. p19 and p12 are bound to the RNA in the ribonucleoprotein inside the capsid. p19 has been shown to bind to specific regions with extensive secondary structure in the RNA, while p12 binds the single-stranded portions (see Bishop, 1978).

The RNA-dependent DNA polymerase (pol) or the reverse transcriptase is also probably associated with the ribonucleoprotein and is necessary for infectivity. It is a dimeric enzyme composed of an α and a β subunit (see Verma, 1977). Studies of tryptic peptides from the two subunits have revealed that the α subunit, approximately 60K, contains a subset of tryptic peptides from the β subunit, approximately 100K. As well as its ability to synthesize DNA on an RNA template, the polymerase has RNase H activity; it can digest the RNA component of an RNA-DNA hybrid molecule. This activity is most likely essential in order to synthesize the double-stranded DNA product. The enzyme has been well characterized *in vitro* but there are still several questions yet to be answered about its function *in vivo*.

The envelope of the virion surrounds the ribonucleoprotein core. The viral membrane is similar to the plasma membrane of the infected cell; in

fact, it is derived from the cell membrane during maturation of the virus particle. The viral envelope contains spikes composed of two important glycoproteins, gp70 and p15E (Leamnson, et al., 1976; Witte, et al., 1977; Mosser, et al., 1977). They are probably integral membrane proteins that exist together in a sulfide bonded, dimer structure with C-termini embedded in the membrane. The N-termini project from the outer surface of the membrane, and are presumably involved in the attachment of the virus to receptors present on the target cell surface. The glycoproteins have antigenic type specificity; they confer the ability to attach to certain cells but not to others presumably by means of their configuration (Duesberg, et. al.,1975; Vogt, 1978). ALSV can be classified into types A-E, with the pheasant viruses comprising two more types (Weiss, 1975). Resistance of a host cell to a particular virus type is a recessive trait due to the absence of the appropriate receptor molecule on the cell surface. The envelope of the virus also probably contains other glycoproteins not encoded in the viral RNA although the viral envelope lacks many of the characteristic membrane markers. The most significant of the host glycoproteins in MuLV virion envelopes are the histocompatibility antigens (Bubbers and Lilly, 1977). Their function in the viral membrane is unknown, but their presence may influence the ability of the immune system of the host to respond to the viral antigens during infection and spread of the virus in the course of disease (Doherty and Zinkernagel, 1975).

ALSV is the only group of retroviruses for which genetics have been very thoroughly characterized (Vogt, 1977). Four complementation groups have been identified. They correspond to the genes: gag, pol, env, and src. Both conditional and non-conditional mutants exist. Oligonucleotide mapping has placed the order of the genes in the 38S RNA as: 5'-gag-pol-env-src-C-3'. C, the common region, is a sequence of less than 1000 bases

which is present in both non-defective (nd) and transformation defective (td) mutants in which part or all of the src gene is deleted (Tal, et al., 1977; Junghans, et al., 1977; Shank, et al., 1978). The function of the common region is unknown. However, it is conserved in many of the exogenous avian retroviruses (Tal, et al., 1977).

Three classes of conditional ALSV mutants have been defined (Tooze, 1974). First, the replication mutants, the R class, are defective in later stages of the life cycle of the virus. They affect the proteins encoded in the structural genes. Infection and transformation can occur but functional virus is not produced. The T class, or transformation mutants, on the other hand, do not affect infection and replication, but these defective viruses are not able to transform the host cell. These mutations all affect the src gene. The final class, the C or coordinate mutants, affects early stages in the life cycle, so that the virus cannot establish infection or transform cells. Examples from this class are mutations in the structural genes, primarily pol, which render the virus noninfectious. Analysis of these mutants has provided a great deal of information about the mechanisms by which ALSV and by analogy other retroviruses replicate in and transform cells.

## Molecular biology of ALSV

### Infectious cycle of ASV

#### Synthesis of viral DNA

As mentioned earlier, entry of the virus particle into the host cells occurs via receptors on the target cell membranes to which the ASV glycoproteins bind. Exactly how the virus enters the cell is not known, but presumably the viral membrane and the cell membrane fuse and the virion core, containing the RNA genome and the reverse transcriptase, enters. Transcription of the RNA by the enzyme begins about 100 bases from the 5'

terminus of the RNA, presumably primed by the 3' end of the $tRNA^{Trp}$ molecule (Dahlberg, et al., 1974). Following the transcription of the 5' terminal 104 bases, the polymerase and the newly synthesized product must "jump" to the 3' end of the same or another viral RNA molecule in the virion. The "jump" is thought to be facilitated by a small region of complementarity, about 20 bases (Haseltine, et al., 1977; Schwartz, et al., 1977), at both ends of the virion RNA. The polarity of the virion RNA and the mRNA is identical; the complementary "minus" strand of the DNA is synthesized continuously along the length of the RNA template. Before "minus" strand synthesis is complete, however, "plus" strand DNA synthesis is initiated (Varmus, et al., 1978). "Plus" strand transcription is discontinuous; the products have an average length of 1000 bases, in contrast to "minus" strand synthesis. But eventually the "plus" strands are ligated to form a full length double-stranded DNA molecule. An important feature of this DNA is that about 300 bases at each end of the molecule are identical ( Hsu, et al., 1978 ; Shank, et al., 1978). The identical regions are complementary to bases at both the 3' and 5' ends of the viral RNA.

The newly synthesized DNA in the cytoplasm is linear, but open circular and closed circular as well as linear viral DNA molecules are found in the nucleus (Guntaka, et al., 1976; Smotkin, et al., 1975). The closed circle, or supercoiled structure may be necessary for integration into the cell DNA (Guntaka, et al., 1975). No virus coded proteins have been implicated in the integration of the viral DNA; the mechanism of integration may be a normal host function. Host DNA synthesis is thought to be necessary for the integration of the viral DNA but the nature of the requirement is not understood (Varmus, et al., 1977).

Synthesis of viral RNA

Transcription of the progeny RNA begins late in infection, probably

after the DNA is integrated, although fragments of cell DNA, transfected but probably not integrated, can be transcribed (Cooper & Okenquist, 1978). However, the transfected DNA may contain all elements necessary to initiate transcription, unlike newly synthesized and unintegrated viral DNA. Viral RNA is synthesized by the cellular RNA polymerase II (Rymo, et al., 1974; Jacquet, et al., 1974). The 38S or genome length RNA is apparently the initial transcript, although longer transcripts have been tentatively identified (Fan, 1977; Stavnezer, personal communication). However, these longer molecules may be continuous transcripts of tandemly linked viral DNA which result from the integration of dimers or oligomers infrequently made early in infection (Kung, personal communication).

Later in infection, three stable species of viral RNA exist in the cytoplasm. They are 38S, 28S, and 21S in size (Hayward, 1977; Weiss, et al., 1977). The 28S and 21S RNAs contain subsets of the sequences present in the 38S RNA. The 28S contains 5'-env-src-C-3', and the 21S RNA contains 5'-src-C-3'. All the species are polyadenylated. In addition, the 5' termini of each of the RNAs contains at least 104 bases from the 5' end of the 38S RNA (Cordell, et al., 1978). The two smaller RNAs may be generated by cleavage of 38S RNA and "splicing" of the 5' "leader" sequence. Exactly how the processing occurs is unknown. However, the specificity of the cleavage may be due to the binding of one of the gag proteins, p19, to the RNA (Leis, et. al., 1978). Alternatively, it might be determined by secondary structure of the RNA or specific endonucleases. Nevertheless, genetic analysis of the viral RNA species has led to the prediction that the 38S RNA is the messenger for the gag proteins, the 28S RNA is the messenger for the env glycoproteins, and the 21S RNA is translated to produce the src gene product (Hayward, 1977; Weiss, et al., 1977). The reverse transcriptase, pol, is synthesized by uninterrupted translation of the

gag and pol genes in the 38S RNA and subsequent cleavage of the long joint poly-protein product (Oppermann, et al., 1977).

Synthesis of viral proteins

The gag proteins have been shown to be produced by the synthesis and cleavage of a precursor polypeptide, $Pr76^{gag}$ (Vogt, et al., 1975). The order of the peptides in the primary translation product is apparently N-p19-(p10)-p27-p12-p15-C. The precursor in the permissive cell has a half-life of approximately 45 min, although in non-permissive cells it is stable (Eisenmann, et al., 1974). The initial cleavage step is probably achieved by a cellular enzyme, but p15 may effect the following cleavages (Von der Helm, 1977). $Pr180^{gag/pol}$ is a long relatively stable polypeptide containing both gag and pol tryptic peptides (Oppermann, et al., 1977). Itis processed in the maturing virion to produce the reverse transcriptase (Witte and Baltimore, 1978).

The synthesis of the env glycoproteins, gp85 and gp37, is not as well understood. A protein $Pr70^{env}$, presumed to be the primary translation product, has been identified (Halpern, et al 1974; Moelling & Hayama, 1977; Buchhagen, & Hanafusa, 1978), but it is very unstable. However, a larger more stable product having the same methionine-containing tryptic peptides as $Pr70^{env}$ but more completely glycosylated, and also found in very brief labeling periods, is $Pr92^{env}$. It is possible that both precursors are glycosylated during translation but infrequently $Pr70^{env}$ is completed before glycosylation to $Pr92^{env}$is finished. Later, processing and cleavage of $Pr92^{env}$ generate gp85 and gp37 (Witte, et al., 1977).

The product of the src gene has been identified as $pp60^{src}$ (Brugge and Erikson, 1977; Purchio, et al., 1977). Since it is labeled in very short periods, it is probably also the primary translation product. In addition, it can be produced by in vitro translation of the viral RNA (Erickson, et al.,

1978; Beemon and Hunter, 1978). It is phosphorylated *in vivo* and also acts as a protein kinase (Collett & Erikson, 1978; Levinson, et al., 1978). It is able to phosphorylate IgG to which it is bound, but the nature of its intracellular substrate(s) is unknown.

Research Plan

The 38S, 28S, and 21S ASV RNAs are present in the cytoplasm and are consequently thought to serve as messengers for the synthesis of viral proteins. The available data suggest that the 5' terminal gene is translated in each of the "messages" (Weiss, et al., 1977); thus, the 38S RNA directs the synthesis of the group specific antigens, the 28S RNA is the mRNA for the envelope glycoprotein, and the 21S RNA is the mRNA for the synthesis of the sarcoma gene product. The synthesis of the RNA-dependent DNA polymerase occurs by uninterrupted translation of the 38S RNA to form a joint product, $Pr180^{gag/pol}$. Earlier work demonstrated that a large fraction of ASV specific RNA was present in polyribosomes (Schincariol and Joklick, 1973). In addition, results of translation of viral RNAs *in vitro* (von der Helm and Duesberg, 1975; Pawson, et al., 1977; Philipsson, et al., 1977; Purchio, et al., 1977; Beemon and Hunter, 1978) and the microinjection of viral RNA into cells (Stacey, et al., 1977) have given credence to this model, but it remains to be domonstrated that each of the size classes of viral RNA serves as messenger RNA *in vivo*.

I wished to determine the sizes of the viral RNAs associated with menbrane-bound and free polyribosomes, in order to investigate the possibility that any of the viral gene products are integral membrane proteins or secretory proteins. Because gp85 and gp 37 are probably integral membrane proteins, I expected to find most of the 28S putative "*env*" RNA in the membrane-bound polyribosomes. I also reasoned that this analysis might generate ideas regarding the location of the *src* gene product.

Immunoprecipitation of polyribosomes synthesizing specific proteins, such as ovalbumin, other egg white proteins, and immunoglobulins, has been effectively employed to enrich for or purify their respective mRNAs (Palacios, 1972; Palmiter, et. al., 1972 ; Groner, et al., 1972; Schechter, 1974). The immunoprecipitation results from the reactions of antigenic determinants on nascent polypeptide chains with antibodies specific for the proteins. I reasoned that it might be possible to characterize or to purify the mRNAs for the various ASV gene products by the combination of this technique and/or molecular hybridization. It should be possible, using fixed Staphylococcus aureus, which binds the Fc portion of the IgG chains (Kessler, 1975), to develop a rapid and presumably efficient method of isolation. The immunoprecipitated RNAs could be analyzed for size and genetic composition by agarose gel electrophoresis and by hybridization with specific cDNAs. Furthermore, the identification of the mRNAs could be supported by translation of the immunoprecipitated RNAs in vitro.

The major objectives in my work were to study the association of the ASV-specific RNAs with polyribosomes, and to establish the identity of the virus-specific messenger RNAs according to size. In addition, I wished to separate the membrane-bound and free polyribosomes in order to obtain more information about the synthesis of viral proteins thought to be synthesized by each of the viral RNA species. Finally, I attempted to use immunoprecipitation of polyribosomes in order to prove the identities of each of the RNAs as functional mRNAs for the synthesis of the proteins encoded by the 5' terminal genes in each of the RNAs.

In an effort to establish the nature of virus-specific messenger RNAs in infected cells, we have characterized the viral RNA contained in total, membrane-bound and free polyribosomes isolated from chicken cells infected with either wild type ASV or deletion mutants of the virus. We increased the resolution and sensitivity of our analyses by using electrophoresis in

slab gels of agarose to fractionate RNA and by transferring the RNA from the gels to chemically derivitized paper for subsequent hybridization with virus-specific cDNAs. Our results conform to the current model for viral gene expression. We could not detect the fourth, small species of viral RNA described previously by other workers (Brugge, et al., 1976), and we found no messenger that might permit the expression of pol by independent initiation of translation. The messenger for env was contained exclusively in membrane-bound polyribosomes, whereas the other viral messengers were found largely or entirely in free polyribosomes. Hence, the env product is likely to be the only viral integral membrane protein, and apparently none of the viral proteins are secretory proteins. My analysis of the RNA isolated from polyribosomes immunoprecipitated with various antisera specific for different viral proteins may be compatible with the model described previously (Weiss, et al., 1977; Hayward, 1977); but because of the nature of the mechanisms by which ASV expresses its gene products, I was not able to unambiguously answer many questions raised by this model.

MATERIALS AND METHODS

Materials. Aminobenzyloxymethyl (ABM) paper was obtained from Enzo Biochem (in Figure 5), or 1-[(m-Nitrobenzyloxy)methyl] pyridinium chloride (NBPC) was obtained from G. Stark, and the ABM paper was made by the method described by Alwine et al. (1977). Proteinase K was purchased from E.M. Laboratories, crystalline trypsin from Worthington, methyl mercury hydroxide from Alpha Division-Ventron Corp., and glyoxal from J.T. Baker.

Cells and viruses. Clones of transformed chicken fibroblasts producing the Prague B (Pr-B), Pr-C, and Schmidt-Ruppin-D (SR-D) strains of ASV, cloned in soft agar following infection at a low multiplicity,were obtained from P. Vogt; the fibroblasts used in this procedure were free of endogenous viral gene products ("chick helper factor" and "group-specific antigen") when tested with conventional assays. The virus produced by these clones was used to establish infection en masse in chick embryo fibroblasts; the cells were then passed at least twice and were fully infected and transformed when used for analysis of viral RNA and polyribosomes. Transformation was monitored by light microscopy. Stocks of transformation defective (td) Pr-B ASV were obtained from P. Vogt and also used to establish infections. Infectious pseudotypes of replication defective (rd) ASV were obtained by superinfecting cells producing noninfectious Bryan ASV with Golden Pheasant virus (GPV), a virus which shares very little or no nucleic acid homology with ASV (Hunafusa and Hanafusa, 1973). A mammaltropic strain of SR-D ASV that infects mammalian cells with relative efficiency was obtained by fusion of infected and transformed NRK cells with permissive cells. The rescued virus was then used to establish infection as before. This strain is denoted $SR\text{-}D_M$ ASV in following discussions.

Preparation of cytoplasmic lysates. Half confluent monolayers of cells were treated with 1μg/ml cycloheximide for 20 min followed by 200 μg/ml cycloheximide for 10 min. The cells were then washed with cold Tris-saline (0.15 M NaCl, 0.005 M KCl, 0.0055 M glucose 0.025 M Tris:HCl; pH 7.3), containing 200 μg/ml of cycloheximide, and removed from plates in Puck's slaine-EDTA (0.14 M NaCl, 0.0054 M KCl, 0.035% $NaHCO_3$, 0.0055 M glucose) plus 20 μg/ml crystalline trypsin, also containing 200 μg/ml cycloheximide. The pellets were then washed two more times with Tris-saline cycloheximide and suspended in ice-cold hypotonic buffer (0.001M NaCl, 0.006 M $MgCl_2$, 0.001 M Tris:HCl; pH 7.4). The cells were allowed to swell for 10 min and disrupted with twenty strokes in a Dounce homogenizer. The nuclei were removed by centrifugation at 4000 rpm for 5 min. The resulting supernatant was made 0.004M dithiothreitol, 1 mg/ml heparin, 0.5 mg/ml yeast RNA, 0.4 M NaCl, 0.05 M $MgCl_2$, and 0.02 M EGTA 0.025 M Tris: HCl; pH 7.4.

Analysis of polyribosomes. For preparation of total cell polyribosomes, 1% (v/v) NP-40 and 0.5% (w/v) sodium deoxycholate were added to the cytoplasmic lysates. For preparation of membrane-bound polyribosomes, the detergents were omitted. The lysates were layered on a 15-40% sucrose gradient with a 55% sucrose cushion in buffer A (0.2 M NaCl, 0.025 M $MgCl_2$, 0.02 M Tris: HCl; pH 7.4) containing 100 μg/ml heparin in an SW41 centrifuge tube. For EDTA treatment, the $MgCl_2$ in the buffers was replaced with 0.01 M EDTA (Buffer B). The lysates were centrifuged as described in the figure legends. Gradients were collected from the bottom and absorbance at 260 nm was continuously recorded by a Gilford spectrophotometer. If intact RNA for size analysis was to be isolated from the fractions, Proteinase K (50 μg) (and EDTA to 0.05 M if the buffers contained $MgCl_2$) were added to each fraction. After 15 min incubation at 37°C, each fraction

was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and the RNA was ethanol precipitated. The precipitates were centrifuged for 20 min at 10,000 rpm and resuspended in $H_2O$. Equal portions of each fraction were hybridized with $^{32}P$-labeled cDNA, or the RNA in the fractions was subjected to size analysis (described below).

Preparative purification of polyribosomes. The method of Palacios, et al. (1972) was modified as follows. Cell lysates were prepared as described above. After addition of buffer and detergents, the lysates were layered on sucrose gradients consisting of 2 ml 2.5 M sucrose, overlayed with 4 ml 1.0 M sucrose, and 1 ml 1.0 M sucrose in Buffer C (0.4 M NaCl, 0.05 M $MgCl_2$, 0.02 M EGTA, 0.025 M Tris: HCl; pH 7.4) or Buffer D (with 0.01 M EDTA replacing the $MgCl_2$ in Buffer C), containing 1 mg/ml heparin, in SW 41 cellulose nitrate centrifuge tubes. The gradients were centrifuged at 38,000 rpm for 90 min. For analytical purposes, the gradients were collected from the top using a Gilson Auto Densi-Flow II C pump, and the fractions were processed as described above. For preparative purposes, the polyribosomes at the 1-2.5 M sucrose interface were collected by removing the sucrose layers above the interface, puncturing the side of the tube just below the interface with a tuberculin syringe fitted with a 19 gauge needle, and withdrawing the visible material. This fraction was then diluted into TE (0.01 M EDTA, 0.01 M Tris:HCl; pH 7.4); SDS was added to 0.5% and Proteinase K to 100 μg/ml. After incubation for 15 min at 37°C, the polyribosomes were extracted with phenol-chloroform-isoamyl alcohol and the aqueous phase was precipitated with two volumes of ethanol. For the experiment in Figure 2, polyribosomes were divided into two parts, diluted with three volumes of buffers with or without EDTA, and resedimented on linear gradients.

Preparation of cytoplasmic RNA. Cytoplasmic RNA was prepared as

described by Weiss et al. (1977).

Fractionation and detection of virus-specific RNA in agarose gels. The RNA was resuspended in $H_2O$ and treated with 1 M glyoxal, 50% DMSO, in 0.01 M phosphate buffer (pH 7.0), as described by McMaster and Carmichael (1977). Alternatively, methyl mercury hydroxide was added to 0.01 M as described by Bailey and Davidson (1976). In either instance the RNA was loaded onto 1.2% agarose gels (in the latter case, 0.005 M methyl mercury hydroxide was present in the gel) and subjected to electrophoresis as described in the figure legends. After transfer of the RNA from the gel to ABM paper, the RNA coupled papers were prehybridized in 50% formamide, 6X SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) 0.01 M Hepes (pH 7.0), 1 X Denhardts (0.02% polyvinylpyprolidone, 0.02% Ficoll, and 0.02% bovine serum albumin) with 1 mg/ml yeast RNA, 100 µg/ml salmon sperm DNA containing 10 mg/ml glycine for 6-12 hours at 41°C. The papers were washed with hybridization buffer without glycine, and hybridized for three days with 1-3,000,000 cpm $^{32}$P-ASV cDNA (Shank, et al., 1978) in the same buffer. The hybridized papers were washed for one hour at room temperature with 2X SSC followed by a wash at 50°C with 0.1X SSC, 0.1% SDS for one hour, rinsed with 0.1X SSC and dried. They were subjected to autoradiography under Kodak X-Omat X-ray film using Lightning-plus screens at -70°C for 2 to 14 days (Shank, et al., 1978).

Preparations of virus-specific cDNAs. $^{32}$P-cDNA$_{B77}$ - was synthesized with detergent activated virions of B77 ASV as described previously (Ringold, et al., 1975). A small amount of sequences in this cDNA were transcribed from the entire genome but most of the DNA had a low genetic complexity complementary to sequences near the 5' terminus of the ASV genome (Friedrich, et al., 1977).

$^{32}$P or $^{3}$H cDNA$_{rep}$ - was prepared by transcription in the presence of

Actinomycin D and purified 35S ASV RNA with AMV polymerase as previously described. The entire viral genome is represented with relative uniformity in this DNA.

$^{3}H$ $cDNA_{sarc}$ - was prepared as described (Stehelin, et al., 1976). Only sequences absent in td Pr-C ASV were represented in this DNA.

$^{32}P$ $cDNA_{rep\ ds}$ (for hybridization of RNA immobilized on ABM paper) - was synthesized in the absence of Actinomycin D as described above (see Shank, et al., 1978).

RNA-DNA solution hybridization. Hybridization mixtures contained 500 to 1500 cpm of cDNA, variable amounts of RNA, 0.6 M NaCl, 0.001 M EDTA, and 0.025 M Tris: HCl; pH 7.4 in 50- or 100 µl reaction volumes. The mixtures were incubated in plastic tubes under mineral oil at 68°C for 20 to 48 hours. RNA-DNA hybridization was measured by digestion with single-strand specific nuclease S1 from Aspergillus oryzae (Leong, et al., 1972).

Quantitation of radioactive virus specific RNA. RNA labeled for 20 hours with $^{3}H$-uridine was isolated by the procedures described above. It was hybridized to unlabeled cDNA as described by Cordell, et al. (1978). After hybridization for 1 to 2 days at 68°C in 0.4 M Na-phosphate buffer, the fractions were treated with RNase A and RNase T1, chromatographed on hydroxyapetite in the presence of 0.2 M phosphate (pH 6.8), 1% SDS, and 8 M urea. The bound material corresponding to the virus specific RNA was eluted with 0.4 M phosphate (pH 6.8). Radioactivity in both bound and unbound fractions was counted in Tritosol (0.3% (w/v) Omnifluor, 27.5% (v/v) Triton X-100, 3.7% (v/v) ethylene glycol, 10.6% (v/v) ethanol in xylene solvent).

Fluorography of agarose gels. After electrophoresis the gels were stained with 10 µg/ml ethidium bromide in 0.5 M ammonium acetate, for

15 min. They were then photographed and soaked twice for 15 min each with acetone. The agarose was impregnated with 5-10% (w/v) Omnifluor in acetone, for two hours, followed by extensive washing with water until the gel no longer smelled of acetone. The impregnated gels were dried under vacuum without heat and then exposed to film at -70°C for 5 to 30 days.

Analysis of virus specific RNA in cylindrical agarose gels. Up to 25 µg of RNA was subjected to electrophoresis in cylindrical 2.0% agarose gels for 4 hours at 50 V in 0.01 M Tris-acetate (pH 7.4), 0.02 M sodium acetate, 0.01 M EDTA (Tal, et al., 1974). The gels were soaked in water after electrophoresis and the $A_{260}$ was recorded by a Gilford spectrophotometer with a linear transport mechanism. The gels were sliced with a Mickle gel slicer, and two 1 mm slices were dissolved at 68 C in a solution containing the appropriate cDNA, calf thymus DNA, yeast RNA, and sodium perchlorate such that the final concentration was 2 M sodium perchlorate. Hybridization continued for one to two days. Prewarmed S1 buffer containing S1 nuclease was added at 1 ml per tube and the tubes were incubated at 50 C for 2 hours. The mixtures were precipitated with 10% trichloroacetic acid, filtered on glass fiber filters, and the filters were counted in Omnifluor-toluene (0.4% (w/v) Omnifluor).

Purification of virus specific RNA by hybridization. RNA labeled with $^{3}$H-uridine was added to a mixture containing 1-2 µg unlabeled cDNA, 1% (w/v) SDS, 50% formamide, and 0.4 M phosphate, pH 6.8. The mixture was hybridized at 41 C for 4-5 hr, in a final volume of 40 µl. One ml of MUP buffer (8 M urea, 1% SDS, 0.2 M phosphate, pH 6.8) was added and the RNA-DNA hybrids were bound to hydroxyapatite in the presence of MUP buffer at 50 C. After several washes with MUP buffer, the temperature was raised to 60 C, and the hybrids were eluted with 1% SDS and 0.4 M phosphate, pH 6.8. The radioactivity in 5 µl aliquots of each fraction was counted in Tritosol, and

the appropriate bound fractions were saved. Chromatography on Sephadex G-50 in the presence of 0.6 M NaCl, 0.001 M EDTA, and 0.1% SDS, 0.02 M Tris:HCl, pH 7.4, was performed. The peak fractions were pooled, and the RNA was precipitated with ethanol in the presence of 50 ug yeast RNA as carrier.

Preparation of immunological reagents. The procedure of Palacios, et al., (1972) for reducing quantities of nuclease in antisera was modified as follows. Whole rabbit antisera were precipitated twice with 40% ammonium sulfate, and then dialyzed overnight at 4 C in 0.015 M NaCl, 0.01 M phosphate, pH 7.2, containing 0.05% sodium azide. The resulting dialysates were clarified at 10,000 rpm for 10 min and applied to columns in the same buffer containing DEAE-Sephadex overlayed with CM-Sephadex (Pharmacia). The $A_{280}$ in each fraction was determined and the first peak from each column was pooled. The pooled fractions were lyophilized and resuspended in water so that the final concentration was 0.15 M NaCl, 0.10 M phosphate, pH 7.2, and 0.5% sodium azide. The resulting preparations of antisera were tested for RNase activity by incubating with 35S $^{32}$P-labeled ASV RNA and subsequent analysis by gel electrophoresis. It was noted that whereas the RNA was degraded to approximately 18S in size, untreated antisera degraded the RNA to approximately 4S. The concentration of the protein was determined by the amido-black method (Schaffner and Weissman, 1973), and the antisera were adjusted so that the concentrations were 2 mg/ml.

Fixed Staphylococcus aureus were prepared according to Kessler (1975) and used at a concentration of 10% (wt/v). The S. aureas preparations did not contain detectable RNase.

Chromatography on oligo-d(T) cellulose. Columns of oligo-d(T) cellulose were prepared by washing with 0.2 N Na)H, followed by equilibrating with Binding Buffer (0.5 M NaCl, 0.001 M EDTA, 0.5% SDS, 0.02 M Tris:HCl, pH 7.4). Samples of RNA were applied to the column in Binding Buffer, the columns

were washed with 0.25 M NaCl, 0.001 M EDTA, 0.5% SDS, 0.02 M Tris:HCl, pH 7.4, and the polyadenylated RNA was eluted with 0.01 M NaCl, 0.01% SDS, 0.01 M Tris:HCl, pH 7.4.

Immunoprecipitation of proteins. Cell lysates were made by resuspending washed cells in Immunoprecipitation buffer (0.15 M NaCl, 0.01 M $MgCl_2$, 0.02% NP-40, and 1 mg/ml ovalbumin) and removing nuclei and cell debris by sedimentation at 10,000 rpm for 5 min. The supernatant was divided into aliquots and the appropriate antiserum was added to each one. Incubation was for 15-30 min on ice and then enough S. aureus was added to bind all the antibody. Incubation continued for 10 min on ice and the mixtures were centrifuged through a layer of 1 M sucrose in High Salt buffer (0.5 M NaCl, 0.01 M $MgCl_2$, 0.02 M Tris: HCl, pH 7.4, 2.0% NP-40). The pellets were washed twice with NMT buffer (0.25 M NaCl, 0.01 M $MgCl_2$, 0.01 M Tris: HCl pH 7.4, 1.0% NP-40, 1mg/ml ovalbumin), and once with water. The antibody-antigen complexes were eluted with Sample buffer (10% glycerol, 5% β-mercaptoethanol, 3.0% SDS, 0.625 M Tris: HCl, pH 6.8). The S. aureus were removed by centrifugation, and the supernatants were subjected to electrophoresis in polyacrylamide gels (described below). When $^{35}$S-methionine labeled products of in vitro translation were subjected to immunoprecipitation, one to three volumes of Immunoprecipitation buffer were added to the aliquots, and the procedure was carried out as above.

SDS-Polyacrylamide gel electrophoresis. Samples of $^{35}$S-methionine labeled proteins were suspended in Sample Buffer and boiled for 2 min. They were then loaded onto 7, 10, or 12.5% polyacrylamide gels as described by Laemmli (1970). Electrophoresis was performed at 15 ma/gel for 4 hours.

Immunoprecipitation of polyribosomes. Polyribosomes were purified as described. After withdrawing the interface between the 1.0 M and 2.5 M sucrose layers, the suspension was diluted with three volumes of Immunoprecipitation

buffer containing 1 mg/ml heparin. Chicken immunoglobulin (purified as described for antisera) was added to a concentration of 100 µg/ml and the mixture was centrifuged at 10,000 rpm for 5 min. Aliquots of the polyribosome supernatant were immunoprecipitated as above, except that the wash with water was omitted. The S. aureus pellets were resuspended in TE buffer with 0.5% SDS. Proteinase K was added to 100 µg/ml and the reactions were carried out at 37 C for 10 min. The RNA was extracted with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with two volumes of ethanol in the presence of 0.2 M sodium acetate, and 50 µg yeast RNA as carrier.

In vitro translation. The micrococcal nuclease treated rabbit reticulocyte lysates were prepared as described by Pelham and Jackson (1976). RNA was added and incubation was carried out in the presence of 19 amino acids (without methionine), ATP, GTP, KCl, $MgCl_2$, tRNA, creatine phosphokinase, spermidine, and $^{35}$S-methionine (final concentration approximately 5 mCi/ml for 2 hours at 28 C.

Preparation of $^{32}$P-labeled 35S RNA. $^{32}$P-labeled 70S ASV RNA was prepared according to Bishop, et al., (1970). An aliquot of the 70S RNA was heated to 100 C, for 2 min and applied to a 5-25% linear sucrose gradient in the presence of 0.1 M NaCl, 0.001 M EDTA, 0.02 M Tris:HCl, pH 7.4, and 1% SDS. 100 µg rabbit reticulocyte ribosomal RNA were added as markers. The gradient was centrifuged at 22,000 rpm for 15 hours at 22 C in an SW 41 rotor (Beckman). The gradient was collected and the $A_{260}$ was continuously recorded with a Gilford spectrophotometer and the fractions containing the RNA 34S or greater were pooled and precipitated with 2 volumes of ethanol in 0.2 M sodium acetate at -20 C.

Radioimmunoassays. p27 was purified from virions by preparative polyacrylamide gel electrophoresis. The protein preparation was iodinated with $^{125}$I by the chloramine T method (Greenwood, et al., 1963). Constant amounts

of the labeled proteins were incubated with increasing amounts of various antisera in 1-200 µl volumes, for approximately 15-30 min. Fixed S. aureus were then added and the mixtures were incubated another 10 min. and centrifuged. The pellets were resuspended in NMT buffer and layered over 200 µl of 1 M sucrose in High Salt Buffer. The 400 µl microfuge tubes were centrifuged at 10,000 rpm for 15 min at 4 C. The tubes were frozen in dry-ice-ethanol, the tips containing the visible pellets were cut off and the radioactivity in each tip was counted in a gamma counter (Searle).

RESULTS

Purification and recovery of the polyribosomes

In order to estimate what fraction of the viral RNA is associated with the polyribosomes in ASV infected cells, I sedimented a detergent-treated cytoplasmic lysate in a linear sucrose gradient. An identical lysate was first treated with EDTA and sedimented in a similar gradient. The results are shown in Figure 2. In my hands, a large portion of the cytoplasmic viral RNA appears to be associated with polyribosome-like structures, because it can be released from the rapidly sedimenting structures with EDTA. I assume that the viral RNAs sedimenting faster than the ribosomal subunits after release with EDTA are molecules bound in ribonucleoprotein particles.

In order to minimize possible problems, such as degradation and aggregation of the polyribosomes and the RNA during the procedure, it was necessary to use a rapid and efficient method of isolation of the polyribosomes. I found that sedimentation through a discontinuous sucrose gradient onto a cushion of dense sucrose (Palacios et al., 1972; Taylor and Schimke, 1973) best fulfilled my requirements. In the experiment illustrated in Figure 3, I sedimented the cytoplasmic lysates treated with and without EDTA through the gradients, fractionated the gradients, isolated the RNA from each fraction, and hybridized the RNA with virus-specific cDNA. The majority of the RNA, both total RNA (panel A) and ASV-specific RNA (panel B), found at the interface between the 1 M and 2.5 M sucrose layers was absent from that region after treatment of the cytoplasmic lysate with EDTA. Quantitative analysis indicated that only 5-10% of the viral RNA was resistant to release with EDTA (data not shown). I also analyzed the purified polyribosome fraction by

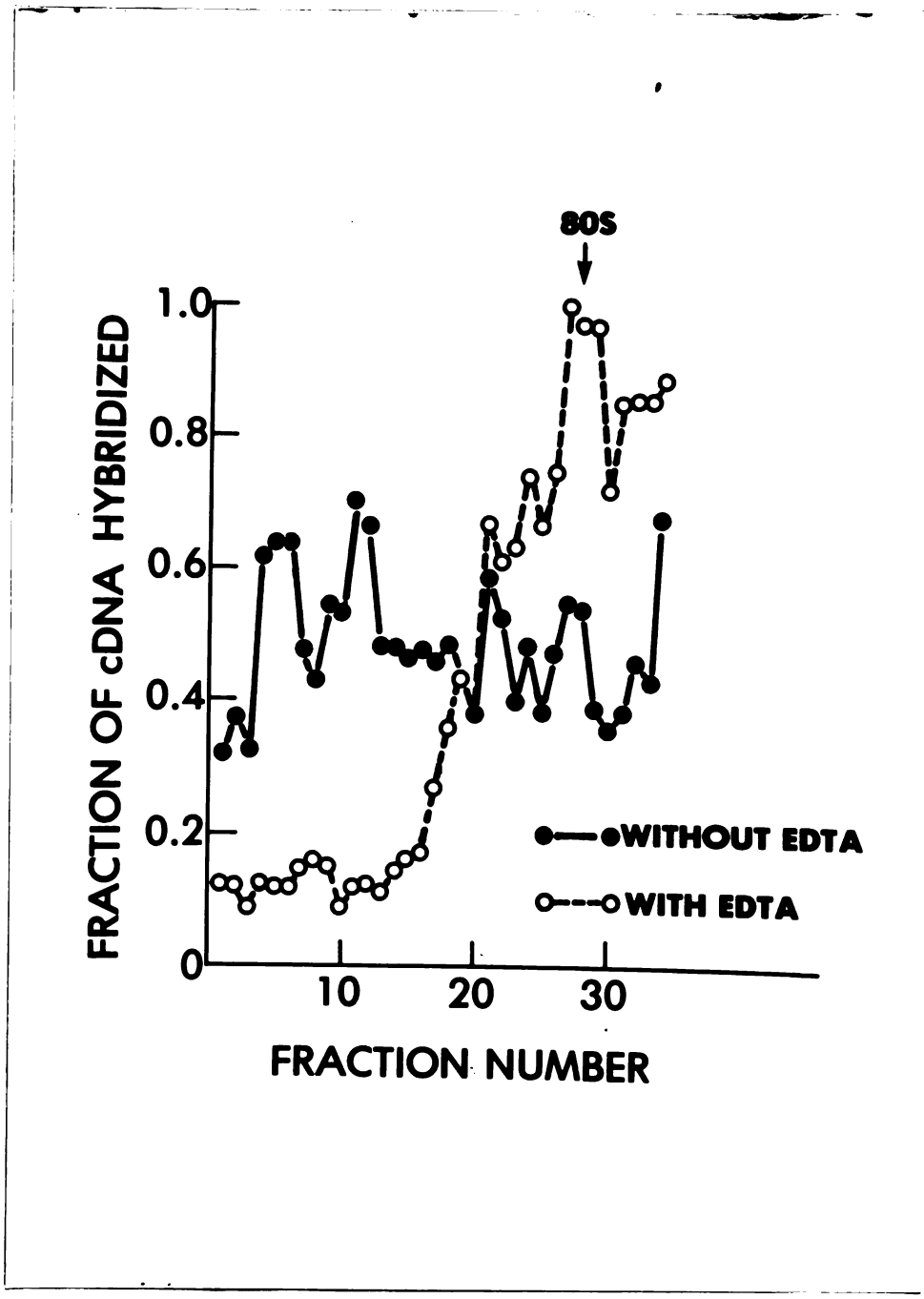


Figure 2. ASV specific RNA in polyribosomes.

Cytoplasmic lysates were prepared as described. Equal portions were treated with and without EDTA. Detergents were then added and the lysates were sedimented through 15-40% sucrose density gradients at 36,000 rpm for 75 min.

at 4 C, using an SW 41 rotor. The viral RNA was located by molecular hybridization of a constant volume from each fraction with $cDNA_{B77}$ as described in Methods. Sedimentation was from right to left.

●——● Sedimentation without EDTA.

o- - - -o Sedimentation with EDTA.

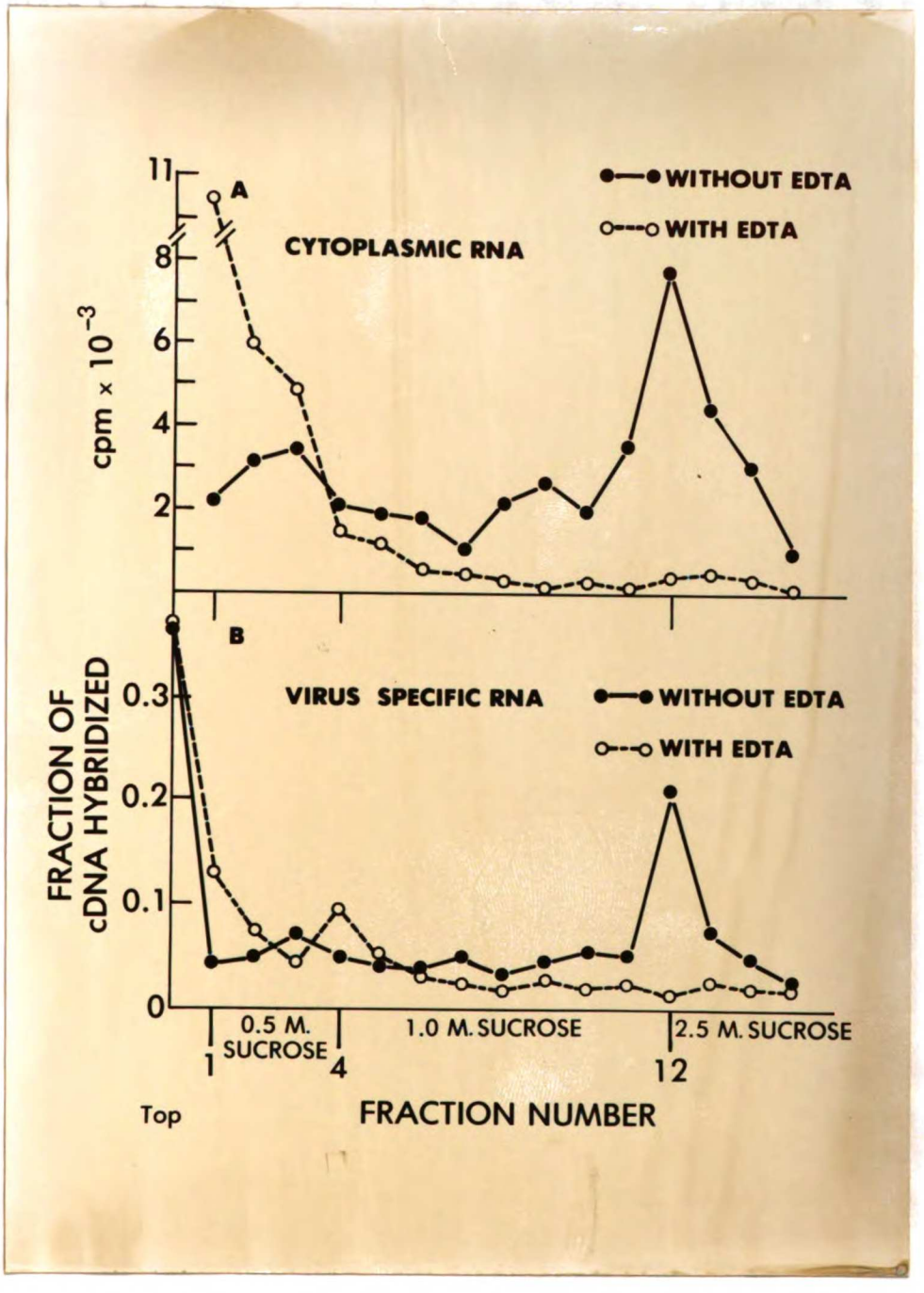


Figure 3. Purification of polyribosomes.

Cells were labeled for several hours with $^{3}$H-uridine (30 µg/ml). Cytoplasmic lysates were then prepared with and without EDTA, treated with detergent, and sedimented through discontinuous sucrose gradients. The gradients were

fractionated by pumping from the top. Equal volumes of each fraction were hybridized with $cDNA_{rep}$ as described in Methods. Sedimentation was from left to right. The $^{3}H$ radioactivity in constant portions of each fraction was determined by counting in Tritosol.

A) Distribution of total cytoplasmic RNA.

B) Distribution of ASV-specific RNA.

●——● Without EDTA.

o- - - -o With EDTA.

resedimenting it on linear sucrose gradients with and without EDTA. Again, most of the viral RNA was released from the polyribosome region when EDTA was present (Figure 4).

In order to measure the recovery of viral RNA in the purified polyribosomes, I prepared polyribosomes from cells that had been labeled for 20 hours with $^{3}$H-uridine. RNA was also isolated from aliquots of the cytoplasmic lysate before purification of polyribosomes. These samples were then analyzed for virus-specific RNA by hybridization and chromatography on hydroxyapatite (Cordell et al., 1978). In the experiment shown in Table I, 42% of the virus-specific cytoplasmic RNA was recovered in the polyribosome fraction. Other experiments gave similar results.

ASV-specific RNA was also quantitated in preparations of unlabeled RNA isolated from the whole cell, cytoplasm, and polyribosome fractions of cells infected with PrC-ASV. In order to do this, increasing amounts of RNA were hybridized with $^{32}$P-cDNA$_{B77}$. In the experiments described here, "$V_o t$" represents the quantity of cells or volume of cell lysate from which the RNA was isolated multiplied by the time of hybridization. The measure is analogous to $C_r t$, where concentration is replaced by another parameter (Ringold et al., 1975). In this way, the quantity of viral RNA in fractions of differing unknown total RNA concentrations could be compared. Table II shows that about 70% of the ASV-specific RNA in the cell was present in the cytoplasm, and approximately 25% of the ASV-specific RNA in the cell was present in purified polyribosomes. Therefore, approximately 36% of the cytoplasmic ASV-specific RNA was recovered with the polyribosomes. This value agrees reasonably with the values obtained by the analysis of labeled RNA.

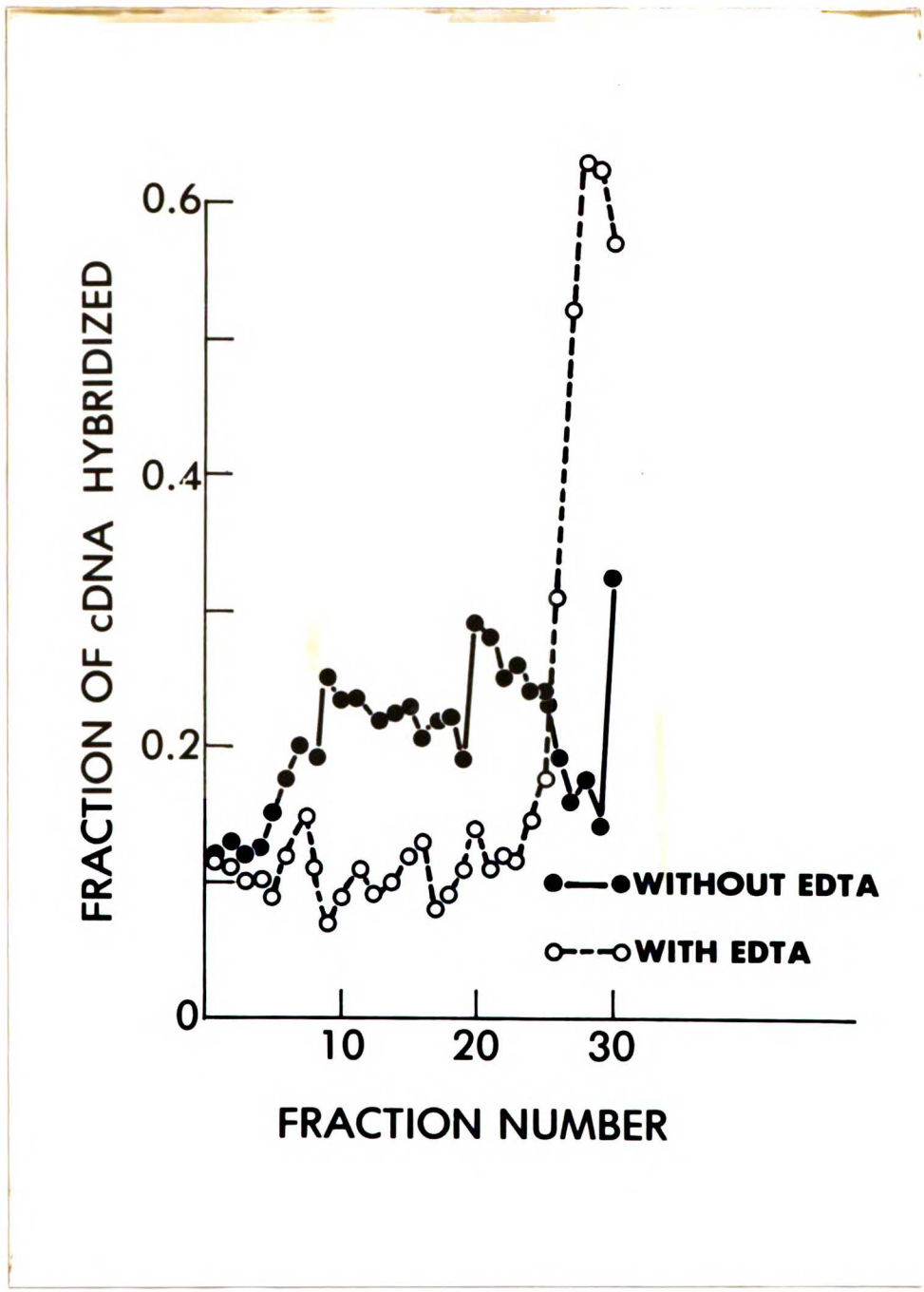


Figure 4. Purified polyribosomes resedimented on linear sucrose gradients. Polysomes were purified in discontinuous gradients; one half of the polyribosomes were diluted into the buffer used in the gradients, and the other half was diluted into the same buffer containing EDTA instead of $MgCl_2$.

The gradients were centrifuged and analyzed by hybridization with $cDNA_{B77}$ as described in Figure 2. Sedimentation was from right to left.


Without EDTA.

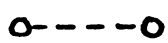
With EDTA.

TABLE 1

Recovery of virus-specific RNA from purified polyribosomes[A]

| Competitor | Cytoplasmic RNA | | | Polyribosome-associated RNA | | | % Recovery[C] |
|---|---|---|---|---|---|---|---|
| | Unbound | Bound | Virus-specific[B] | Unbound | Bound | Virus-specific | |
| - | 1,083,456 | 3648 | 10,694 | 360,618 | 1354 | 4393 | 42 |
| + | 1,060,128 | 1270 | | 378,344 | 124 | | |

[A]ASV infected chicken embryo fibroblasts were labeled for 20 hours with 300 μCi/ml $^{3}$H-uridine. RNA was isolated from the cytoplasm and from purified polyribosomes of equal numbers of the labeled cells. Aliquots of each RNA fraction and a small amount of [$^{32}$P] 70S ASV RNA were hybridized for ∿ 40 hours in the absence and presence of 1 μg 38S ASV RNA competitor. Following hybridization the mixtures were digested with RNase, hybrids bound to hydroxyapatite columes in 0.2M phosphate pH 6.8, 8 M urea and 1% SDS, and eluted with 0.4 M phosphate pH 6.8. Unbound and bound radioactivity were counted in Tritosol.

[B]Cpm virus-specific RNA were calculated by correcting the bound cpm minus competed background for 24.5% (cytoplasmic RNA) and 28.1% (polyribosome associated RNA) hybridization of the [$^{32}$P] labeled 70S ASV RNA internal standards.

[C] $\frac{\text{Virus-specific cpm in polyribosomes}}{\text{Virus-specific cpm in cytoplasm}} \times 100$

TABLE II

Recovery of virus-specific RNA from purified polyribosomes

| RNA isolated from:[a] | $V_o t_{1/2}$ [b] | % Recovery of ASV RNA |
|---|---|---|
| Whole cell | 0.27 | 100 |
| Cytoplasm | 0.38 | 71 |
| Polyribosomes | 1.1 | 25 |

[a] RNA was extracted from identical numbers of cells after the various steps in the purification of polyribosomes. The RNA was hybridized in increasing concentrations with $cDNA_{B77}$ for 20 hours at 68° C.

[b] $V_o t_{1/2}$ = number of cells from which RNA was extracted x $10^{-7}$ x time, at the point when the hybridization was 50% complete.

Size of the viral RNA associated with polyribosomes

RNA from purified polyribosomes was subjected to electrophoresis in an agarose gel. Cytoplasmic RNA was run in a parallel lane. The method of Alwine et al. (1977) was used to transfer the RNA from the gel and immobilize it on ABM paper; then the paper was hybridized with ASV-specific cDNA. I am certain that most of the bands visible after hybridization and autoradiography correspond to the viral RNA species. However, two bands, present in every sample apparently correspond to the ribosomal RNAs, because they comigrate with the rRNAs seen when the gels were stained with ethidium bromide and because they were present in uninfected cells (Figure 5A). Additional bands were detected only when RNA from infected cells was analyzed, and these RNAs migrated with rates similar to those described previously (Weiss et al., 1977). In addition, when cDNAs specific for the env, src, or 3' sequences were used for hybridization in similar experiments. the ribosomal RNAs were not detected. Also, the deletion of specific genes from the RNA species in cells infected by the defective viruses did not affect the migration of the rRNA, but did affect the viral RNA migration.

In cells infected by ndASV, three viral RNA species are detected in both the cytoplasm and the polyribosomes (Figure 5B-E). The molecular weights of the viral RNAs were 3.3, 1.9, and 1.1 x $10^6$; the values were derived by comparison with the migration of the chicken 28S and 18S ribosomal RNAs which have known molecular weights of 1.59 and 0.7 x $10^6$ respectively (Attardi & Amaldi, 1970). Data in the following sections will further substantiate these findings. The genetic compositions of the RNAs were defined by earlier work, but to ascertain that the smallest RNA

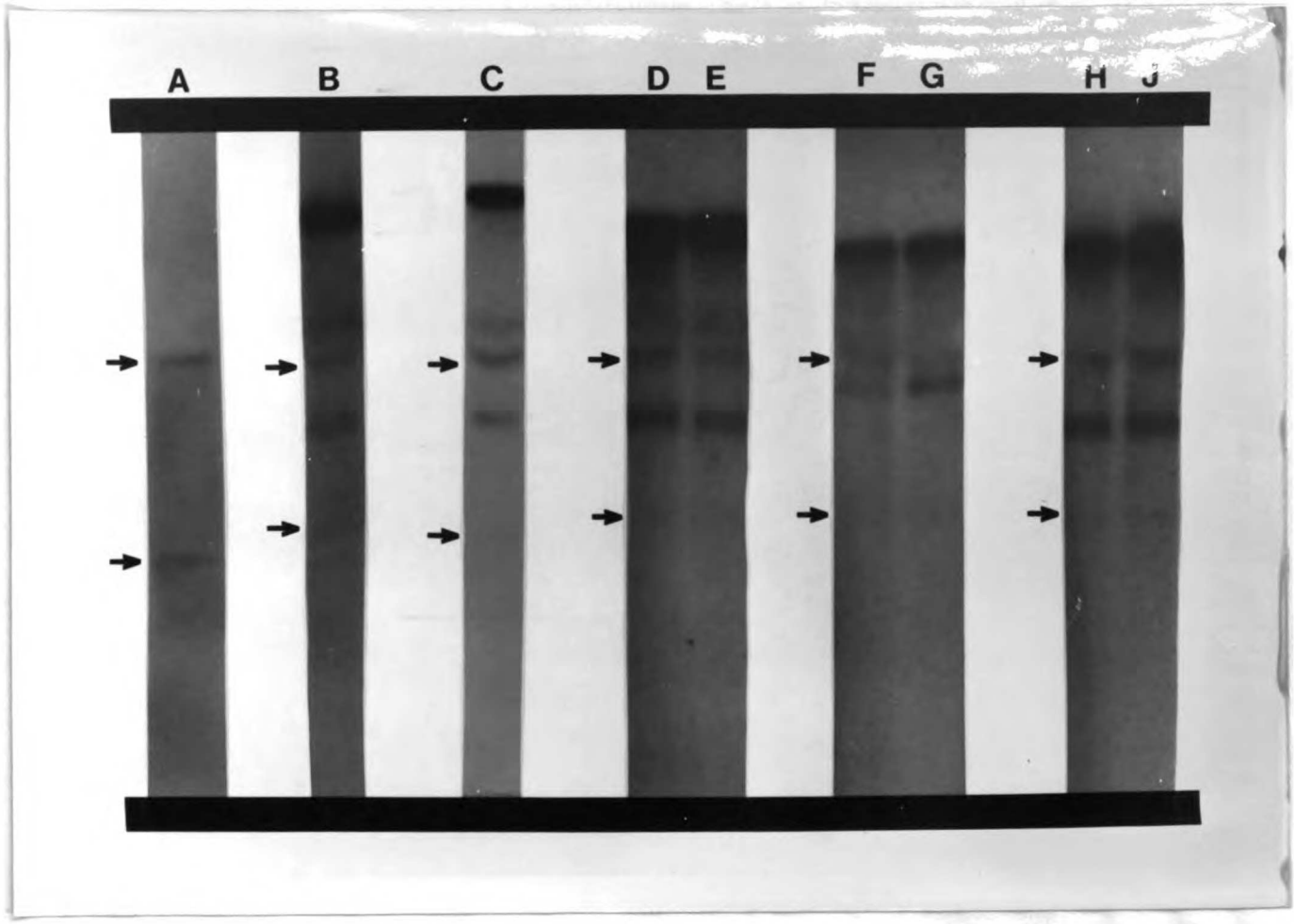


Figure 5. Sizes of virus-specific RNAs associated with polyribosomes.

Figure 5. Sizes of virus-specific RNAs associated with polyribosomes. RNAs were isolated from the cytoplasm and from purified polyribosomes, fractionated by electrophoresis in denaturing agarose gels, transferred to ABM paper following electrophoresis (Alwine, et al.,1977), and hybridized with $cDNA_{rep}$ as described in Methods. Hybridization of cDNA to RNA was detected by autoradiography.

A) Cytoplasmic RNA from uninfected chicken embryo fibroblasts.

B) Cytoplasmic RNA from chicken embryo fibroblasts infected with Pr-B ASV.

C) Polyribosome-associated RNA from chicken embryo fibroblasts infected with Pr-B ASV.

D) Cytoplasmic RNA from chicken embryo fibroblasts infected with Pr-B ASV.

E) Polyribosome-associated RNA from chicken embryo fibroblasts infected with Pr-B ASV.

F) Cytoplasmic RNA from chicken embryo fibroblasts infected with td Pr-B ASV.

G) Polyribosomes-associated RNA from chicken embryo fibroblasts infected with td Pr-B ASV.

H) Cytoplasmic RNA from chicken embryo fibroblasts infected with Br-ASV(GPV).

I) Polyribosome-associated RNA from chicken embryo fibroblasts infected with Br-ASV(GPV).

corresponded to the putative src mRNA I exploited the existence of deletion mutants for src (tdASV) and env (rdASV). The deletion of either gene eliminates one of the two small RNA species, and only one subgenomic viral RNA is observed in cells infected by either tdASV or rdASV. Each of these subgenomic RNAs has a sedimentation coefficient of 21S (Weiss, 1977). I found that the td "env" mRNA (Figure 5F,G) migrated at 1.4 x $10^6$, whereas the rd "src" mRNA (Figure 5H,J) migrated at 1.1 x $10^6$, the same size as the nd src mRNA. The genome sizes were determined to be 3.3 x $10^6$ for ndASV RNA, 2.9 x $10^6$ for tdASV RNA, and 2.8 x $10^6$ for rdASV RNA (data not shown) in reasonable agreement with published values (Duesberg and Vogt, 1973). I conclude that in chicken cells productively infected with ndASV all three major size classes of viral RNA function as messenger RNAs.

I also wished to assess the genetic composition of the mRNAs with the use of specific cDNAs. In the following experiments, I fractionated the polyribosome-associated RNA according to size in non-denaturing agarose gels. The gels were sliced and each fraction was hybridized in solution to $cDNA_{B77}$ and $cDNA_{sarc}$ in the same reaction mixture. The results are shown in Figure 6. $cDNA_{B77}$ hybridized significantly with the 3.3, 1.9, and 1.1 x $10^6$ RNA species in cells infected with cloned PrB-ASV (panel A, Figure 6). In contrast, the pattern of hybridization with $cDNA_{B77}$ was much more heterogeneous when the RNA from cells infected with an uncloned stock of PrC-ASV was analyzed with $cDNA_{B77}$ (Figure 6B), whereas $cDNA_{sarc}$ hybridized mainly to the species present in cells infected with a cloned virus. The results suggest that the RNA in PrC-ASV infected cells may be a mixture of RNAs derived from both nd and td viruses. Therefore, two extra RNAs, approximately 2.9 and 1.4 x $10^6$ in

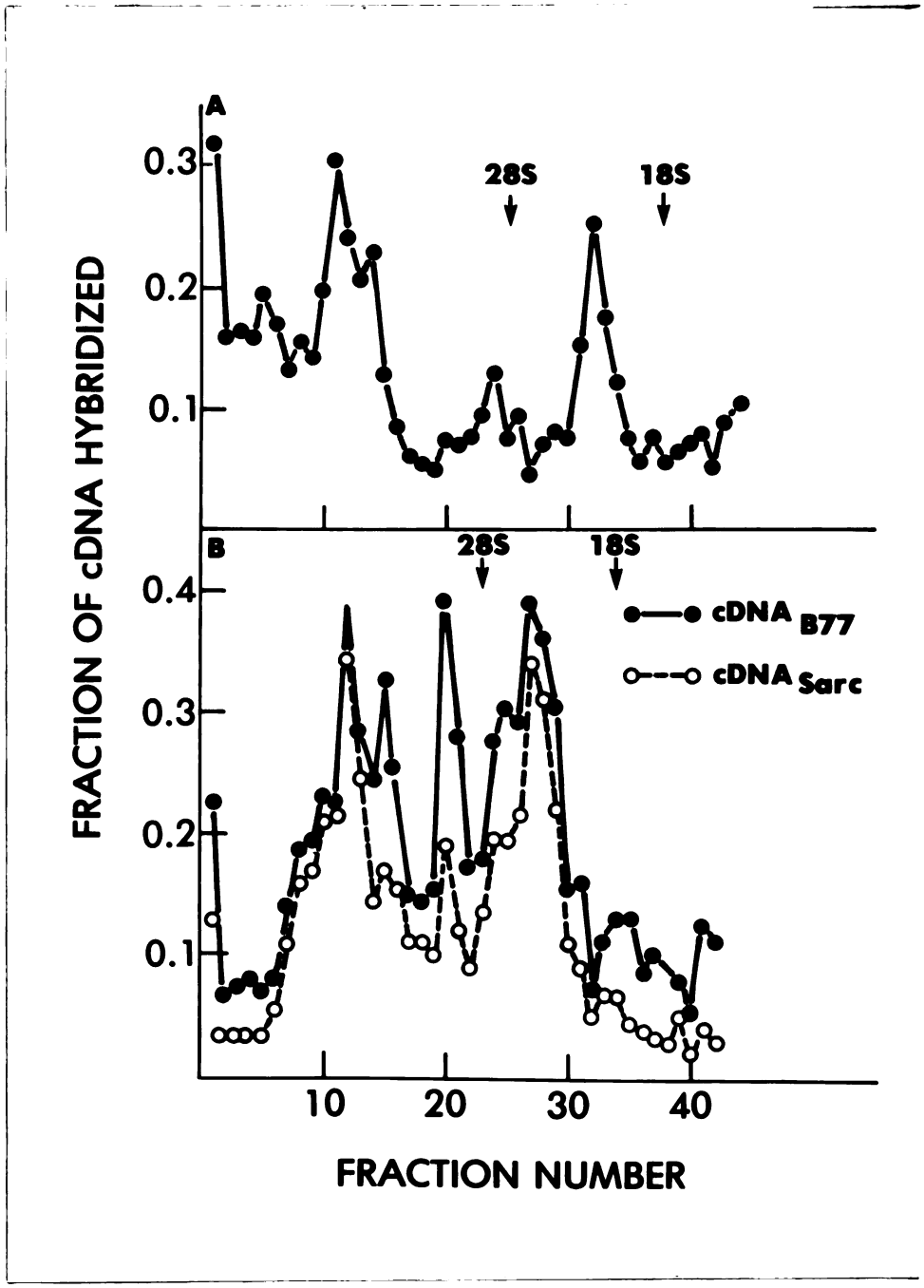


Figure 6. Electrophoresis of virus-specific mRNA in agarose gels.

Figure 6. Electrophoresis of virus-specific mRNA in agarose gels. Electrophoresis in agarose gels and location of virus-specific RNA by molecular hybridization were performed as described in Materials and Methods. The 18S and 28S rRNAs were located by scanning for absorbance at 260 nm.

A. Polyribosome associated RNA (20 μg) was isolated from cells infected with Pr-B ASV Clone 19. The RNA was subjected to electrophoresis in noadenaturing conditions in a 2% agarose gel for 5 hours. Hybridization with $cDNA_{B77}$ was in 100 μl volumes for 20 hours.

B. Polyribosome associated RNA (25 μg) was isolated from cells infected with Pr-C ASV. The RNA was subjected to electrophoresis as in panel A for 4 hours. Hybridization with $^{32}P\text{-}cDNA_{B77}$ and $^{3}H\text{-}cDNA_{sarc}$ was in 200 μl volumes for 36 hours.

molecular weight, hybridizing to a greater extent with $cDNA_{B77}$ than with $cDNA_{sarc}$, were observed. These results show that the 1.9 and 1.1 x $10^6$ mRNAs contain sarc sequences and are therefore probably identical to those RNAs previously found in the cytoplasm (Weiss et al., 1977; Hayward, 1977).

Viral RNA in membrane-bound and free polyribosomes

Proteins that are secreted through the plasma membrane or cell surface proteins that are anchored in the membrane and are exposed to the external environment are as a rule synthesized on membrane-bound polyribosomes (Blobel and Dobberstein, 1975; Wirth et al., 1977). I wished to determine if any of the viral RNA species were associated with membrane-bound polyribosomes, in order to learn whether the viral proteins, $pp60^{src}$ in particular, were possible candidates for secretory or cell surface proteins. I sedimented a cytoplasmic lysate through a linear sucrose gradient layered over a 55% sucrose cushion. Membrane-bound polyribosomes sediment rapidly to the 55% sucrose cushion, while the free polyribosomes are retarded in the linear sucrose gradient above the cushion (Wirth et al., 1977). A detergent treated lysate, a lysate treated with EDTA, and a lysate treated with both detergent and EDTA were sedimented on parallel gradients. The RNA from each fraction was hybridized with ASV-specific cDNA. Figure 7 shows the patterns obtained. The gradients and cDNA were annealed beyond the linear range of hybridization in the free polyribosome region in order to more accurately estimate the amount of viral RNA in the membrane regions. The membrane fractions contained a small quantity of virus specific RNA, part of which was resistant to release by EDTA. Detergent treatment of the

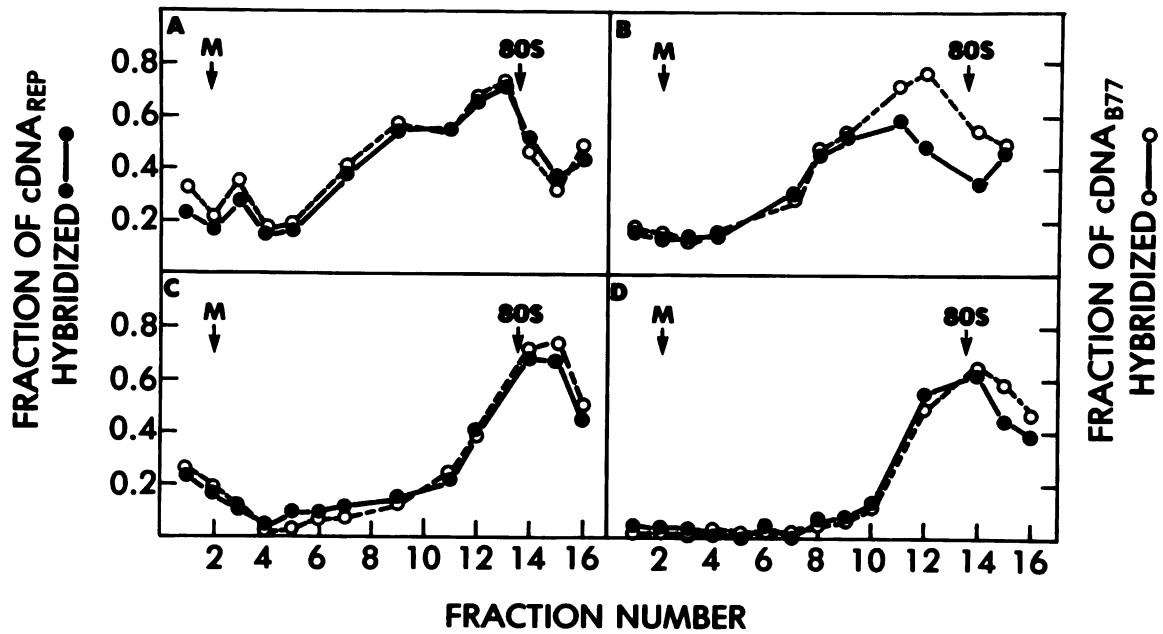


Figure 7. ASV-specific RNA in membrane-bound and free polyribosomes. Cytoplasmic lysates were prepared and sedimented at 32,000 rpm for 60 min at 4°C through 15-40% sucrose gradients on a 55% sucrose cushion as described in Methods. Membranes sediment rapidly to the bottom of the gradient (see Results). The gradipnts were fractionated from the bottom and RNA was extracted from each fraction.

●——● Hybridization with $cDNA_{rep}$.

○- - -○ Hybridization with $cDNA_{B77}$.

Hybridization was performed as in Figures 1 and 3. Sedimentation was from right to left.

A) Cytoplasmic lysate without detergent or EDTA.

B) Cytoplasmic lysate with detergent.

C) Cytoplasmic lysate with EDTA.

D) Cytoplasmic lysate with detergent and EDTA.

cytoplasmic lysates prior to centrifugation appeared to remove some of the viral RNA from the membrane fraction, and treatment with both EDTA and detergent removed almost all of the viral RNA from the membrane region of the gradient. In order to find out which of the viral RNA species were membrane-bound, I determined the sizes of the RNAs in each fraction of the control gradient. The autoradiogram was also overexposed in the free polyribosome region in order to visualize all the bands in the membrane fractions. I found that all size classes were present in the membrane fractions (Figure 8, #1-3). However, the putative env mRNA was greatly enriched with respect to the other viral RNA species and barely detectable in the free polyribosome region of the gradient (Figure 8, #4-12). In contrast, the other two ASV-specific RNAs were primarily associated with the free polyribosomes. I then determined the effect of treating the cytoplasmic lysate with EDTA prior to centrifugation by analyzing the RNAs from each fraction of the gradient in Figure 4C (Figure 9). In addition, to more accurately quantitate the effect of EDTA, the RNAs from fractions 1 and 2 in Figures 8 and 9 were analyzed in parallel lanes in the same gel and hybridization (Figure 10). Only the env mRNA was diminished in the membrane fractions after sedimentation in the presence of EDTA, whereas the bulk of the genome length RNA and the putative src mRNA that had been present in the membrane region without EDTA treatment remained in that region. The genome length RNA appeared to sediment faster after treatment with EDTA, an observation I cannot explain. In addition, because of slight degradation of the RNA, I was unable to determine the sizes of the ASV-specific RNA after treatment with detergent. However, I believe that only the env mRNA is present in membrane-bound polyribosomes

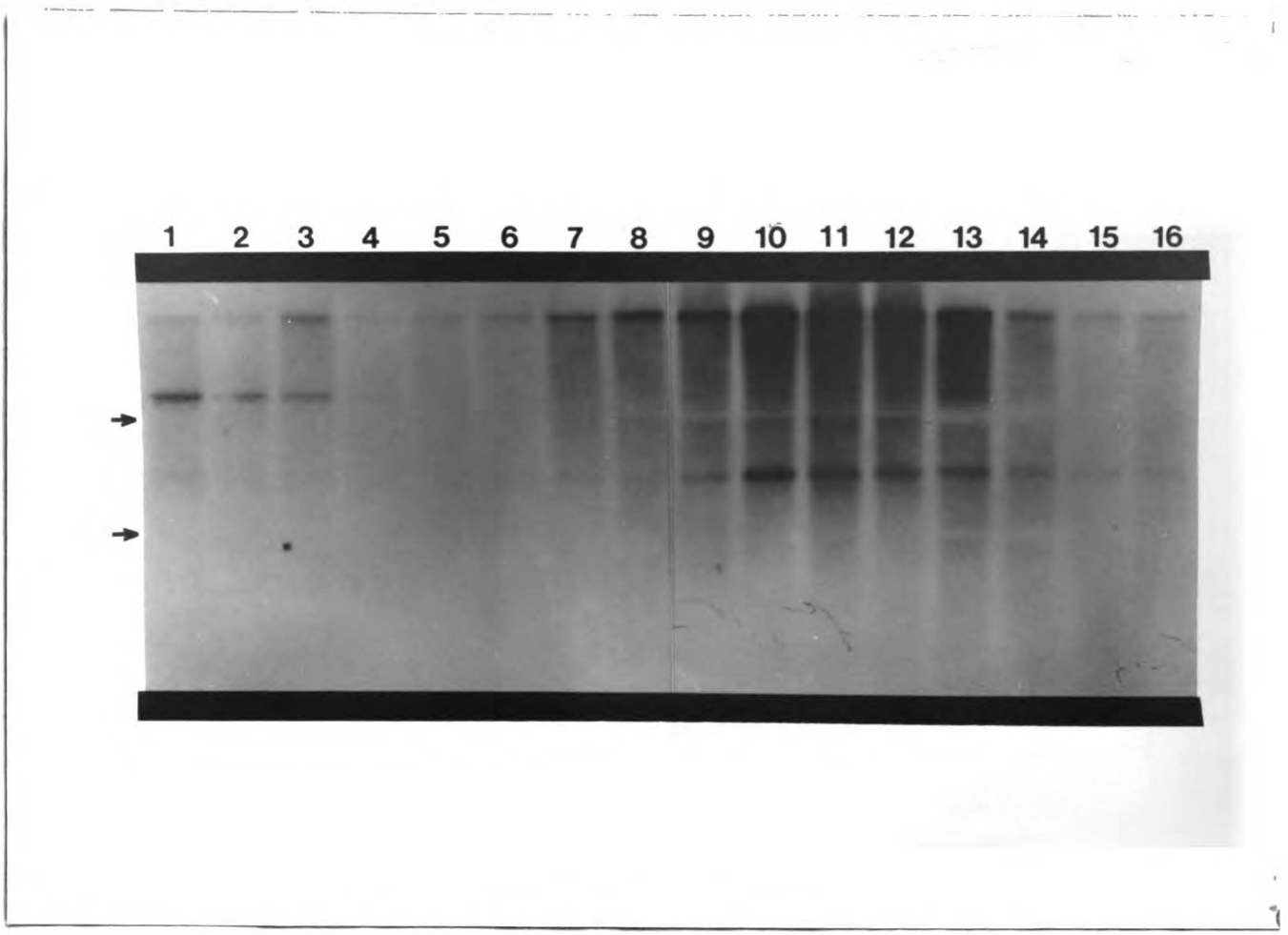


Figure 8. Sizes of ASV specific RNAs in membrane-bound and free polyribosomes. RNA from equal volumes of each fraction of the gradient in Figure 7A was subjected to electrophoresis, transfer, and hybridization as described in Figure 5. The numbers of the lanes correspond to fraction numbers in Figure 7A.

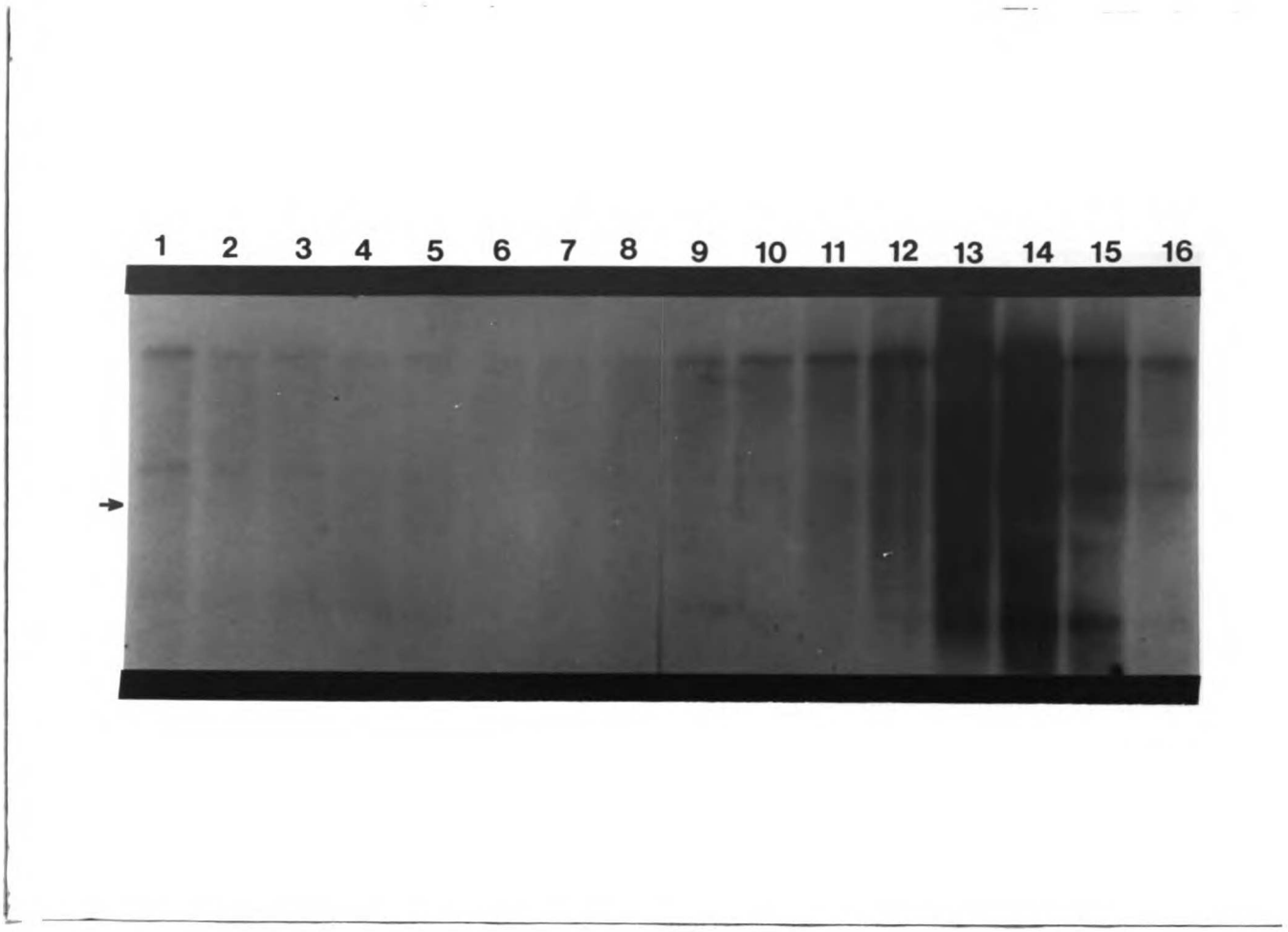


Figure 9. Sizes of ASV specific RNAs after treatment of cytoplasmic lysate with EDTA and sedimentation in a linear gradient. RNA from the gradient in Figure 7C was analyzed as in Figure 8.

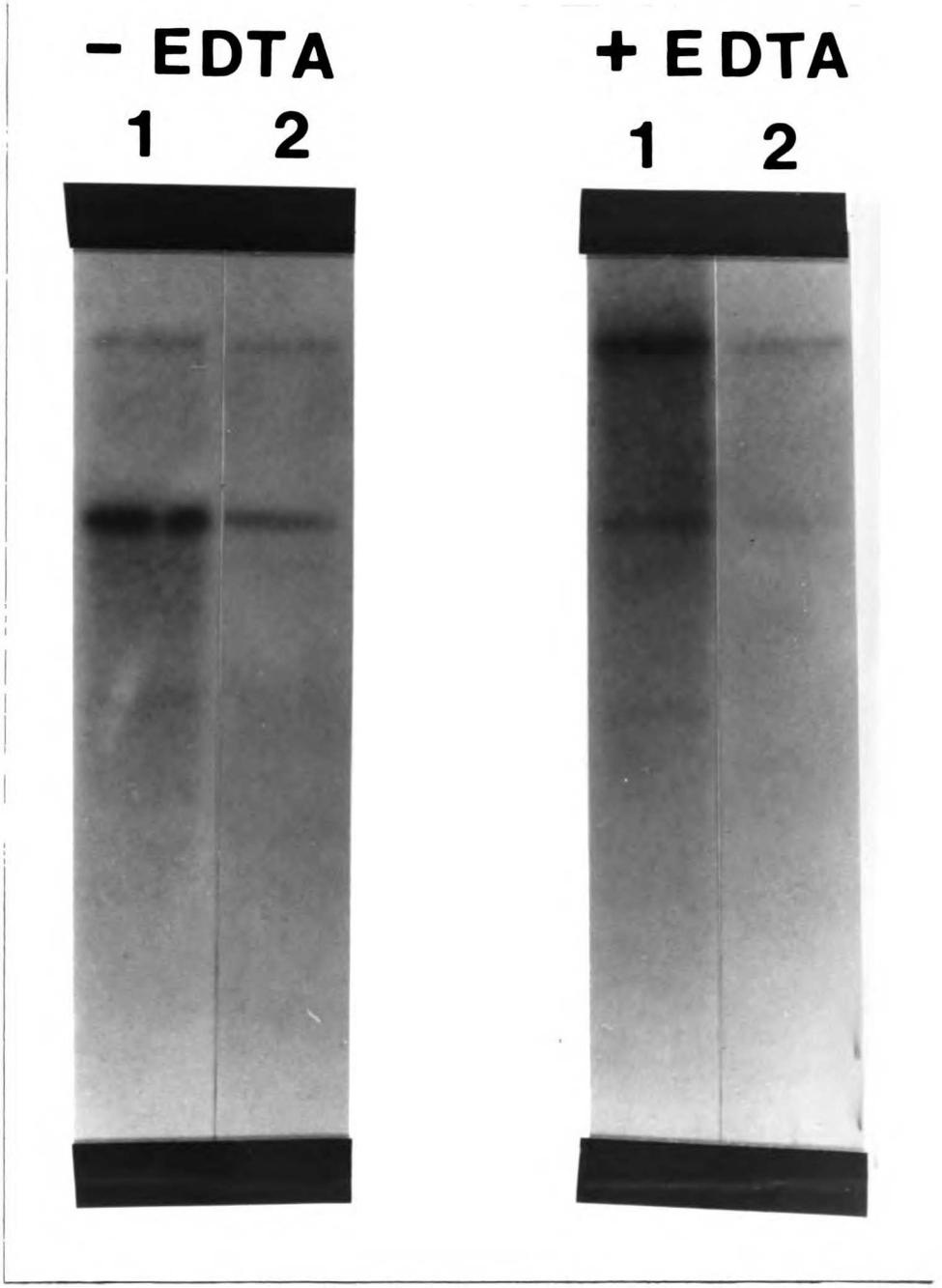


Figure 10. Sizes of ASV-specific RNA from fractions 1 and 2 from the gradients in Figures 5 and 6 were analyzed as in Figure 5.

because essentially all of the env mRNA was membrane-bound, whereas little, if any, of the other two viral mRNAs were bound in polyribosomes associated with the membranes.

Immunoprecipitation of polyribosomes

In order to characterize the ASV-specific mRNAs by immunoprecipitation of polyribosomes, I considered it necessary to demonstrate that: 1) a significant fraction of the polyribosome associated ASV-specific RNA could be precipitated with the appropriate antisera, 2) the immunoprecipitation of polyribosomes was specific with respect to the various antisera that I used, and 3) that the immunoprecipitated RNA was intact, so it could be subjected to size analysis.

A. Quantitation of immunoprecipitated RNA

The specificity and efficiency of immunoprecipitation with respect to the ASV RNA was analyzed by hybridization with $cDNA_{B77}$ and measured by $V_o t_{1/2}$ values (described earlier). Table III shows that up to 75% of the polyribosome-associated RNA could be precipitated with anti-$ASV_C$, an antiserum which precipitates the products of the gag, pol, and env genes (see below). Antisera with more restricted specificities precipitated less ASV-specific RNA, while normal rabbit serum precipitated less than 1% of the ASV-specific RNA.

RNA that had been isolated from cells labeled for 20 hours with $^3$H-uridine was also analyzed for ASV-specific sequences (also shown in Table III). The results agree reasonably with the data derived from $V_o t$ analysis. Other experiments gave similar results, except that occasionally up to 30% of the ASV-specific RNA (see Figure 20) could be precipitated with antiserum directed against reverse transcriptase

TABLE III

Recovery of ASV-specific RNA after immunoprecipitation of polyribosomes

| RNA isolated from polyribosomes immunoprecipitated by: | $V_o t_{1/2}$[a] | % Recovery of ASV RNA | Cpm ASV RNA[b] | % Recovery of ASV Cpm |
|---|---|---|---|---|
| Total (no antiserum) | 0.45 | 100 | 13,092 | 100 |
| Anti-$ASV_C$ | 0.65 | 69 | 9,753 | 75 |
| Anti-p27 | 2.0 | 22 | 8,582 | 65 |
| Anti-pol | 9.0 | 5 | 391 | 3 |
| TBR[c] | - | - | 3,978 | 30 |
| TBR + V [d] | - | - | 290 | 2 |
| NR | - | - | 82 | 0.6 |

[a]RNA was isolated from polyribosomes from equal numbers of cells before and after immunoprecipitation with various antisera. $V_o t_{1/2}$ values were determined by hybridization of $cDNA_{B77}$ as in Table II.

[b]RNA was isolated from the polyribosomes of equal numbers of cells labeled with $^3$H-uridine for 20 hours. The polyribosomes were immunoprecipitated with different antisera. The RNA was analyzed by hybridization with unlabeled cDNA as in Table I.

[c]TBR = tumor bearing rabbit. Antisera was raised by injecting newborn rabbits with Sr-D ASV to induce tumors. The antiserum contained antibodies to the src gene product and other viral proteins (H. Oppermann, personal communication)

[d]B77 ASV was added during immunoprecipitation of polyribosomes with TBR serum.

(anti-pol). A curious aspect of these experiments was that much greater quantities of antisera were required to immunoprecipitate the polyribosomes than was expected from analysis of amounts of p27 determinants in the polyribosomes. Perhaps, the titers of the antibodies which recognize the nascent polypeptides present on polyribosomes are lower than those for determinants on the mature proteins.

B. Sizes of immunoprecipitated RNAs

The RNAs isolated from polyribosomes immunoprecipitated by anti-$ASV_C$, anti-p27, anti-pol, and normal rabbit serum were analyzed by electrophoresis in non-denaturing agarose gels. Following electrophoresis the gels were scanned for absorbance at 260 nm and sliced. Each fraction was hybridized for a constant time with $cDNA_{B77}$. The results are shown in Figure 11. Anti-$ASV_C$ precipitated polyribosomes containing both the 3.3 and 1.9 x $10^6$ RNAs (Figure 11A), anti-p27 (anti-gag) (Figure 11B), and anti-pol (Figure 11C) appeared to precipitate polyribosomes containing mainly the 3.3 x $10^6$ RNA, whereas normal rabbit serum precipitated only a small amount of high molecular weight RNA, presumably in aggregates (Figure 11D). However, the majority of the gels that I analyzed by this technique were impossible to interpret, because the RNA was either aggregated or degraded.

In order to overcome the problems of degradation and aggregation, I decided to analyze the RNA isolated from smaller quantities of cells labeled with $^3$H-uridine. When the immunoprecipitated RNA was selected for poly(A) by binding to oligo-d(T) cellulose and then hybridized to unlabeled cDNA, it appeared to be highly enriched for virus-specific RNA. The results are shown in Table IV. The RNAs precipitated by the specific antisera were from 20-60% pure with respect to viral RNA. The

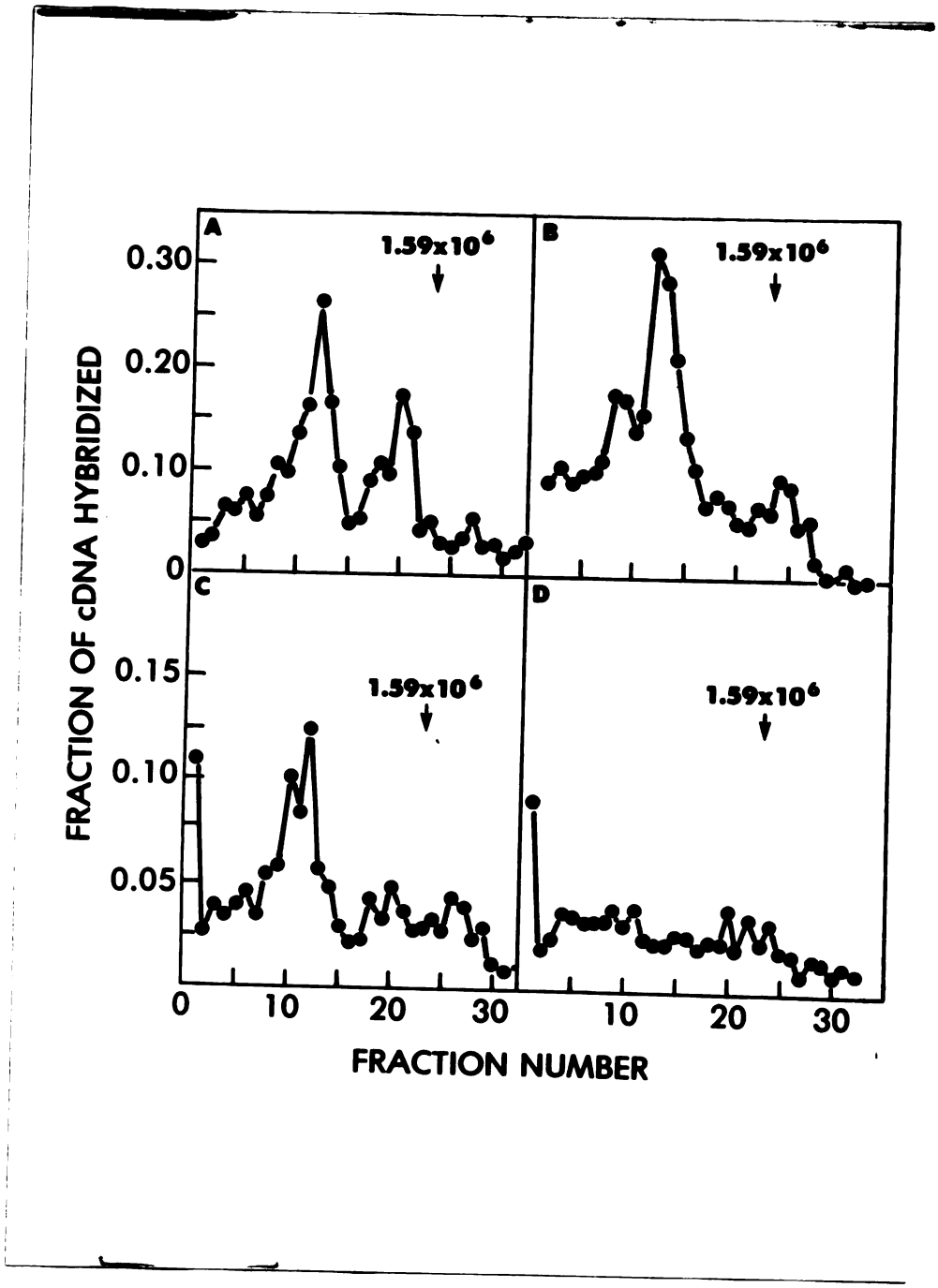


Figure 11. Electrophoresis of RNA isolated from polyribosomes immunoprecipitated with specific antisera. The RNA was analyzed as in Figure 6. A) RNA isolated from polyribosomes from $1.8 \times 10^7$ cells immunoprecipitated by anti-$ASV_c$. Electrophoresis under nondenaturing conditions was for 5

hours. Hybridization with $cDNA_{B77}$ was for 18 hours.

B) RNA was isolated by immunoprecipitation of polyribosomes from $1.8 \times 10^7$ cells using anti-p27. Electrophoresis was performed as in panel A, and hybridization was for 19 hours.

C) RNA was isolated as in panels A and B except that anti-pol serum was used. Hybridication was for 40 hours.

D) RNA isolated as in panels A, B, and C except that normal rabbit (NR) serum was used as a control. Hybridization was for 40 hours.

TABLE IV

Purity of RNA isolated from immunoprecipitated polyribosomes

| RNA isolated from polyribosomes purified by: | Total Cpm | ASV specific cpm[b] | % ASV specific cpm |
|---|---|---|---|
| Total (no antiserum) | 180,912 | 13,092 | 7.2 |
| Hybridization | 10,038 | 6,972 | 70 |
| Anti-$ASV_C$ | 20,411 | 9,753 | 48 |
| Anti-p27 | 14,885 | 8,582 | 58 |
| Anti-pol | 1,870 | 391 | 21 |
| TBR | 9,151 | 3,978 | 44 |
| TBR + V | 706 | 290 | 41 |
| NR | 539 | 82 | 15 |

[a]Cells were labeled for 20 hours with $^3$H-uridine and RNA was isolated from the polyribosomes by immunoprecipitation with the various antisera from total polyribosomes, or from polyribosome associated RNA by hybridization with unlabeled ASV-specific cDNA.

[b]ASV-specific cpm were determined by hybridization to unlabeled cDNA and analyzed as in Table I.

major contaminant appeared to be 18S rRNA (see below). I therefore subjected the RNAs to electrophoresis in 1.2% agarose gels under denaturing conditions, and analyzed the gels by fluorography. RNA purified by preparative hybridization to unlabeled cDNA was used as marker and analyzed in a parallel lane. Figures 12 and 13 show the patterns obtained in two experiments. Different exposures are shown in order to compare the sizes of the RNAs immunoprecipitated by different antisera. Cells infected with a mammaltropic strain, SR-$D_m$ - ASV, were analyzed in Figure 12. It was this virus that was used to induce tumors in newborn rabbit in order to raise antisera against the src gene product (H. Oppermann, personal communication). I wished to analyze the RNA produced in cells infected by this virus because the sera would presumably have greatest reactivity with the homologous src gene product. Unfortunately, the cells contained an excess of $2.8 \times 10^6$ RNA presumably from a defective virus containing a deletion of an unknown nature. However, the hybridization-purified viral RNA (lane M) showed that the 1.9 and $1.1 \times 10^6$ RNAs were still present associated with polyribosomes in these cells. In addition, there did not appear to be an extra small RNA species migrating at about $1.4 \times 10^6$, which would correspond to the small RNA found in tdASV infected cells (Figures 5 and 6). For these reasons, I believe it is possible that the defective RNA contains a deletion in the env gene and not the src gene, although the hypothesis has not been adequately tested. When anti-$ASV_C$, anti-p27, and anti-pol were used to immunoprecipitate the polyribosomes, the genome length RNAs were enriched with respect to the 1.9 and $1.1 \times 10^6$ RNAs (Figure 12). In contrast, when TBR serum (from rabbits bearing SR-$D_m$-ASV induced tumors) containing antibodies to gag, pol, env, and src products, was

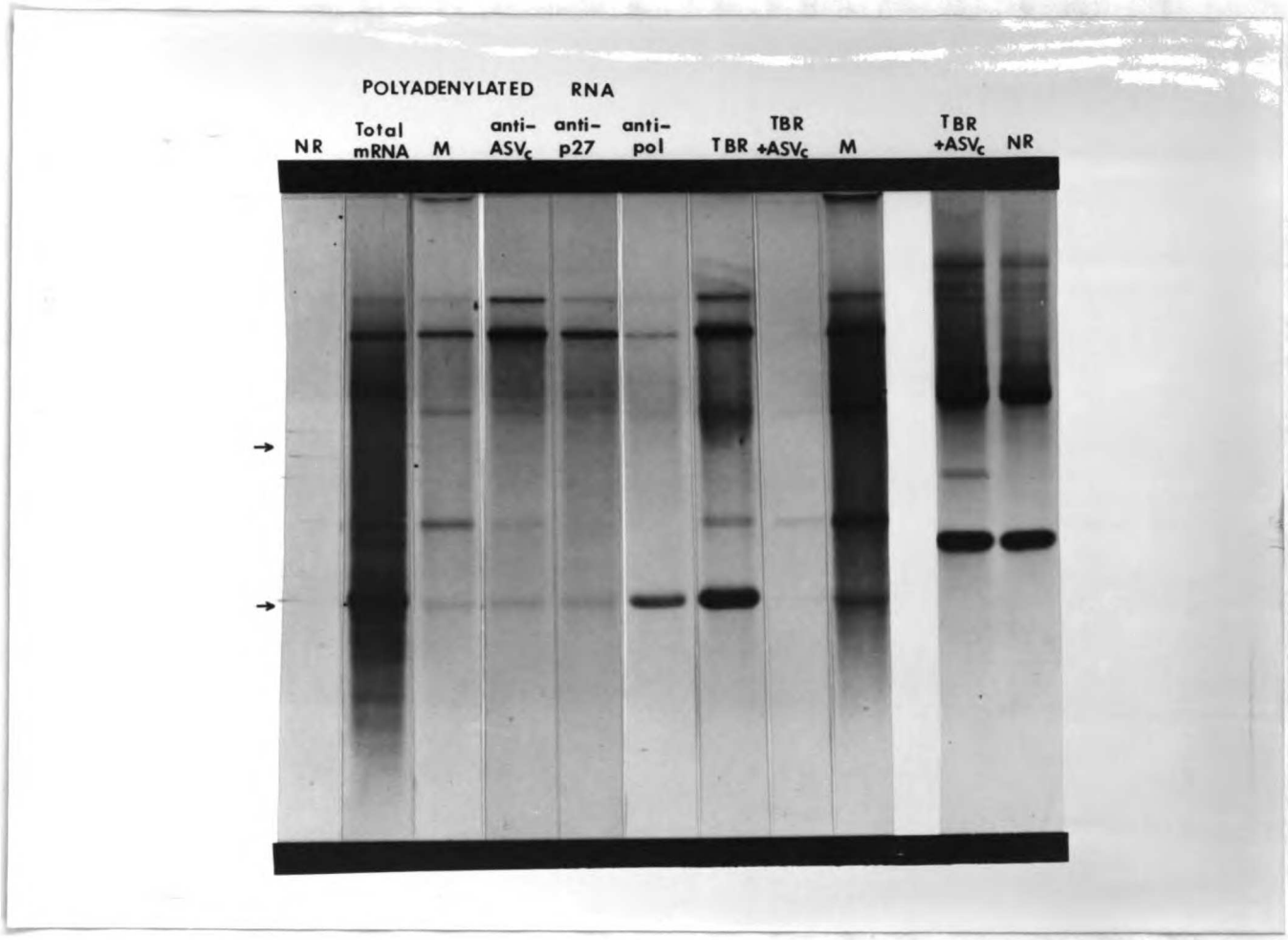


Figure 12. Analysis of RNA from polyribosomes immunoprecipitated with specific antisera. The RNA polyribosomes were isolated from SR-$D_m$ ASV infected cells. Total mRNA was isolated, ASV RNA was purified by hybridization to unlabeled cDNA (lane M) as described in Materials and Methods, and the other RNAs were isolated from polyribosomes after immunoprecipitation with several antisera. Polyadenylated RNA (in left 9 lanes) was selected by oligo-d(T) cellulose chromatography as described. Electrophoresis was for 12 hours at 70 V in a 1.2% agarose gel in the presence of 5 mM Methyl mercury hydroxide. Electrophoresis of RNA not selected for poly (A) was at 60 V for 12 hours. The gels were impregnated with Omnifluor and the RNA was visualized by flurography at -70° C as described in

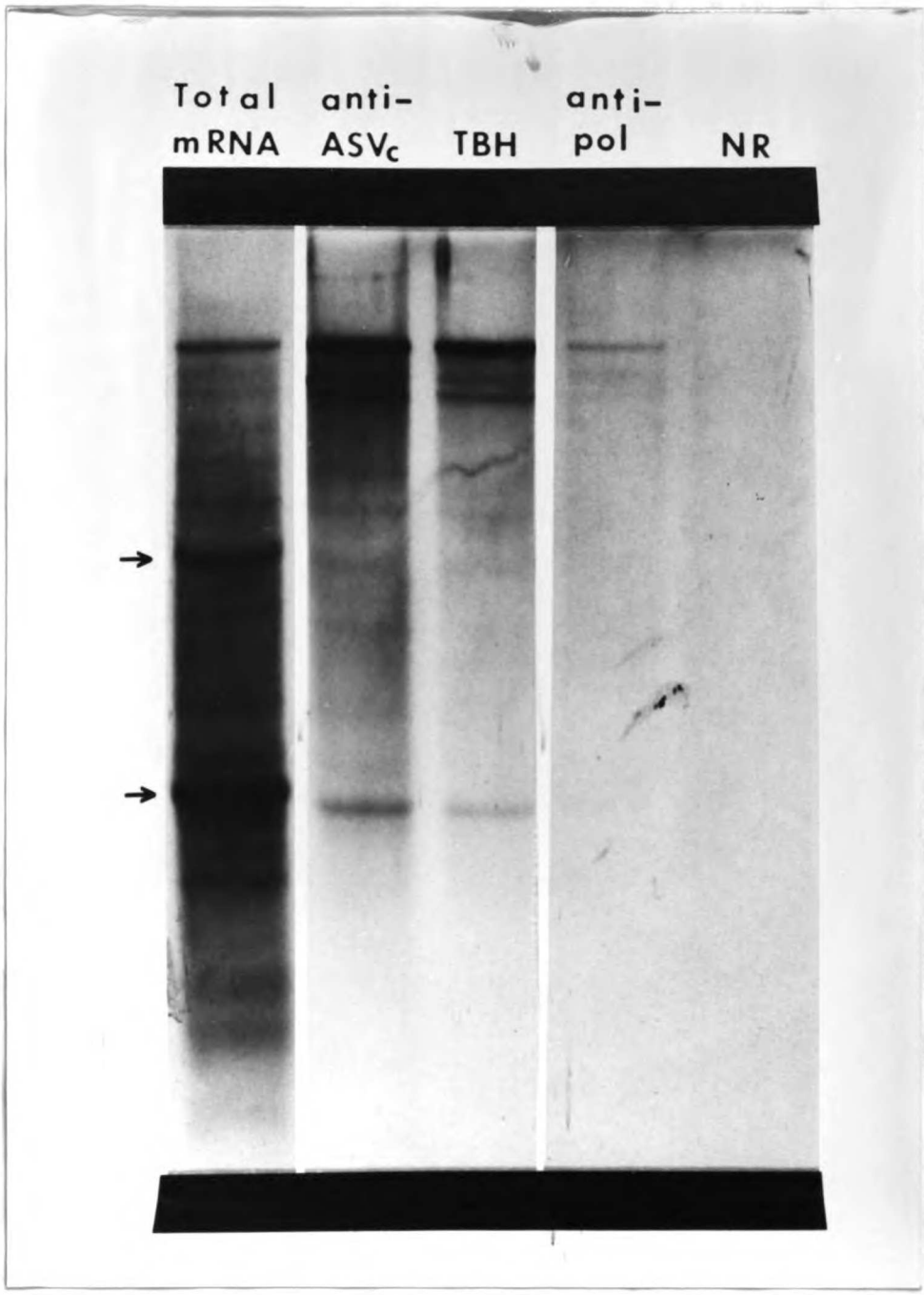


Figure 13. Analysis of RNA from polyribosomes of cells infected with cloned SR-D ASV. RNA was immunoprecipitated as in Figure 12 and analyzed by electrophoresis under similar conditions.

used, the smaller messages were immunoprecipitated in greater amounts with respect to the genome length RNAs (lane TBR). In addition, when purified B77-ASV was used to compete the immunoprecipitation of the structural protein determinants, only the 1.1 x $10^6$ message and perhaps a small amount of the 1.9 x $10^6$ message remained (lane TBR+$ASV_C$). This could be seen more clearly before the RNA was selected on oligo-d(T) cellulose (compare two lanes on right, Figure 12). A small amount of the 1.9 x $10^6$ message remained in the TBR+$ASV_C$ immunoprecipitation. This may be explained by differences in the antigenic determinants between the glycoproteins of the B77 and SR-D ASVs. The gp85 from B77-ASV may not contain all the antigenic determinants which the TBR serum recognizes on the intracellular env product present in $SR\text{-}D_m$ ASV infected cells. As a corollary, many of the $SR\text{-}D_m$ env determinants may not have been recognized by the anti-$ASV_C$ serum, made against B77-ASV. This may be the reason why the 1.9 x $10^6$ message was not specifically enchanced in the anti-$ASV_C$ immunoprecipitated RNA. In the experiments of Fig. 13, polyribosomes from cells infected with cloned SR-D ASV were immunoprecipitated with anti-ASV, TBR (anti-gag), anti-pol and NR. These results support my earlier results where the 3.3 x $10^6$ RNA was selectively precipitated with anti-pol serum.

I used antibodies directed against purified glycoproteins on several occasions in an attempt to immunoprecipitate the 1.9 x $10^6$ mRNA in the absence of the 3.3 x $10^6$ mRNA, but I did not observe any significant precipitation. Perhaps the nascent protein is not recognized by antisera directed against the purified mature protein. This seems likely because an in vitro translation product, precipitable with anti-$gp85^{env}$ serum, has been extremely difficult to identify (Pawson et al.,

1977; Beemon and Hunter, 1978; S. Weiss, personal communication). In addition, the titer of this antiserum for the glycoprotein products in infected cells is much lower than the same titer in anti-$ASV_C$, where the glycoprotein was not purified from the other viral proteins (H. Oppermann, personal communication).

It was apparent in the gels shown in Figures 12 and 13, that the immunoprecipitation of polyribosomes by antisera directed against protein products of ASV was highly specific for the viral RNA species. However, because of the presence of the subgenomic RNAs in the immunoprecipitation by anti-p27 and anti-$ASV_C$, the results were difficult to interpret. The anti-$ASV_C$ and the anti-p27 sera do not contain antibodies against the product of the src gene (see below). I could not explain the presence of the $1.1 \times 10^6$ message in the RNA isolated from polyribosomes immunoprecipitated with these two antisera by such an immune reactivity. There were three possible mechanisms which could explain my findings.

One theory which could explain the immunoprecipitation of the subgenomic messages by anti-p27 was that the amino-termini of the primary translation products of the gag, env, and src genes were identical. The N-terminus of $Pr76^{gag}$ contains the tryptic peptides of p19 (Vogt, et al., 1975). It is known that the anti-p27 serum reacts with p19 (see below). The N-termini could be identical if translation of the RNAs began in the 5' leader sequences. However, this is unlikely for several reasons which will be discussed in a later section.

A second possibility to explain the precipitation of the small messages by anti-p27 was that the gag proteins bind to the RNA. Two proteins, p19 and p12, are known to bind to the viral RNA (Leis et al.,

1978). I attempted to examine this possibility by adding labeled 35S RNA to a cytoplasmic lysate from chronically infected cells and immunoprecipitating with various antisera. Less than 2% of the radioactivity was precipitated and in no case was it more than two to three-fold over that precipitated by normal rabbit sera. In another experiment, RNA was isolated from polyribosomes immunoprecipitated before and after treatment with EDTA or puromycin. The RNA was quantitated by $V_0t$ analysis. After addition of EDTA, less than 10% of the RNA precipitated before its addition, remained. Similar results were obtained with puromycin. I believe that binding of gag proteins to the RNA probably does not account for the immunoprecipitation.

Artifactual processing during the isolation procedure could also account for the presence of the small messages in the anti-p27 immunoprecipitates. Leis et al. (1978), had previously observed processing in vitro of the genome length RNA into small heterogeneous species. More recently, Hayward (personal communication) found that the genome length RNA can be specifically cleaved in vitro by cytoplasmic preparations from infected cells. It is possible that an enzyme(s) could be associated with the purified polyribosomes after isolation, and that the smaller RNAs could be generated during isolation. Therefore, I incubated labeled 35S ASV RNA with a preparation of polyribosomes, recovered the RNA, and analyzed it by electrophoresis in a denaturing agarose gel. Figure 14 shows that after incubation at 25 and 37 C an heterogeneous population of RNAs migrating with a mean molecular weight of $1.9 \times 10^6$ was observed. In addition, the reaction appeared to be temperature dependent, since little or no processing occurred at 0 C. In this experiment, I did not observe a significant generation of an

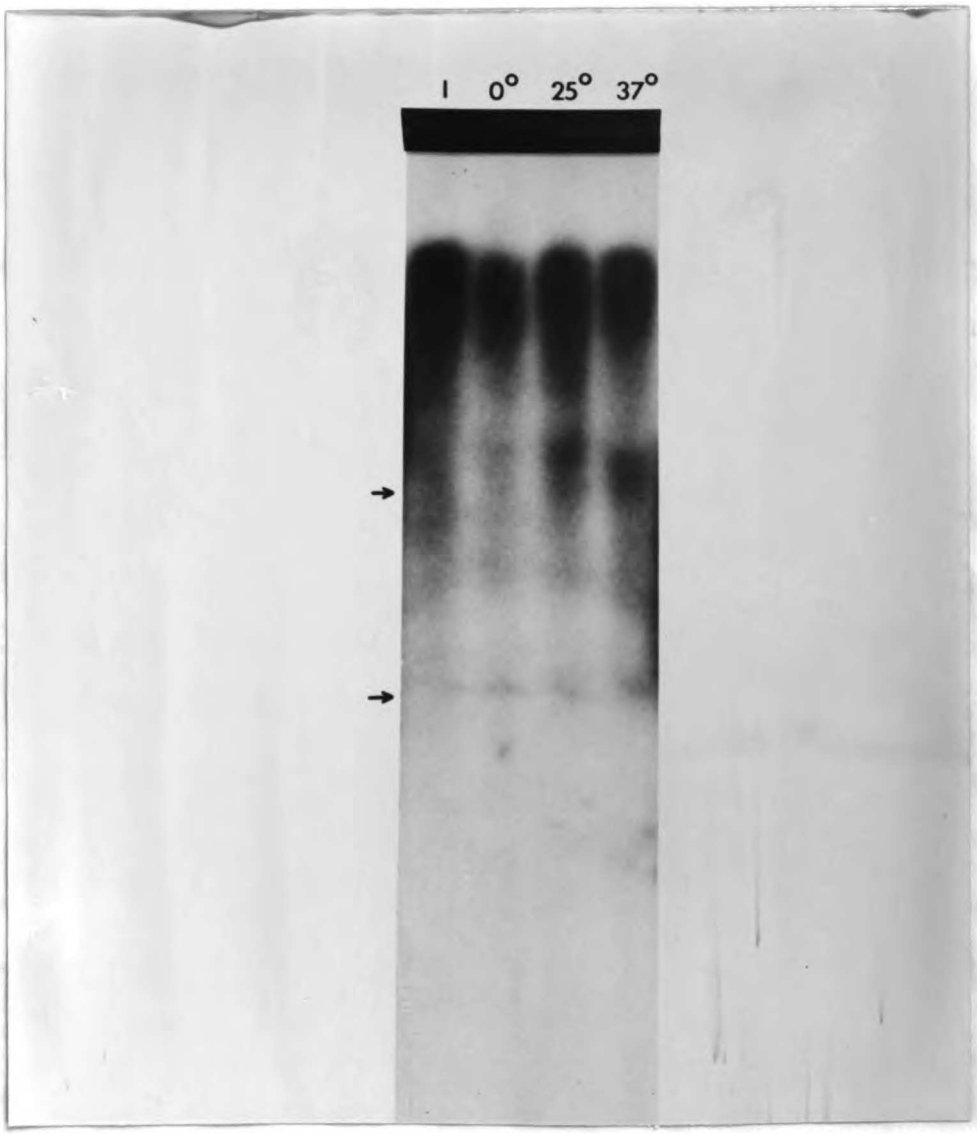


Figure 14. In vitro processing of ASV RNA. $^{32}P$ labeled 35S ASV RNA was prepared as described in Materials and Methods. Polyribosomes were isolated from PrB ASV infected cells as described. Aliquots of the labeled RNA were untreated, or incubated with the polyribosomes at 0, 25 or 37 C for 30 min. The labeled RNA was re-isolated and analyzed by electrophoresis in a 1.2% agarose gel in the presence of 5 mM methyl mercury hydroxide as in Figure 12. The gel was stained with ethidium bromide to localize the 28S and 18S rRNA bands (indicated by arrows). The labeled RNA was visualized by autoradiography of the wet gel using a Lightning Plus screen at -70° C for four days.

RNA migrating at 1.1 x $10^6$, perhaps because the reactions were terminated too quickly. However, since Hayward has observed the processing of RNA to the size of the putative src message in vitro, I believe that in vitro processing of the immunoprecipitated RNA could account for my findings.

C. Specificity of antisera for viral proteins

In order to document the specificities of the polyribosome immunoprecipitations described in the previous sections, I examined the reactions of the antisera with viral proteins by several different methods. This was necessary because most of the antisera were made against only partially purified viral proteins.

1. A radioimmunoassay using $^{125}$I labeled p27 was performed to determine whether the anti-pol serum was contaminated with antibodies against p27. Figure 15 shows that the titer of antibodies directed against p27 in the anti-pol and anti-gp85 sera were at least three orders of magnitude lower than in the anti-$ASV_C$ or anti-p27 sera. A similar radioimmunoassay for p19 also ruled out contamination of the anti-pol serum by antibodies directed against p19 (H. Oppermann, personal communication). However, these experiments did not rule out contamination of anti-pol with antibodies against other gag proteins.

2. Specificity of antisera for purified viral protein.

To more accurately assess the immune reactivities of the antisera, I labeled cells infected with ASV with $^{35}$S-methionine for several hours, collected the medium, and pelleted the labeled virus. The virus was resuspended in immunoprecipitation buffer and aliquots of the preparation were immunoprecipitated with different antisera. The immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis

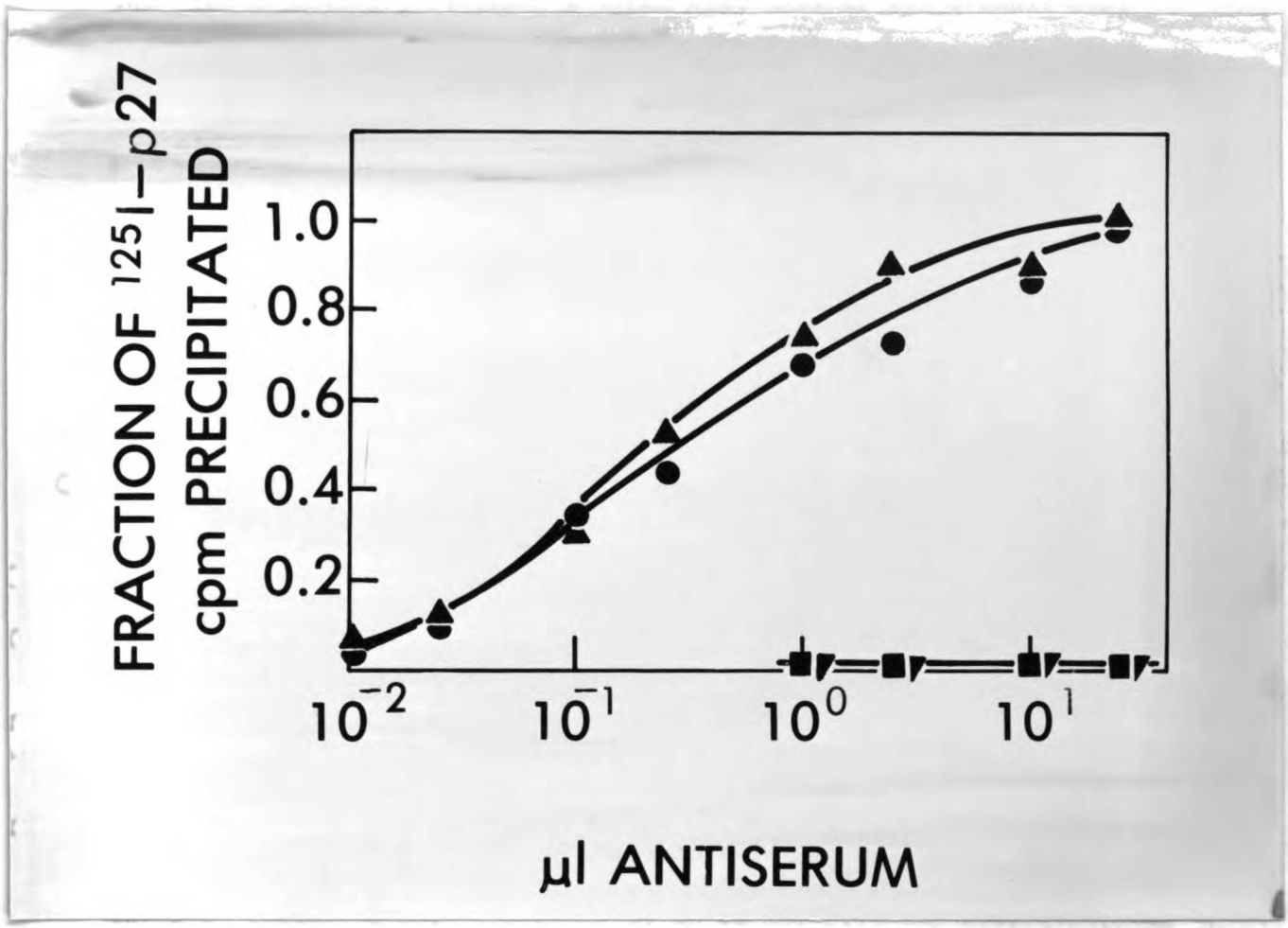


Figure 15. Titration of antisera against $^{125}$I-p27. Radioimmunoprecipitation of p27 was performed as described in Materials and Methods. Increasing amounts of antisera were added to constant amounts of $^{125}$I-p27.

- ● Anti-$ASV_C$
- ▲ Anti-p27
- ■ Anti-pol
- ▼ Normal Rabbit

and autoradiography. Figure 16 shows that even at the highest concentrations used, the anti-pol which efficiently precipitated the reverse transcriptase, did not immunoprecipitate the small gag proteins in amounts greater than that precipitated by normal rabbit serum.

3. Reactivity against intracellular viral proteins.

Cells infected with Pr-C ASV were labeled for a short period with $^{35}$S-methionine, so that the precursor proteins, which are presumably more similar in antigenic activity to the nascent polypeptide chains than are the mature viral proteins, would be predominantly labeled. The cells were disrupted with immunoprecipitation buffer and the proteins were precipitated and analyzed as before. Figure 17 shows that anti-p27 and anti-$ASV_C$ precipitated large quantities of $Pr76^{gag}$, whereas anti-pol precipitated only a small quantity of this protein over background. All three antisera precipitated $Pr180^{gag/pol}$. The results were analyzed by scanning the autoradiogram with a densitometer. The scan indicated that a maximum of 2% of the Pr76 immunoprecipitated by the two anti-gag specific antisera was precipitable by the anti-pol serum. This experiment also demonstrated the specificity of the anti-p27 serum for the gag proteins, because the antiserum even at the highest concentrations did not precipitate any $gp92^{env}$, the only env product labeled in this experiment. The broad band located above $Pr76^{gag}$ was not characterized but was presumably related to the gag proteins, because it was precipitated with antibodies raised in a hamster bearing a tumor induced by rdASV, in which the env gene is deleted. In addition, anti-$gp85^{env}$ precipitated only $gp92^{env}$. None of the antisera used in this experiment contained antibodies against the product of the src gene, $pp60^{src}$ (H. Oppermann, personal communication). It was clear,

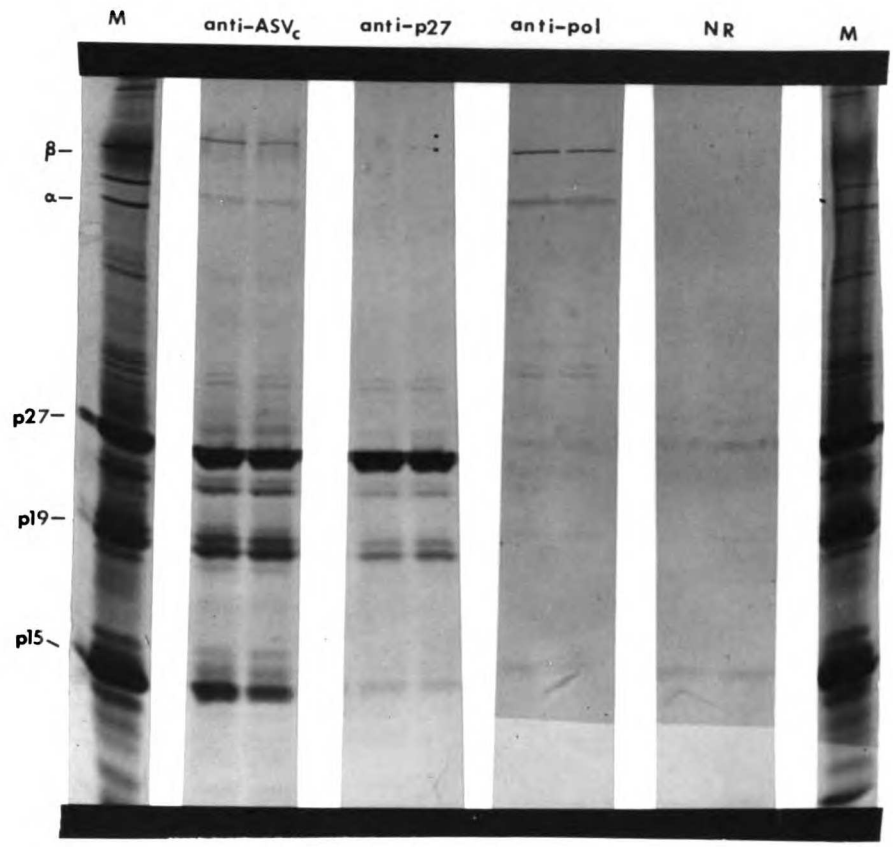


Figure 16. Specificity of antisera for purified viral proteins. $^{35}$S-methionine labeled virus was prepared by collecting medium from labeled cells and pelleting the virus for 60 min at 30K in the SW41 rotor. The virus was resuspended in Immunoprecipitation Buffer and increasing amounts of antisera were added to aliquots. Immunoprecipitation was carried out as described in Materials and Methods. The immunoprecipitates and aliquots of the labeled virus were subjected to electrophoresis in 13% polyacrylamide gels in the system described by Laemmli (1970). Only the highest concentrations of antisera are shown here. Autoradiography was for two weeks with No-screen X-ray film.

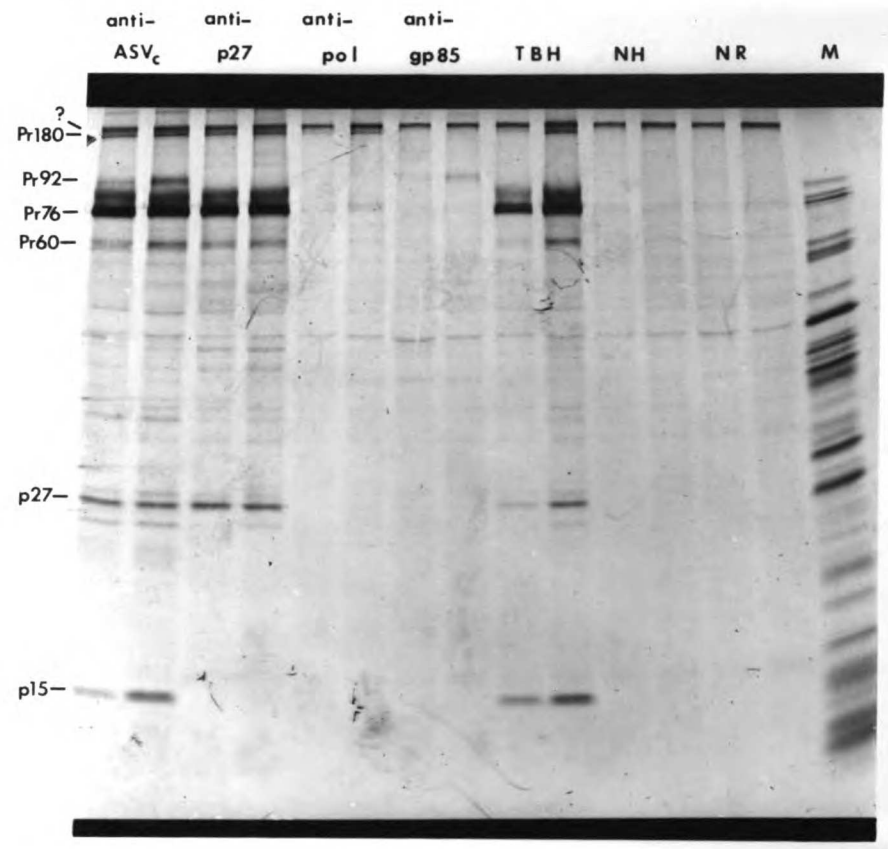


Figure 17. Immunoprecipitation of labeled cytoplasmic proteins. Cells infected with Pr-C ASV were labeled for one hour with $^{35}S$ methionine. Cytoplasmic lyastes were prepared and immunoprecipitations were carried out as described in Materials and Methods. The immunoprecipitates were analyzed in 12% SDS poly-acrylamide gels and visualized by autoradiography for one week. Bacteriophage T4 proteins were used as markers for molecular weights (Lane M).

however, that further definition of the specificity of the anti-pol serum was necessary.

In other experiments, it had been demonstrated that purified virus preparations could compete the reactions by antibodies in the anti-pol serum with $Pr76^{gag}$ (Oppermann et al., 1977). The competition does not affect the precipitation of $Pr180^{gag/pol}$, because the gag proteins are in vast excess with respect to the reverse transcriptase in the virion particles. In an effort to find out whether the antisera would have the same specificities under the conditions used for immunoprecipitation of polyribosomes, I labeled cells with $^{35}$S-methionine for a short period and performed the experiment under those conditions. Aliquots were precipitated with anti-$ASV_C$, anti-p27, and anti-pol. An amount of viral protein sufficient to compete the reaction of anti-p27 completely (see Figure 20A), was added with the anti-pol serum. Normal rabbit serum was the control. It was observed in Figure 18 that all three specific antisera again precipitated $Pr180^{gag/pol}$, but only anti-$ASV_C$ and anti-p27 precipitated $Pr76^{gag}$. In addition, anti-pol did not precipitate the smaller gag proteins in contrast to the other two antisera.

4. Immunoprecipitation of in vitro translation products.

Since in vitro translation products might be expected to be the proteins most similar to the nascent polypeptides of any proteins available to me, I immunoprecipitated the translation products of virion RNA with anti-$ASV_C$ and anti-pol in the presence of viral protein or avian myeloblastosis virus (AMV) reverse transcriptase. The immunoprecipitates were analyzed as before. The results in Figure 19 show that anti-ASV precipitates both $Pr76^{gag}$ and $Pr180^{gag/pol}$ from the extract.

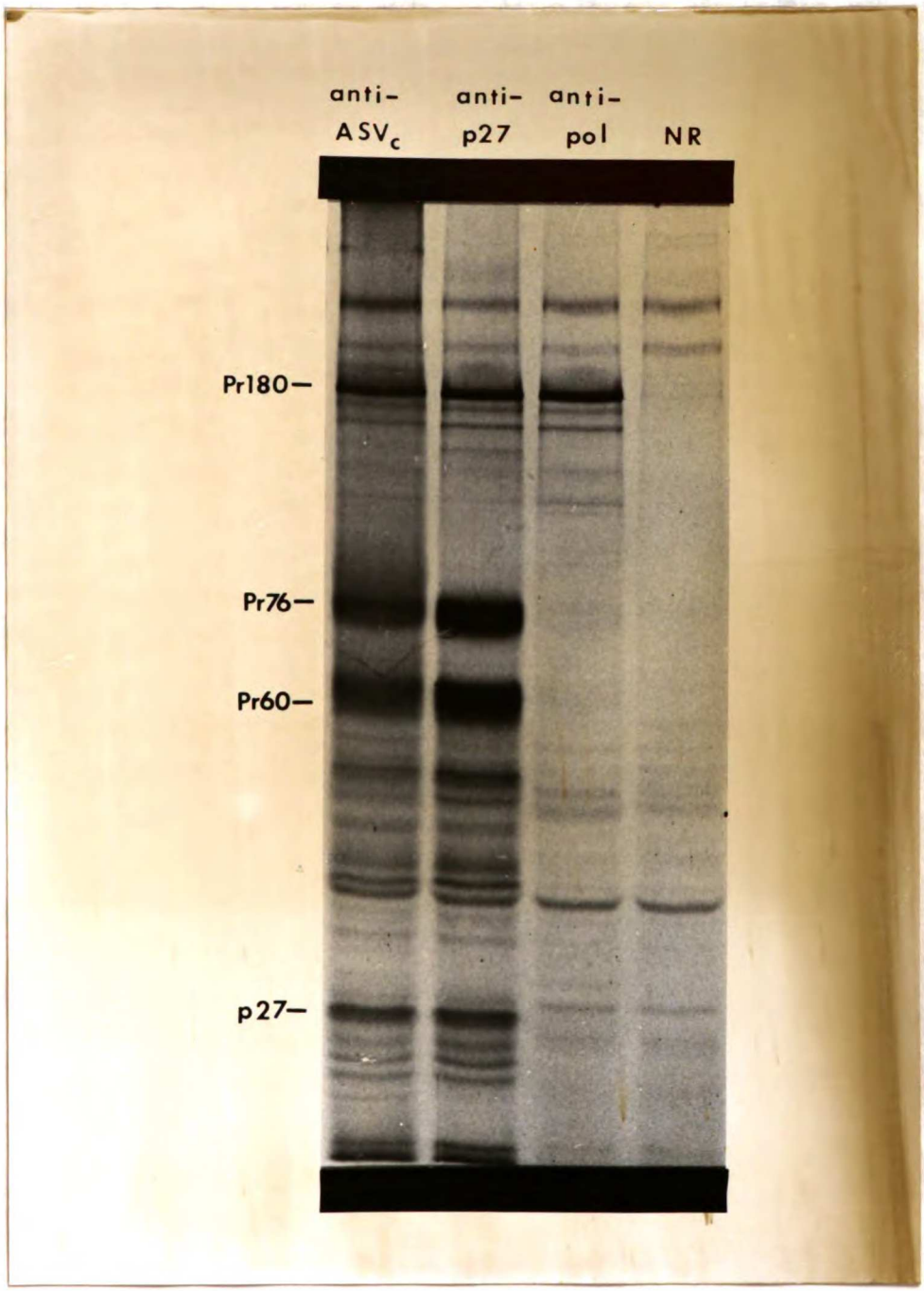


Figure 18. Specificity of anti-reverse transcriptase (anti-pol). Cytoplasmic lysates were prepared and immunoprecipitated as in Figure 17, except that an amount of B77-ASV protein sufficient to completely eliminate precipitation of viral proteins by p27 was present in the immunoprecipitation by anti-pol. Quantities of antisera were the same as those used for

the immunoprecipitation of polyribosomes. The immunoprecipitates were analyzed in 7.5% polyacrylamide gels as described. Autoradiography was for two weeks.

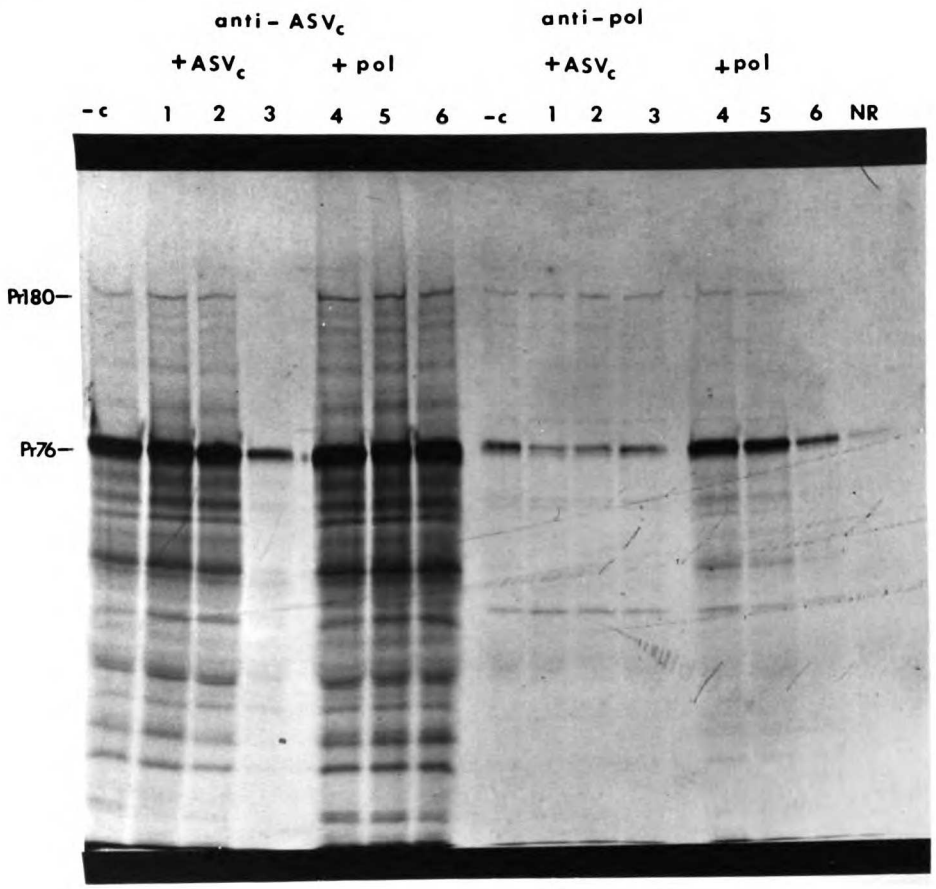


Figure 19. Immunoprecipitation of in vitro translation products of 35S ASV RNA. 35S ASV RNA was prepared by first isolating 70S RNA from 2 hour rapid harvests of PrB ASV (Bishop, et al., 1970). The 70S ASV RNA was then denatured and sedimented on a gradient as described in Materials and Methods for the isolation of labeled 35S RNA. This RNA was translated in the micrococcal nucelase treated rabbit reticulocyte system for two hours at 28° C. The $^{35}$S-methionine labeled products were divided into aliquots and immunoprecipitated with anti-ASV$_C$, anti-pol, or Normal rabbit (NR) sera. In lanes 1, 2 and 3 of each antisera increasing amounts (0.5, 1.0, and 10 µg) of B77-ASV were added to the immunoprecipitations. In lanes 4, 5 and 6 of each antiserum increasing amounts of AMV polymerase

(0.15, 0.75 and 1.5 μg) were added to the immunoprecipitations, which were then carried out as described in Figure 18. The immunoprecipitates were analyzed in 7.5% polyacrylamide gels. Autoradiography was for three weeks.

Both of these proteins are diminished in the immunoprecipitates in the presence of the highest concentration of competing viral protein, but no competition of the immunoprecipitation was observed when AMV polymerase was added. The anti-pol serum also precipitated both $Pr76^{gag}$ and $Pr180^{gag/pol}$. However, $Pr76^{gag}$ was present in much lower quantities than in the anti-$ASV_C$ immunoprecipitates. Surprisingly, the addition of viral protein did not significantly compete the precipitation of Pr76 by anti-pol. But, when an excess of AMV polymerase was added, much greater amounts of Pr76 were precipitated whereas Pr180 immunoprecipitation was diminished. It is possible that the unlabeled AMV polymerase while competing anti-pol precipitation was associating in vitro with the labeled Pr76, and that the Pr76 was immunoprecipitated by virtue of this association.

D. The specificity of the anti-pol serum in the immunoprecipitation of polyribosomes

Because the experiments using labeled proteins did not conclusively rule out an artifactual result in the immunoprecipitation of the genome length RNA by the anti-pol serum, I attempted to analyze the immunoprecipitation of the RNA in polyribosomes directly.

1. Competition with purified proteins of polyribosome immunoprecipitation.

I labeled cells to a steady state with $^3$H-uridine, isolated the polyribosomes, and immunoprecipitated aliquots with constant amounts of anti-p27 or anti-pol in the presence of increasing amounts of virus or purified reverse transcriptase. The polymerase was judged to be pure by polyacrylamide gel electrophoresis (H. Oppermann, personal communication). The immunoprecipitated RNA in each sample was

analyzed for virus specific RNA by hybridization with unlabeled cDNA. Figure 20 shows the results obtained. The virus protein completely competed the reaction of anti-p27, whereas reverse transcriptase did not significantly affect the reaction. On the other hand, the purified virus did not compete the reaction with anti-pol while the reverse transcriptase partially competed the reaction. The partial competition was observed in three other experiments, but complete competition was never observed and a definite plateau was never achieved. The serum may contain a low titer of antibodies against some gag proteins, but these can be competed without an appreciable effect on the precipitation of polyribosomes with the antibodies directed against reverse transcriptase. Alternatively, it is known that MuLV reverse transcriptase can bind to p30 (Bandyopadhay, 1977) therefore, the added reverse transcriptase may have associated with nascent Pr76 molecules and even large quantities of free reverse transcriptase failed to completely eliminate the precipitation of the molecules associated with Pr76.

2. In vitro translation of immunoprecipitated RNA.

The mechanism of translation of $Pr180^{gag/pol}$ has been a conundrum. It has been shown that Pr180 contains all the major methionine-containing peptides present in the β subunit of reverse transcriptase as well as those present in $Pr76^{gag}$ (Oppermann et al., 1977). Suppressor tRNA for the amber nonsense codon is able to enhance the synthesis of Pr180 in translation systems supplied with MuLV virion RNA (Philipsson et al., 1977). However, it was shown that when ASV RNA was translated in the presence of the amber suppressor tRNA, a molecule of 80K rather than 180K was generated (Weiss et al., 1978). It was unlikely then, that classical tRNA suppression is responsible for the "read-through" synthesis of $Pr180^{gag/pol}$. If the putative gag and

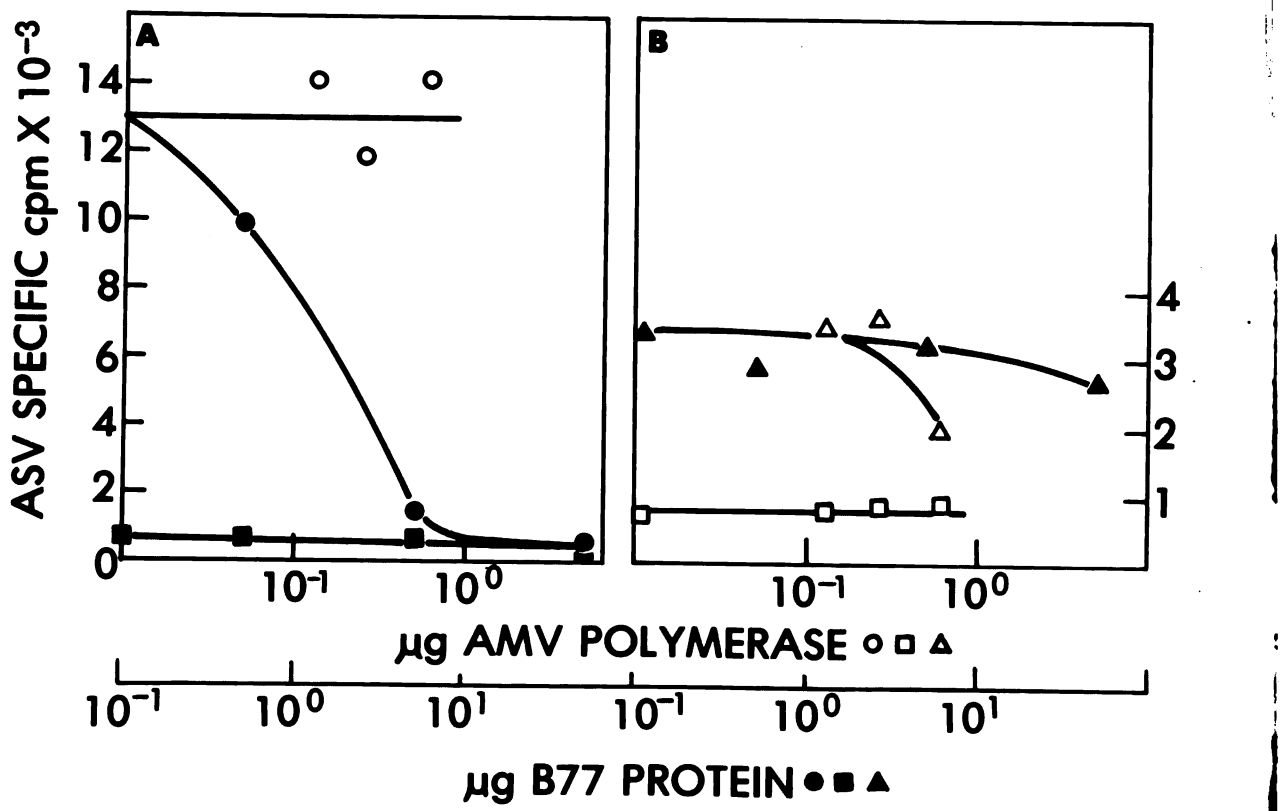


Figure 20. Competition of immunoprecipitation of antisera with ASV protein and with purified reverse transcriptase. Polyribosomes were isolated from Pr-C ASV infected cells that had been labeled with $^{3}$H-uridine for 20 hours. The polyribosomes were immunoprecipitated as described in Materials and Methods except that competitor B77-ASV protein or reverse transcriptase (obtained from R. Swanstrom) were added in increasing amounts to constant amounts of antisera and polyribosomes in the immunoprecipitations. After the RNA was isolated, it was hybridized with unlabeled cDNA and analyzed by chromatography on hydroxyapatite as described in Materials and Methods. A) Polyribosomes were immunoprecipitated by anti-p27 in the presence of

B77-ASV (●) or reverse transcriptase (o). Normal rabbit serum was the control (■).

B) Polyribosomes were precipitated by anti-pol in the presence of added B77-ASV (▲) or reverse transcriptase (Δ). Normal rabbit serum (□) was the negative control.

gag/pol mRNAs are identical, then another hypothesis, such as suppression by frame-shifting tRNA or "read-through" mediated by a protein or other factor, must be invoked to explain the phenomenon. On the other hand, if the messages are different in nucleotide sequence then translation of Pr180 could be explained by the deletion of specific termination codons at the end of the gag gene. There is a possible precedent for this kind of "splicing out" of termination codons in the messages for the various forms of T/t antigens in SV40 infected and transformed cells (Berk and Sharp, 1978). I tested the hypothesis by translating 35S ASV RNA in vitro, immunoprecipitating the polyribosomes formed, isolating the RNA, and re-translating the RNA. If the mRNA for the synthesis of Pr180 were different in nucleotide sequence than the mRNA for the synthesis of Pr76, then when it is translated in the absence of mRNA for Pr76 only Pr180 should be produced. Also, if the immunoprecipitation of polyribosomes efficiently isolated the proposed mRNA for Pr180, a significant amount of Pr180 approaching the amount made in the translations of other immunoprecipitated RNAs, should have been observed. Figure 21 shows that RNA immunoprecipitated by the anti-pol serum could act as template for the synthesis of Pr76, although much less was made when the in vitro system was supplied with this mRNA than when it was supplied with RNAs precipitated by anti-$ASV_C$ or by anti-p27. Significant quantities of completed Pr180 were not observed in this experiment.

Because I knew that the RNA precipitated from polyribosomes purified from the infected cell was intact and relatively pure after immunoprecipitation, I purified large quantities of RNA by precipitating with anti-$ASV_C$, anti-p27, anti-pol, and normal rabbit sera. Again, I translated these RNAs in the micrococcal nuclease treated rabbit

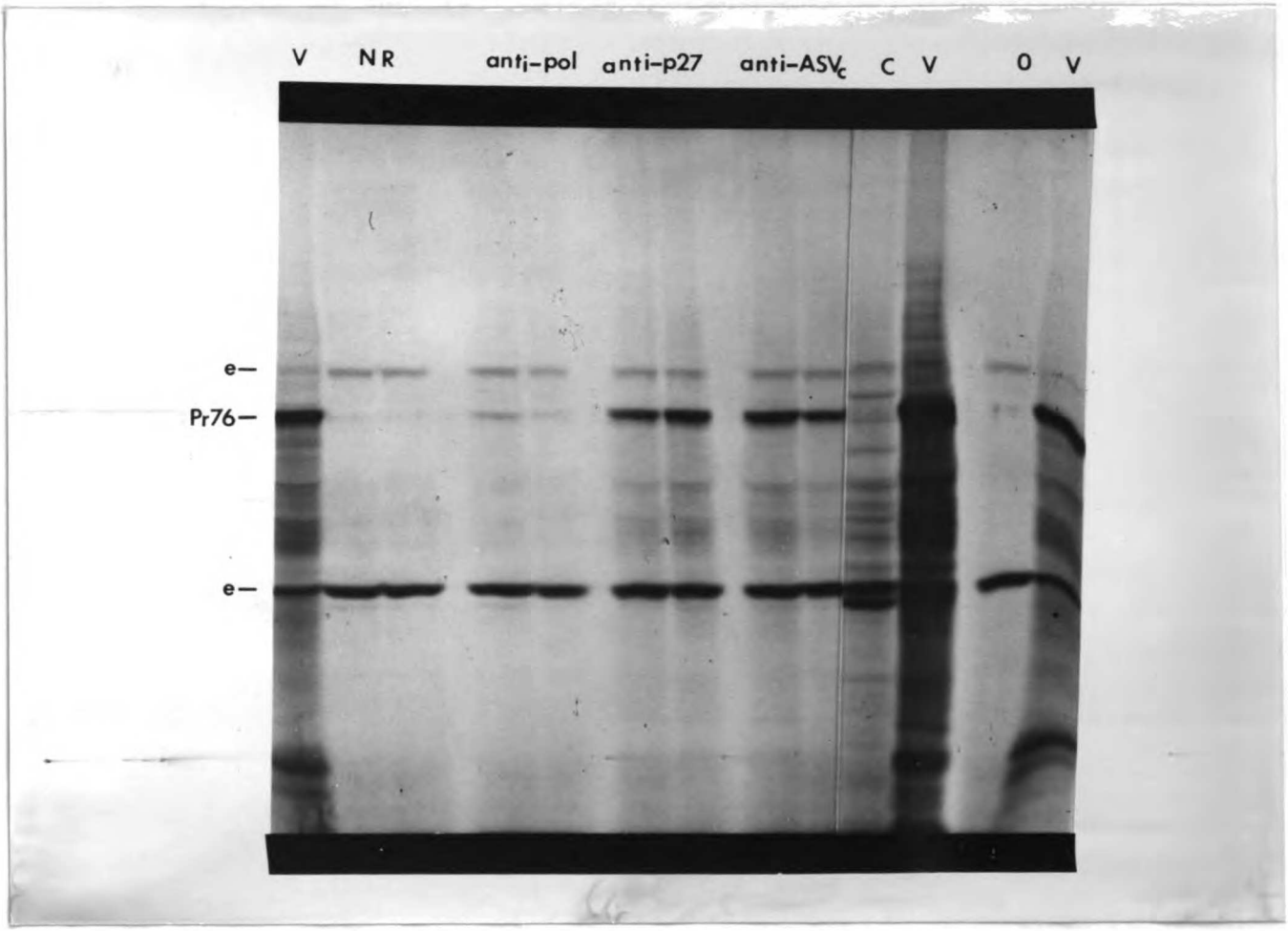


Figure 21. Translation of RNAs immunoprecipitated from an in vitro translation system. 35S Pr-B ASV RNA was translated in the micrococcal Nucelase treated rabbit reticulocyte system for 15 min. Then cycloheximide was added at 1 µg/ml for 5 min and at 100 µg/ml for another 5 min. Antiserum was added to the extract aliquots and immunoprecipitations were carried out as described for the polyribosomes in Materials and Methods. Instead of yeast RNA, HeLa cell 18S RNA (obtained from P. Hackett) was added as carrier in the ethanol precipitation. The isolated RNAs were precipitated two more times with ethanol and then retranslated in the rabbit reticulocyte system. The products of these translations were analyzed by electrophoresis in a 7.5% acrylamide gel.

V = 35 S RNA not immunprecipitated

NR, anti-pol, anti-p27, and anti-$ASV_C$ = RNAs immunoprecipitated by these various antisera and retranslated

C = HeLa 18S RNA

0 = no RNA added to the translation system

e = endogenous proteins

reticulocyte system. The labeled translation products were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. Figure 22 shows that both Pr76 and Pr180 could be translated from the anti-$ASV_C$ and anti-p27 immunoprecipitated RNA. In contrast, a significant amount of Pr76 but very little Pr180 were made using the anti-pol precipitated RNA as mRNA in the translation. Immunoprecipitation of the *in vitro* translation products documented the identities of the Pr76 and Pr180 (Figure 23). Precise quantitations of the Pr180 could not be made because there was not sufficient radioactivity in the band, but it appeared that the ratio of the Pr76 to Pr180 might be similar in the products of the translation from the RNA precipitated by anti-pol as the ratios observed when virion RNA, or RNA precipitated by anti-$ASV_C$ or anti-p27 were translated. I believe these results argue that nonsense codons are not deleted from the Pr76 mRNA to generate a specific mRNA for Pr180. Unfortunately, because of the peculiarities involved in the immunoprecipitation of polyribosomes by anti-pol, this conclusion cannot be considered definitive.

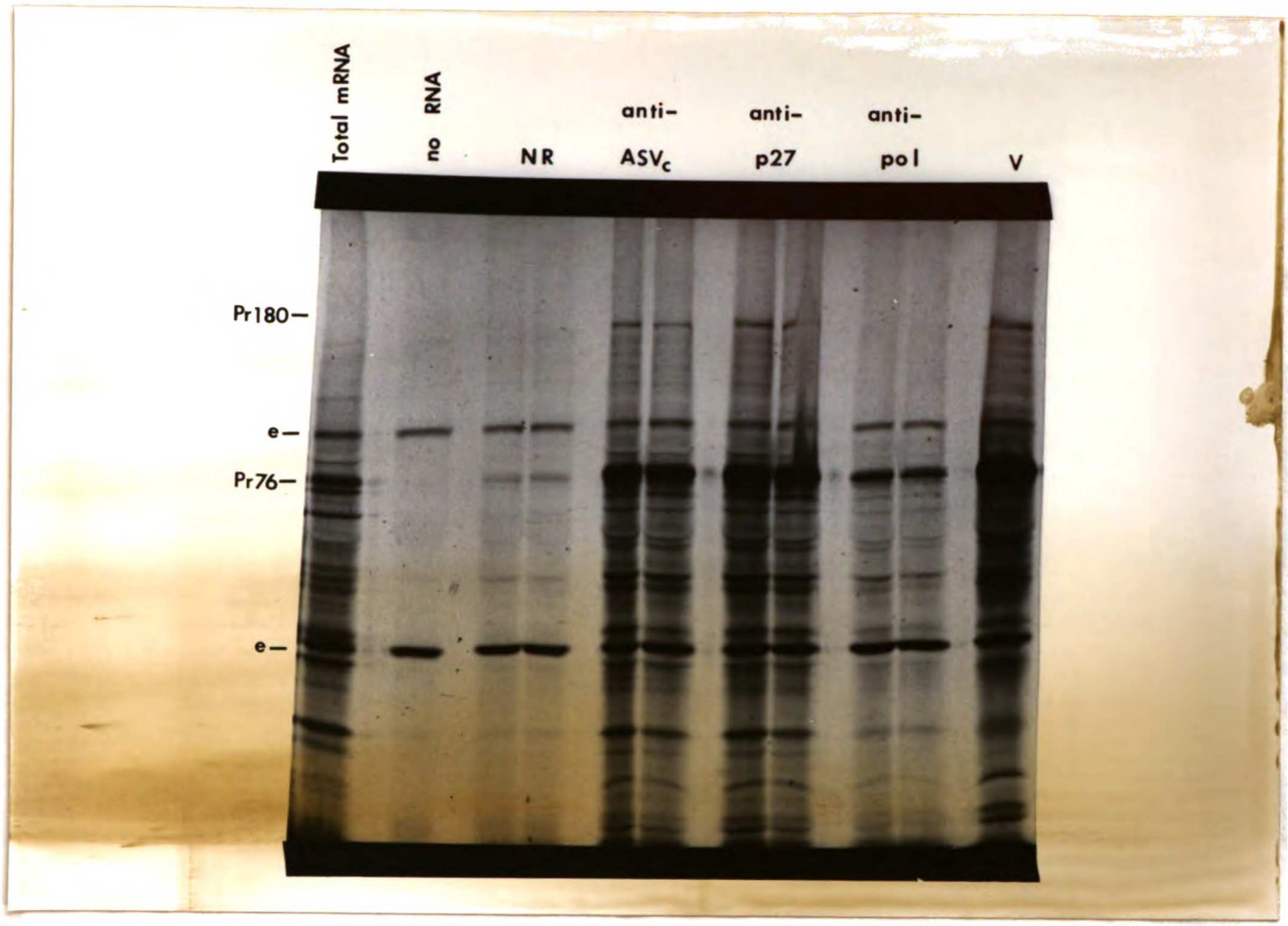


Figure 22. Translation of immunoprecipitated mRNAs. RNA was isolated from the polyribosomes of Pr-C ASV infected cells by immunoprecipitation with various antisera. No carrier RNA was added during ethanol precipitations. In addition, the RNAs were washed with 2.5 M LiCl to remove any heparin or possible contaminants that inhibit translation. These RNAs were then translated as in Figure 21 in the rabbit reticulocyte system. The products were analyzed on a 7.5% acrylmide gel and autoradiography was for 11 days.

V = 35S viral RNA added

e = endogenous proteins

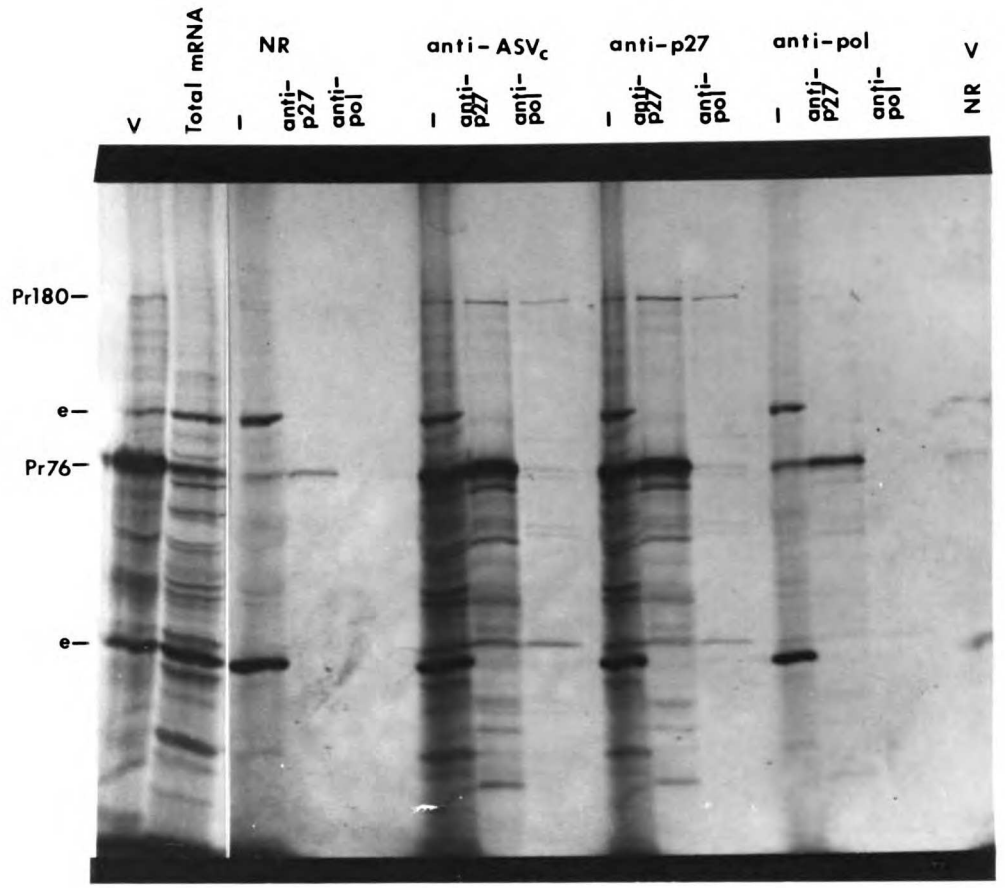


Figure 23. Immunoprecipitation of products of in vitro translation of mRNAs. The products of translation shown in Figure 22 were immunoprecipitated with anti-p27 and anti-pol sera as described in Materials and Methods. They were analyzed on a 7.5% polyacrylamide gel and autoradiography was for 30 days.

V = 35S viral RNA added

e = endogenous bands

DISCUSSION

Association of viral RNA with polyribosomes

Analysis of the genetic composition of the stable ASV-specific RNA species in cells productively infected by ndASV and the characterization of $Pr180^{gag/pol}$ led to predictions that the 3.3 x $10^6$ genome length RNA is the messenger for the group specific antigens (gag) and the RNA dependent DNA polymerase (pol), the 1.9 x $10^6$ RNA is the messenger for the virion envelope glycoprotein (env), and the sarcoma (src) gene product is translated from the 1.1 x $10^6$ viral RNA. My present data provide direct evidence that each RNA acts as a messenger in the infected cell. These findings conform to previous demonstrations that RNA from each size class can be translated into the appropriate viral gene product in vitro (von der Helm and Duesberg, 1975; Pawson, et al., 1977; Purchio, et al., 1977). In the discussions that follow, I will refer to these RNAs as the gag/pol message, the env message, and the src message.

Viral RNA may exist in two separate intracellular pools, one for mRNA and one for RNA destined to become virion RNA (Levin and Rosenak, 1976; Paskind, et al., 1975), but it is not known what determines how the RNA will function. For the following reasons, I assume that specific differences in nucleotide sequences between the viral mRNA and provirion RNA do not play a role in the binding of the viral RNAs to ribosomes, or in the segregation of the viral RNAs into provirion structures. First, all three of the virus-specific RNAs can be packaged and exported from the cells in virion particles (Hayward and R. Swanstrom, personal communications). Therefore, the structure required for packaging may exist in the mRNAs as well as in provirion RNA. Second, virion RNA can be used as template for the translation of all the major viral polypeptide precursors present in the infected cell (Purchio, et al., 1977; Beemon and Hunter, 1978;

Pawson, et al., 1977). Third, in acute infections, virion RNA can be translated before the synthesis of proviral DNA is complete and before transcription of progeny RNA begins (Gallis, et al., 1976). Fourth, I did not observe any differences in the sizes of the virus specific RNAs in my analyses when comparing the RNA from purified polyribosomes and the RNA in the total cytoplasm or in the total cell. It is conceivable that segregation of the RNA to be packaged depends initially on association of mature viral proteins or $tRNA^{Trp}$ with the RNA, or perhaps on chemical modifications of the RNA.

Analysis of polyribosome associated RNA in cells infected by td- and rdASV

Greater resolution of the subgenomic RNAs was achieved by the method of analysis used in this work than in previous studies. In particular, I was able to distinguish the src message and the td env message (by size alone) in my experiments (Figure 5). The deletion of the src gene apparently reduces the size of the env message and eliminates the src message. It remains possible that the size of the td env message varies according to the size of the src deletion. Nevertheless, these results may indicate that the RNA can be processed only to produce a functional message. For example, even though only sequences affecting the src gene are deleted in tdASV, a smaller mRNA derived from the ndASV src mRNA with only the sequences flanking the deletion was not observed. The deletion of the env gene, however, eliminates only the $1.9 \times 10^6$ env message and does not detectably change the size of the src message.

Is there a specific message for the RNA-dependent DNA polymerase?

The model for the synthesis of the viral gene products proposed that the genome length RNA is the template for read through translation of Pr180, and that this large gag/pol polypeptide is the functional precursor for the virion reverse transcriptase (Oppermann, et al., 1977; Witte and

Baltimore, 1978). In my experiments, I was still unable to detect a species of viral RNA which contains the sequences 5'-pol-env-src-C-3' and would presumably be the template for the translation of the mature reverse transcriptase. Because the deletion of the gag gene would probably result in an RNA species approximately the size of the tdASV or rdASV, I expected to distinguish such a message from the genome length RNA. In addition, I oberved that my method of analysis can detect defective ASV RNA when it is present at a level of 5-10% of the ndASV RNA in a mixed infection (e.g., after two or more passages of a cloned virus stock). Furthermore, it is apparent that the sensitivity of the method is sufficient to detect a shorter minor RNA component at a level much lower than 5%. The absence of a detectable pol message in my experiments is consistent with the inability of investigators in several laboratories to find intracellular mature reverse transcriptase (Oppermann, et al., 1977; Jamjoom, et al., 1977). I, therefore, do not believe that a specific message with pol at its 5' terminus exists. It is possible that a 38S message differing in nucleotide sequences for the translation of the gag/pol precursor Pr180. Such an mRNA could have a small deletion affecting the termination codons at the end of $Pr76^{gag}$ (Weiss, et al., 1978), analogous to the "spliced" mRNA for the SV40 T antigen (Berk and Sharp, 1978). The deletion if it exists must be very small, because in several analyses, I have not separated subpopulations from the 38S RNA species (see further discussion below). However, my current results leave this possibility open to investigation.

Is there an ASV RNA species smaller than $1.1 \times 10^6$?

The common region (C) is a region of approximately 500 kilobases adjacent to the 3' end of the viral RNA (Shank, et al., 1978; Junghans, et al., 1977). The sequence is shared by both nd and td ASV. A viral mRNA smaller

than the $1.1 \times 10^6$ putative src message might be expected if C codes for a protein. The existence of a major class of ASV-specific RNA sedimenting at approximately 16S has previously been reported (Brugge, et al., 1976). I did not find any viral RNA migrating at a size corresponding to 16S in any of my experiments. Moreover, even with the use of a cDNA which specifically recognizes approximately 300 bases at the 3' end of the viral RNA (Tal, et al., 1977), I have not identified such an RNA species in any of the cells studied (N. Quintrell, personal communication). I conclude that an RNA species smaller than the src mRNA is not a major stable component of the viral RNA in ASV infected fibroblasts.

Viral RNA in membrane-bound and free polyribosomes

Many glycoproteins, membrane proteins, and proteins that are transported across the cell membrane are known to be synthesized on membrane-bound polyribosomes (Blobel and Dobberstein, 1975, Wirth, et al., 1977). The signal hypothesis states that association of the mRNA and ribosomes with the membranes occurs by virtue of a leader sequence at the N-terminus of the polypeptide which may be removed during maturation of the protein (Blobel and Dobberstein, 1975). In these cases, the polyribosomes are bound to the membranes primarily via the nascent polypeptide, although secondary interactions between the ribosomes and the membrane may exist. Therefore, when the polyribosomes are disrupted, the messenger RNA is completely released from the membranes. The synthesis of the ASV glycoproteins may occur by the mechanism just described. A precursor, $Pr92^{env}$, appears in a very short labeling period and is glycosylated, probably while being translated, although synthesis of a smaller, unstable precursor has been reported (Moelling and Hayami, 1977; Buchhagen and Hanafusa, 1978). $Pr92^{env}$ may be synthesized on the membrane-bound polyribosomes, and later cleaved into the mature glycoproteins, gp85 and gp37 (England, et al., 1977),

found in the viral envelope.

In my investigation of the subcellular location of the polyribosomes containing the viral RNA species, I found that the gag/pol message and the src message were associated primarily with the free polyribosomes (Figure 8). Because the group specific antigens and the polymerase are not integral membrane proteins, but comprise the virion cores, the finding of the gag/pol message in the free polyribosomes was not unexpected. In contrast, the precursor, Pr60, to the murine leukemia virus (MuLV) group specific antigens has been reported to be synthesized on membrane-bound polyribosomes (Gielkens, et al., 1976). A glycosylated form of Pr60 is present in MrLV infected cells (Ledbetter and Nowinski, 1977; Snyder, et al., 1977) whereas there has been no such equivalent form of Pr76 observed in ASV infected cells. The occurrence of this glycosylated, membrane-bound form of Pr60 may reflect the discrepancy between the two systems.

The indication that the src message is not predominantly associated with the membrane-bound polyribosomes, on the other hand, is intriguing. It was recently suggested that pp60 was synthesized on membrane-bound polyribosomes because a small polypeptide was removed from the product of in vitro translation of 20S virion RNA after incubation of the lysate with dog microsomes (Kamine and Buchanan, 1978). These investigators have suggested that $pp60^{src}$ may be secreted from the cell because it was protected from digestion with proteases by membrane vesicles, and because other investigators have found cell surface changes which could be associated with the function of src (Pastan and Willingham, 1978; Todaro, et al., 1977). I believe this interpretation is not correct since I have found virtually all of the src mRNA associated with free polyribosomes (Figure 8). In addition, the pp60 is a very stable protein and therefore, not likely to be secreted; and other investigators have not detected such differences in the molecular weights of the 60K in vitro translation product of the src

gene and the $pp60^{src}$ identified *in vivo* (Purchio, et al., 1977; Erikson, et al., 1978; Beemon and Hunter, 1978; Weiss, personal communication). In summary, my data imply that the *src* protein, pp60 is neither an integral membrane protein nor exported through the membrane. The $pp60^{src}$ could be acting freely in the cytoplasm or in the nucleus, and it remains possible that pp60 could be attached post-translationally to the inner surface of the plasma membrane.

In contrast, the *env* message appeared to be associated with membrane-bound polyribosomes because it was removed from the membrane fractions of the gradient when the lysate was treated with EDTA (Figures 8-10). I observed that the *env* message was found almost exculsively in the membrane fractions, considerably enriched with respect to the other two viral RNA species (Figure 8). This finding concurs with the model for virion assembly that gp85 is an integral membrane glycoprotein inserted into the membrane prior to virus assembly (Bolognesi, et al., 1978).

Another hypothesis could explain the binding of mRNAs for membrane-bound or secretory proteins to the membranes during translation. This model states that the mRNA is attached to the membranes via specific sequences at the 3' end, the binding occurs before the ribosomes begin translation, and is resistant to disruption by EDTA (Milcarek and Penman, 1974). Because the three ASV mRNAs probably have very similar or identical 3' sequences (Cordell, et al, 1978; Mellon and Duesberg, 1977) and yet are synthesized on the two different classes of polyribosomes, I do not believe that a 3' sequence contained in the *env* mRNA but not in the *gag/pol* mRNA or *src* mRNA mediates the membrane binding. In addition, because EDTA removed the bulk of the *env* mRNA from the membranes, I believe the hypothesis is ruled out for the ASV mRNA.

I observed that at least the bulk of the *gag/pol* and *src* RNAs found in the membranes (Fractions 1-3, Figure 8) were not released from that

region of the gradient with EDTA (Figures 9,10). I assume that the RNAs present in the membranes after EDTA treatment are not being actively transcribed, but are possibly bound to the membrane via viral structural proteins that serve to package the RNAs into provirion particles. Because I cannot detect viral RNA in the heavy fractions after treatment with both EDTA and detergent (Figure 7 and unpublished observations) I assume that these viral ribonucleoproteins are membrane-bound. I conclude that only the env message, not the gag/pol or src messages, appears to be present in membrane-bound polyribosomes, because the env mRNA was present almost exclusively in the membrane fractions and it was diminished in those fractions after treatment with EDTA. Therefore, only $Pr92^{env}$ and its products are membrane-bound, whereas $pp60^{src}$ does not appear to be an integral membrane protein or a secretory protein.

The structure of ASV mRNAs

The virus specific mRNAs in cells infected by ASV have features which are common to other eukaryotic mRNAs. First, ASV mRNAs are polyadenylated; but, as in other cases, the function of the poly(A)is unknown (Weiss, et al., 1977; Hayward, 1977). Second, viral mRNAs and virion RNA possess 5' terminal capped structures (Furuichi, et al., 1975; Collett, et al., 1977; Mellon and Duesberg, 1977; Cordell, et al., 1978). Capped ends apparently perform some function in the initiation of translation because addition of 7-methyl guanosine, an analog of the cap structure, to in vitro translation systems partially inhibits the translation of capped RNAs while it does not affect translation of messages lacking the cap (Hickey, et al., 1976). Third, the viral RNA also contains methylated bases in internal sequences of the 3' half of the genome (Furuichi, et al., 1975; Taylor and Illmensee, 1975).

Perhaps the most puzzling feature of ASV mRNAs, as well as those of

MuLV, adenovirus, and SV-40, is the presence of "leader sequences" at the 5' termini of the messages (Rothenberg, et al., 1978; Cordell, et al., 1978; Berget, et al., 1977; Chow, et al., 1977; Mellon and Duesberg, 1977; Gelinas and Roberts, 1977). In ASV mRNAs, the sequence is at least 104 bases long (Cordell, et al., 1978). My determinations of the sizes of the mRNAs combined with the data of Wang, et al., (1976) and Shank, et al., (1978) may allow for a sequence of up to 600 nucleotides at the 5' terminus of the env mRNA, which is not translated into the glycoprotein (see Figure 24). For example, the env mRNA is approximately 5600 bases long, but the env gene appears to begin about 5000 bases from the 3' end.

However, the boundaries of the deleted sequences in defective mutants, type specific variability found in the env gene, and the mutations affecting the function of the env and src genes, all of which have been used as criteria for mapping the coding sequences of the genes, may not exactly coincide with the boundaries of the sequences present in the mRNAs. As shown in Figure 5, deletion of the env gene in rdASV does not demonstrably affect the size of that src mRNA, even though the ndASV src mRNA contains some sequences detected by the env cDNA (my unpublished data, and N. Quintrell, personal communication). The deleted sequences might be small enough so that a size difference would not be detected (200 bases or less) or they may be replaced by a sequence from a different location. The env specific sequences in the src mRNA may be an untranslated region between the leader sequence and the coding sequences. A similar region may be present in the rdASV src mRNA, but comprised of different sequences, to account for the finding that both nd and rdASV src mRNAs are the same size. It might be suggested that more than one "splicing" event may be involved in the processing of ASV mRNAs. Such an hypothesis may also account for the identical sizes of the mRNAs for src.

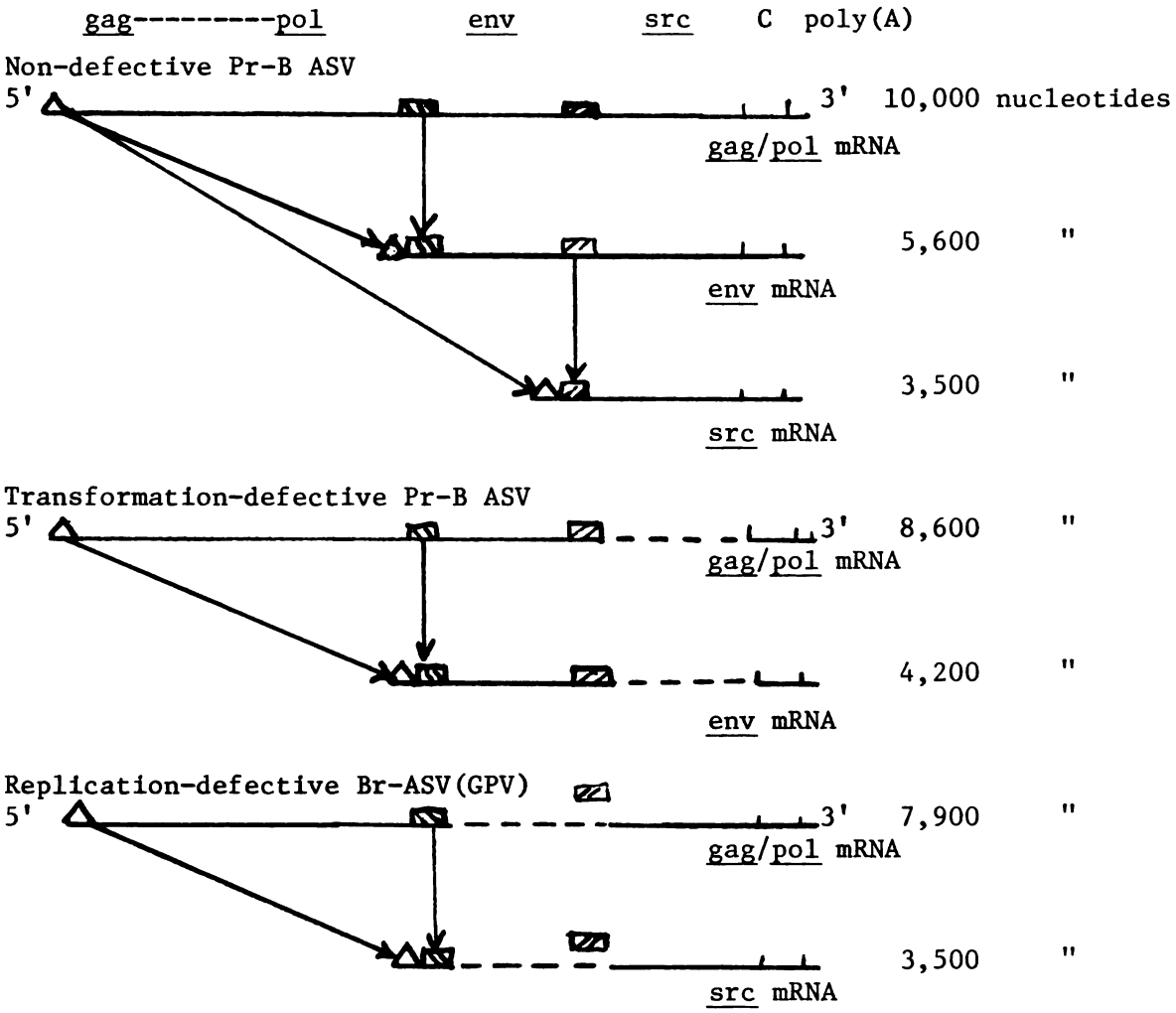


Figure 24. Possible structures for ALSV mRNAs.

Leader sequence

Untranslated sequences ?

Deleted sequences

What is the leukosis-specific function?

None of the identified gene products of ALSV or coding sequences in the RNA have been implicated in the induction of leukosis. The "leuk" function is probably not encoded in the src gene, since tdASVs can cause leukosis (Biggs, et al., 1973). It is possible that the common sequence, C, is responsible for the induction of this disease. If it is responsible, however, the nature of the expression of the C region is unknown. One obstacle in the study of leukosis has been the lack of an in vitro assay for the function. It is conceivable, that leuk is expressed only in lymphoid cells and not in fibroblasts at all. A mechanism which may account for the expression of leuk in lymphoid cells would be the presence in those cells of a processing enzyme to generate a small mRNA in such cells that is not present in ASV infected fibroblasts (see Figure 5). A clue that the C region may be involved in the leuk function is the fact that the 3' sequences of avian retroviruses which have the ability to induce leukosis are considerably different from the 3' sequences of the endogenous virus of chickens which does not cause this disease (Coffin, et al., 1978).

Alternatively, the leuk function may be encoded in the env gene. Experiments with MuLVs have implicated the analogous gene in pathogenesis of the viruses in mice (Hartley, et al., 1977; Troxler, et al., 1977). Because the envelope genes of endogenous viruses, which do not cause leukosis, are different from the env genes of those which do, such a possibility remains viable (Fujita, et al., 1978).

Immunoprecipitation of polyribosomes

I believe that my data showing the immunoprecipitation of polyribosomes containing ASV mRNAs are consistent with, but do not prove, the model for translation of the ASV gene products (Weiss, et al., 1977; Hayward, 1977). For instance, the gag gene products appear to be translated from the 38S

ndASV RNA *in vivo*, as well as *in vitro*, because the 38S or 35S RNAs are the major RNAs isolated by immunoprecipitation with anti-*gag*. In addition, the *src* and *env* gene products appear to be translated from the 21S and 28S mRNAs respectively (see Figure 11-13). However, it was difficult to obtain unambiguous data, because of the presence of smaller RNAs that were precipitated in the presence of the large RNA, even using monospecific sera.

In order to explain the presence of the subgenomic messages immunoprecipitated by the anti-*gag* sera, I tested several different hypotheses. One possibility was that all the primary translation products contained the same N-terminal amino acids, which would occur if the leader sequence contained the site for initiation of translation. However, antisera containing activity against p19, the N-terminal protein in $Pr76^{gag}$ (Palmiter, et al., 1978), did not precipitate the *env* glycoprotein precursor, $Pr92^{env}$, or the *src* gene product, $pp60^{src}$ (see Figures 17-18). The pp60 and Pr92 are not likely to be products from which N-termini have been cleaved, since both of the proteins are labeled in a short period and because an active *src* product with a mobility identical to the *src* product found *in vivo*, can be synthesized *in vitro* (Erickson, et al., 1978; S. Weiss, personal communication). A contradictory result has been obtained by other investigators, where a putative *src* gene product can be cleaved *in vitro* by addition of microsomes during translation to produce a slightly smaller molecule (Kamine and Buchanan, 1978). However, this result may be an artifact because the *src* mRNA does not appear to be translated on membrane-bound polyribosomes *in vivo* (Figure 8). Indeed, presumably because only the glycoprotein contains an N-terminal signal sequence, only the *env* mRNA, and not the *gag/pol* and *src* mRNAs, is found on membrane-bound polyribosomes. Therefore, at least the *gag/pol* and *src* gene products

appear to differ from the env gene product at the N-terminus. Data from other investigators also indicates that identical N-terminal peptides on the primary translation products for the three mRNAs cannot account for my findings. First, no products of the src gene, immunoprecipitable with both antibodies directed against src and antibodies directed against the structural proteins, have been observed. Second, $pp60^{src}$ can be translated from RNA fragments which lack the 5' terminal leader sequence (Erickson, et al., 1978). Third, circumstantial evidence indicates that leader sequences in the adenovirus mRNAs may not contain ribosome binding sites (Dunn, et al., 1978). In addition, preliminary evidence suggests that the ribosome binding sequences for each of the three viral RNA species are distinct (B. Cordell, personal communication).

A second explanation for the presence of the env and src mRNA in anti-gag immunoprecipitates, was contamination of anti-p27 with antibodies to both env and src gene products, and of anti-$ASV_C$ with antibodies to src. This was improbable because the anti-p27 did not precipitate any $Pr92^{env}$ or $pp60^{src}$, and the anti $ASV_C$ did not precipitate pp60 under any circumstances (Figures 17-18).

An hypothesis which was more difficult to rule out was that small gag proteins are bound to the RNAs and the antibodies precipitate the polyribosomes by virtue of the bound ptoteins as well as the nascent chains. It is known that p19 binds to double-stranded regions and p12 binds to single-stranded regions in virion RNA (Leis, et al., 1978; Fleissner and Tress, 1973; Sen and Todaro, 1977). I do not think that precipitation of the message is entirely due to the presence of these proteins for the following reasons. When puromycin or EDTA was used to disrupt polyribosomes prior to immunoprecipitation, less than approximately 10% of the viral RNA was precipitated when compared with the untreated polyribosomes. Second,

the polyribosomes were isolated under conditions which the small proteins bind less avidly (Leis, et al., 1978). Third, when labeled viral RNA was added to a cytoplasmic lysate, less than 2% of the radioactivity could be precipitated by anti-gag antisera, and only 2-3 fold over background. In addition, when cells were labeled for one hour with $^{35}$S-methionine and polyribosomes were isolated, no mature gag proteins could be detected with the polyribosomes, even though significant amounts were present in the cytoplasm. Therefore, I believe that the majority of the viral RNA is not precipitated by this mechanism, but small amounts of env and src mRNAs may be immunoprecipitated for that reason.

Another way in which the small messages could be generated in the immunoprecipitations of polyribosomes containing the 38S RNA is by processing of the RNA during isolation. Figure 14 shows that processing enzymes may be associated with the polyribosomes, and that such enzymes could cleave the RNA specifically in vitro. Microinjection experiments showed that the processing of genome length RNA to the size of the env mRNA probably occurs in the nucleus (Stacey and Hanafusa, 1978). The results of Hayward (personal communication) where the greatest processing activity was found in the cytoplasm, and my results, can be explained by leakage of processing enzymes from the nucleus during isolation. Therefore, I believe that it is possible that the antisera specifically immunoprecipitated the viral polyribosomes by virtue of the nascent polypeptide chains, but the data could not be unambiguously interpreted.

The mechanism for translation of Pr180$^{gag/pol}$

My data show that 38S or 3.3 x $10^6$ MW RNA can be isolated from polyribosomes immunoprecipitated by antibodies directed against the reverse transcriptase. I believe that contaminating anti-gag antibodies in the anti-pol serum are not responsible for this immunoprecipitation because

amounts of gag proteins, sufficient to completely eliminate precipitation of ASV RNA by anti-p27 do not affect the immunoprecipitation by anti-pol (Figures 18-20). However, it is not certain that the anti-pol antiserum actually immunoprecipitates the polyribosomes synthesizing Pr180. As shown in Figure 19, the Pr180 may bind to $Pr76^{gag}$; and if the nascent proteins bind, then the anti-pol may precipitate polyribosomes synthesizing Pr76 by this association or if small amounts of mature Pr180 bind to the nascent Pr76. Nevertheless, in some experiments 30% (see Figure 20) of the virus-specific RNA could be isolated from polyribosomes precipitated by the anti-pol serum in the presence of competing gag proteins. It is difficult to imagine that all of the 38S RNA precipitated by anti-pol in these experiments would have been obtained by such a mechanism.

Pr180 is a joint product of the gag and pol genes of ASV. Since it contains the major tryptic peptides of Pr76 and presumably the same amino terminus, some mechanism must be used in order to suppress termination of translation at the end of Pr76 and read-through. One or more nonsense codons may be deleted at the end of the coding sequences for Pr76, producing an mRNA specific for Pr180. Analogous spliced mRNAs exist for the T antigens of SV-40 (Berk and Sharp, 1978). If such a "spliced" mRNA exists, the spliced-out region would probably be very small, since the Pr180 produced by suppression of the nonsense codon with amber tRNA in in vitro translation of MuLV RNA co-migrates exactly with the "non-suppressed" Pr180 and the Pr180 found in vivo (Philipsson, et al., 1977). In my translation experiments where RNAs isolated from polyribosomes precipitated by both anti-gag and anti-pol were tested, I did not observe that the "pol" RNA directed the synthesis of only Pr180. Pr76 and Pr180 were both synthesized when the translation system was supplied with the "pol" mRNA (Figures 20-23). I believe that the weight of evidence with regard to the nature of the mRNA

which serves to direct the synthesis of Pr180, suggests that it is identical to the mRNA for the Pr76. However, I could not exclude possible artifacts which could account for my findings.

If the mRNAs for Pr76 and Pr180 are identical in nucleotide sequence, then a mechanism other than classical prokaryotic tRNA suppression probably mediates the read-through of the termination codons at the end of the coding sequences for Pr76 (Weiss, et al, 1978). No tRNA suppressors or other mechanisms for suppression of termination codons have been observed in normal eukaryotic cells (Capecchi, et al., 1977). However, frame-shifting tRNA suppression may operate in some circumstances in prokaryotic mRNA translation. An example of this, is the frame-shift read-through of the QB replicase by $tRNA^{tyr}$ (Gesteland, personal communication). Other possibilities for suppression may include influences of secondary structure in mRNAs on the operation of ribosome and actions of specific proteins on the ribosomes. One of these mechanisms may be involved in the read-through of the termination codons of Pr76.

ASV-Viral genes and cellular genes

In general, the mechanisms used by avian sarcoma virus to express the products of three of its four genes conform to ideas previously offered for the expression of other eukaryotic and animal virus genes. The structural proteins in the core of the virus are processed from a large polyprotein precursor, the primary translation product of the gag gene (Vogt, et al., 1975). Such polyproteins were first identified in picornavirus infected cells, and may be found for other eukaryotic genes as a mechanism of generating several protein products from a single messenger RNA (Holland and Kiehn, 1968; Jacobson and Baltimore, 1968).

The glycoproteins of ASV appear to be translated from a separate mRNA, probably because fewer glycoprotein molecules than core protein molecules

are required for virion assembly. In addition, the glycoprotein precursors are synthesized on membrane-bound polyribosomes and are probably inserted into the membrane at this stage, as suggested by my work (Figure 8). The separate mRNA may be needed to insure that gag proteins and possibly the src protein are not inserted into membranes. Glycoproteins from other viruses, e.g. the vesticular stomatitis virus glycoprotein, are also synthesized on membrane-bound polyribosomes and later processed into one or more mature glycoproteins (Lodish & Froshauer, 1977). The Sindbis virus glycoproteins are exceptional in that they are synthesized by an mRNA which codes for a core protein as well as two glycoproteins, and only after the synthesis of the core protein is complete and the core protein is cleaved from the precursor, do the glycoprotein precursors bind to membranes (Wirth, et al., 1977).

The src gene product is also probably synthesized from a separate mRNA as suggested by earlier work (and see Figure 12). Again, the relative need for the protein may be reflected by the quantity of the mRNA present.

The products of all three genes discussed above are translated from the 5' gene of their respective mRNAs. It was suggested by Jacobson and Baltimore (1968), that initiation of protein synthesis could only occur at the 5' end of the mRNA. Certainly, ASV gene expression in vivo appears to conform to this rule. It was unexpected, however, that initiation of translation would occur as far from the 5' capped ends (at least 104 bases, Cordell, et al., 1978) as it must (Palmiter, et al., 1978). In fact, as pointed out already, the 5' untranslated sequence of the src mRNA could be as large as 1000 bases (see Figure 24). Untranslated sequences of over 100 bases have been observed in several mRNAs, e.g. those for adenovirus proteins, globin, immunoglobulin light chain (Berget, et al., 1977; Chow, et al., 1977; Kinniburgh, et al., 1978; Tonegawa, et al., 1978). It

was speculated that the 5' untranslated sequences participate in binding to ribosomal subunits by small regions of complementarity with sequences at the 3' end of 18S ribosomal RNA (Shine and Dalgarno, 1974). Such a small region is found near the 5' end of the RNA in ASV (Shine, et al., 1977; Haseltine, et al., 1977), as well as in several other viral and eukaryotic cell RNAs (Hagenbuchle, et al., 1978). However, the significance of this site in ASV is now unclear.

It is not known whether the ASV genome contains intervening sequences which can be spliced out to form linked coding sequences for the various protein products, apart from the processing of the two subgenomic messengers by splicing out entire genes. Intervening sequences, or exons, have been observed in several eukaryotic genes (Breathnach, et al., 1978; Kinniburgh, et al., 1978; Brack and Tonegawa, 1977; Berk and Sharp, 1978). This kind of phenomenon does not appear to be likely in the ASV genome because the virion RNA and fragments derived from it can be translated in vitro (Beemon and Hunter, 1978; Erickson, et al., 1978). However, since mRNA can be packaged this finding does not rule out the possibility that intervening sequences are present.

Much information regarding the expression of eukaryotic genes has been accumulated over a very short period of time. The ASV system appears to conform to the ideas derived from these studies. However, an outstanding problem, yet to be solved, is the mechanism for translation of $Pr180^{gag/pol}$, as discussed earlier. Perhaps, an as yet unrecognized mechanism, such as a low level of a normal tRNA binding to a nonsense codon for reading through a termination signal, can be demonstrated in the study of this problem.

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