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The mechanism of the reversion of avian sarcoma virus-transformed baby hamster kidney cells

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THE MECHANISM OF THE REVERSION OF AVIAN SARCOMA VIRUS-TRANSFORMED BABY HAMSTER KIDNEY CELLS

by

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DISSERTATION

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Abstract

Clones of avian sarcoma virus (ASV)-transformed baby hamster kidney cells (BHK-21) and subclones reverted to normal phenotype were found to contain one copy of integrated virus-specific DNA per diploid cell by measuring the influence of unlabeled DNA from hamster cells upon the reassociation kinetics of labeled ASV polymerase product (slowly reassociating DNA). To detect viral RNA, highly labeled, single-stranded DNA synthesized by the DNA polymerase of avian sarcoma virus (ASV cDNA) was employed in the hybridization analysis with unlabeled hamster cellular RNA. I have observed that in the transformed clones, virus-specific DNA is transcribed into RNA at low levels (at least 100-fold lower than the concentration of viral RNA in permissive chick cells); in the revertant clones, viral RNA is detected, but at a still lower concentration (from 2- to 85-fold lower than the concentration of viral RNA in transformed hamster clones). The amount of viral RNA in these clones correlates with the frequency with which ASV can be rescued after fusion with permissive chick embryo cells. Treatment of a revertant clone with 5-bromodeoxyuridine increased the concentration of viral RNA and the frequency

of rescue.

To ask whether the reversion phenomenon is due simply to the quantitative reduction in viral RNA, or whether additional qualitative differences in the metabolism of viral RNA serve to differentiate those phenotypes, I have compared several characteristics of ASV RNA transcripts in transformed and reverted cells. Using hybridization analysis with highly labeled ASV cDNA, I have observed that viral RNA transcripts in both transformed and reverted hamster cells contain poly(A) sequences, and associate with polyribosomes. Viral RNA in cells of both phenotypes appears to be 3-fold more abundant in the cytoplasm than in the nuclei. Furthermore, in the transformed clone, whole-cell RNA contains the 35S and 24S species of ASV RNA, whereas cytoplasmic RNA contains only the 24S species (polyadenylated); in the reverted clone, the 24S species of ASV RNA is present in whole-cell RNA and in polyadenylated cytoplasmic RNA, but it is not clear whether whole-cell RNA of the reverted cells contains 35S viral RNA.

Using a hybridization reagent specific for the nucleic acid sequences required for the transformation by avian sarcoma viruses ($cDNA_{sarc}$), I have shown that polyribosomal

RNA in both transformed and reverted cells contains transformation-specific sequences; however, the concentration of those sequences reflects the overall concentration of viral RNA in these cells. Thus I can find no qualitative difference in the pattern of viral gene expression in these phenotypically varied cells, and I conclude that the quantitative differences in the concentration of viral RNA are sufficient to produce the marked phenotypic variations among them.

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Abstract

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I. Introduction

I.1. A Brief Description of Avian Sarcoma Viruses.

Although the first avian sarcoma virus (ASV) was isolated and shown to be an etiological agent of a solid tumor by Rous as early as 1911 (Rous, 1911), ASV did not attract much attentions in the scientific community until several decades later. The association of other viruses with a large variety of neoplasms has now been established in many species of animals (Gross, 1970; Tooze, 1973). Since those viruses contain RNA as their genome (Duesberg, 1970), a general name, RNA tumor viruses, was given to distinguish them from DNA tumor viruses. The discovery that the virions of RNA tumor viruses contain reverse transcriptase (RNA-directed DNA polymerase) which transcribes viral RNA into DNA (Baltimore, 1970; Temin & Mizutani, 1970) has had a tremendous impact upon RNA tumor virus research. The enzyme fulfilled the requirement of Temin's "provirus hypothesis" (Temin, 1971, 1972; also see section I.2.) and enabled the in vitro synthesis of labeled virus-specific DNA as hybridization reagents to detect unlabeled virus-specific nucleic acids in cells. (Gelb et al., 1971; Varmus et al., 1972a, b).

ASV contains a native complex of single-stranded RNA sedimenting at 60-70S with a mass of 9-12 x 10^6 daltons; the RNA dissociates into two to four 35S subunits of 2.5-3.5 x 10^6 daltons upon denaturation (Duesberg, 1968; Erikson, 1969; Kung et al., 1974). The 35S subunits of ASV RNA are identical; therefore the genome is diploid or polyploid (Billeter et al., 1974; Beemon et al., 1974). ASV 35S RNA contains approximately 10,000 nucleotides for at least four identified viral functions (Duesberg et al., 1974; Baltimore, 1974) and a segment of poly(A) sequences with about 200 adenylate residues at the 3' end of viral genome (Lai & Duesberg, 1972; Wang & Duesberg, 1974). Chromatographic comparisons of ribonuclease T1-resistant oligonucleotides from the labeled non-defective ASV 35S genome with those from the genomes of its derived deletion mutants have allowed mapping of two viral functions in relation to the poly(A) sequences at the 3' end of 35S RNA (Wang et al., 1975, 1976). The tentatively accepted map (Fig. 1) of a subunit of ASV RNA is as follows: a region of up to 600 nucleotides ("C") is shared by the same strain of the non-defective and deleted transformation-defective pairs of viruses; the src gene, specifying sarcoma-specific

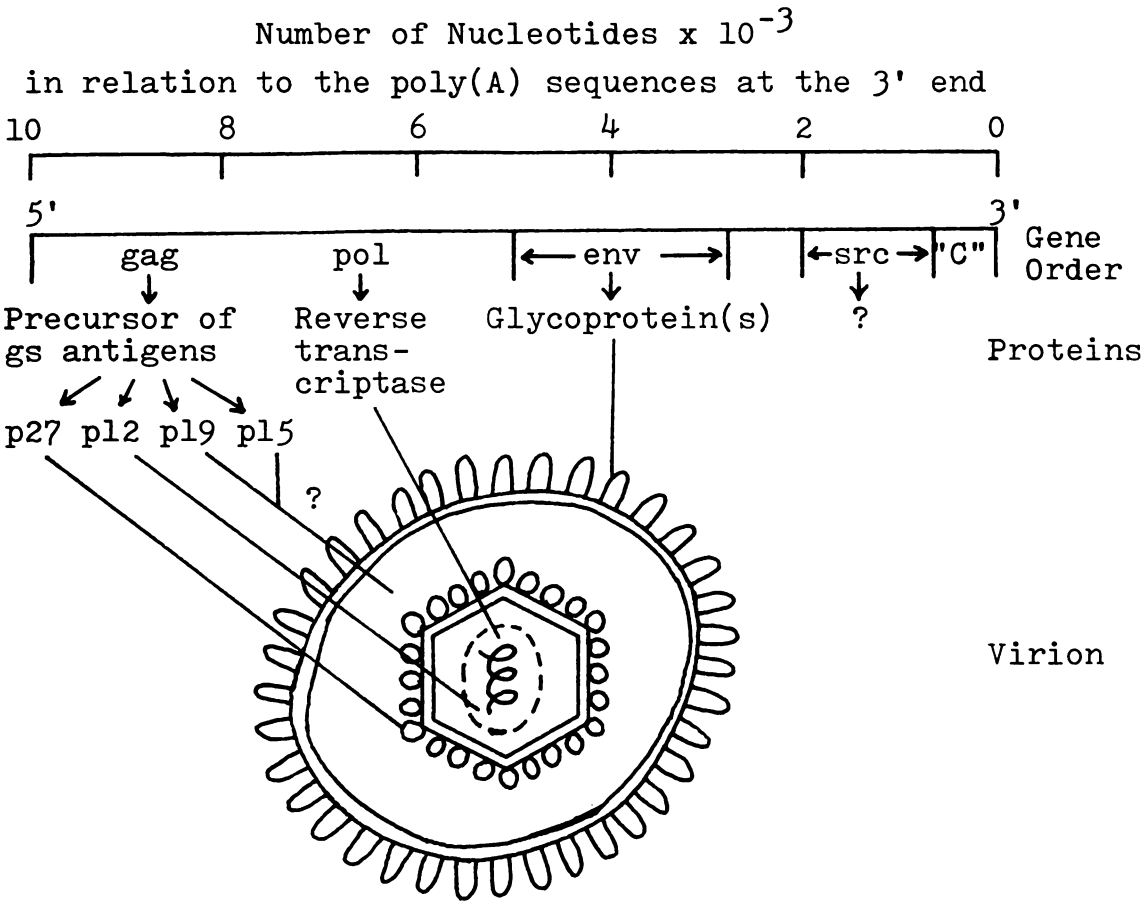

Fig.1. Tentative map of ASV genes along a 35S RNA genome and the locations of virus-coded proteins in a virion. "C" is a region shared by the same strain of non-defective and deleted transformation-defective pairs of viruses. The gene order is discussed in the text. The proteins of gag gene are named for the approximate molecular weight in thousands.

Ref. (1) Map of ASV genes: Wang et al., 1975, 1976; Duesberg et al., 1976.

(2) Locations of proteins: see reviews by Bishop & Varmus, 1975; Baltimore, 1974.

(3) Precursor-product relationship of gs antigens: Eisenman et al., 1974.

sequences ("sarc") of the transforming viruses (Duesberg & Vogt, 1973a, b), is located at about 600-2,000 nucleotides; the env gene, coding for the glycoprotein(s) of viral envelope, is located at about 2,800-5,000 nucleotides. (Wang et al., 1975, 1976). The tentatively proposed order for the last two genes is as follows: the pol gene, coding for the RNA-directed DNA polymerase (reverse transcriptase) is located next to the env gene and the gag gene, coding for the group-specific (gs) antigens of avian viruses is located at the remaining 5' end of a 35S ASV RNA (Duesberg et al., 1976).

I.2. Virus-Cell Interaction in Transformation.

ASV efficiently infects and replicates in the cultures of permissive cells (including chick, duck, quail and turkey)(Temin, 1971). Concomitantly, the infected cells transform into a neoplastic state in many ways analogous to the malignant sarcoma induced by ASV in animals. Several aspects about the mechanism of the transformation induced by ASV infection appear to be relevant in pursuing the mechanism of how a transformed cell reverts to a normal phenotype. First, the transformation of ASV-infected cells does not depend on the replication of

infectious ASV in those cells. This is supported by the existence of:(1) Temperature-sensitive replication-defective mutants that can transform fibroblasts but fail to synthesize infectious virus at nonpermissive temperatures (Vogt et al., 1972; Friis & Hunter, 1973); (2) replication-defective transforming virus such as Bryan strain Rous sarcoma virus (RSV) which is able to transform cells but produces only noninfectious particles in the absence of a helper virus (Hanafusa et al., 1963); and (3) ASV-transformed mammalian cells (nonpermissive) which usually fail to support the replication of ASV (Temin, 1971). Second, the continuous presence and expression of the src gene seems to be required for the maintenance of transformation. This is indicated by: Temperature-sensitive mutants that are defective in transformation alone and can replicate normally at the nonpermissive temperatures (Martin, 1970; Kawai & Hanafusa, 1971; Bader, 1972; Wyke, 1973).

Third, viral information required for the transformation is introduced and maintained in the cell as "DNA provirus" (Temin, 1971). Temin proposed that the RNA tumor viruses transform cells and replicate through the agency of a DNA copy of the viral genome, synthesized early in viral

infection and then integrated into the DNA of host chromosomes (Temin 1971, 1972). The evidence which formed the basis of his proposal has been reviewed frequently (Bishop & Varmus, 1975; Temin & Baltimore, 1972; Temin, 1971). The salient points are as follows:(1) Infection and transformation of cells by RNA tumor viruses require virus-specific DNA synthesis during the first 12 h of the infectious cycle (Bader, 1965, 1966; Temin, 1968). (2) Production of virus requires continuing DNA-dependent RNA synthesis (Bader 1965; Temin, 1963) and structural integrity of the virus-specific DNA (Balduzzi & Morgan, 1970; Boettiger & Temin, 1970). (3) The genome of RNA tumor viruses is perpetuated as a stable genetic property of infected cells even in the absence of detectable viral replication (Coffin, 1972; Svoboda & Dourmashkin, 1969; Rowe, 1971). The provirus hypothesis requires an enzymatic mechanism capable of copying RNA template into DNA, a requirement fulfilled by the discovery of an RNA-directed DNA polymerase in virions of RNA tumor viruses (Baltimore, 1970; Temin & Mizutani, 1970; Temin & Baltimore, 1972).

I.3. Revertants of ASV-Transformed Hamster Cells.

Morphological reversion of ASV-transformed hamster

cells was first observed by Macpherson (Macpherson, 1965). He reported that some clones of baby hamster kidney cells (BHK-21) transformed by Schmidt-Ruppin strains of Rous sarcoma virus (SR-RSV) spontaneously revert to a normal phenotype at variable rates upon subcloning. The revertants of ASV-transformed hamster cells in many aspects resemble the uninfected BHK-21 cells (Table 1). Revertants have also been isolated from populations of cells transformed by murine sarcoma virus (Fischinger et al., 1972) and by DNA tumor viruses, SV40 and polyoma (Pollack et al., 1968; Marin & Littlefield, 1968) (also see IV.2. in discussion). Since most reverted cells are less tumorigenic than their parental transformed cells, it is the goal of this investigation to find out what made a malignant cancer cell convert into a less malignant state. The scheme of the possibilities to account for the reversion of ASV-transformed hamster cells is shown in Table 2.

I.4. Outline of the Approach.

To detect virus-specific nucleic acids in hamster cells, labeled single- and double-stranded DNAs synthesized by the endogeneous activity of RNA-directed DNA polymerase of the purified ASV were employed in hybridization analysis.

Table 1. Initially reported properties of ASV-transformed hamster cells and revertants.[a]

Phenotype	Transformed	Reverted
Morphology	round, spindle	as long fibroblast
Orientation	random	parallel
Soft agar cloning	yes	no
Tumorigenicity		
in chickens	high efficiency	rare
in hamsters	high efficiency	as low as BHK-21
Avian group-specific antigens[b]	detectable	non-detectable
Rescue of ASV by cocultivation with chick cells	yes	no[c]

[a]Macpherson, 1965, 1966, 1971.

[b]Detected by complement fixation test.

[c]However, rescue of transforming ASV from the revertants was reported later by Boettiger using inactivated Sendai virus to generate heterokaryons of revertants and chick cells (Boettiger, 1974).

Table 2. Scheme for the possible mechanisms of reversion.

I. Direct mechanisms: due to the abnormality of provirus.
   (A) Loss of provirus
      (a) Episomal segregation of nonintegrated provirus
      (b) Chromosomal loss of integrated provirus
   (B) Mutation of provirus
      (a) Point mutation
      (b) Deletion of transformation-specific sequences

II. Indirect mechanisms: due to cellular regulation of expression of normal provirus.
   (A) Repression of transcription of provirus: due to the different integration site of provirus or to the variation of host enzymes, factors and so forth.
      (a) Complete repression: absence of any virus-specific RNA.
      (b) Partial repression: some virus-specific RNA is still transcribed.
         (1) Qualitative regulation: reducing the sequence complexity of virus-specific RNA.
         (2) Quantitative regulation: reducing the concentration of virus-specific RNA.
   (B) Post-transcriptional regulation of processing of virus-specific RNA.
      (a) Qualitative regulation: reducing relative sequence complexity of virus-specific RNA in cytoplasm vs. nuclei.
      (b) Quantitative regulation: reducing relative concentration of virus-specific RNA in cytoplasm vs. nuclei.
   (C) Translational regulation: reducing the amount and/or fidelity of synthesis of virus-specific proteins (particularly the src protein).
   (D) Different host responses to "transforming protein": increased cellular resistance to the src gene product.

The in vitro enzymatic product of ASV consists of relatively short chains of DNA (about 100-1,000 nucleotides) transcribed in large measure from a very limited portion of the 70S RNA template molecule (Varmus et al., 1971; Garapin et al., 1973).

In chapter 1, I show that both the transformed and reverted cells contain one copy of integrated provirus DNA per diploid cell. The complete viral genome appears to be present in the reverted cells, since fully infectious virus can be rescued from the reverted cells (Boettiger, 1974). Thus, the possibility of having lost the provirus in the reverted cells is ruled out. The proviral DNA in both cell types is transcribed into viral RNA, but the concentration of viral RNA in the reverted cells is lower than in the transformed cells. Furthermore, the concentration of viral RNA in those clones correlates with the frequency that transforming ASV can be rescued from those cells (Boettiger, 1974). The rescue of the transforming ASV also ruled out the possibility of mutated provirus in the reverted cells.

In chapter 2, several characteristics of ASV RNA transcripts in transformed and reverted clones are compared.

In brief, viral RNA transcripts in both cell types contain poly(A) sequences and associate with polyribosomes. No difference has been observed between the transformed and reverted cells in terms of the relative intracellular distribution of viral RNA and the size of viral RNA in the cytoplasm. Furthermore, polyribosomal RNA in both cell types contains transformation-specific sequences; however, the concentration of those sequences reflects the overall concentration of viral RNA in these cells. Hence, no qualitative difference in the pattern of viral gene expression in these phenotypically varied cells has been found. It appears that the quantitative differences in the concentration of viral RNA are sufficient to produce the marked phenotype variations among them.

In the last chapter, I describe an analysis of the complexity of the viral RNA transcripts in hamster cells. The attempt to measure the extent of the transcription from the provirus in reverted cells was not successful because of insufficient amounts of viral RNA. The preliminary complexity study of viral RNA in transformed hamster cells suggests that the nuclei contain the complete RNA transcript (as 35S RNA) from the provirus, but the major viral RNA in

the cytoplasm appears to be a partial transcript (as 24S RNA). Other minor species of viral RNA which appears to be different from the major viral RNA is also present in the cytoplasm. Several possibilities to account for the minor species of viral RNA in the cytoplasm of transformed hamster cells are discussed in section 3 of chapter 3.

II. Materials and Methods

II.1. Viruses and Cells.

Schmidt-Ruppin strain of RSV (SR-RSV, subgroup A) and B77 strain of ASV (B77, subgroup C) were grown in chick embryo cells and purified as described (Bishop et al., 1970).

Normal, transformed, and reverted BHK-21 cells are described in Table 3 and were grown in monolayer culture in Dulbecco's modified Eagle's Medium (Grand Island Biological Co.) supplemented with 3% calf serum.

II.2. Preparation of cell DNA.

The technique used has been described elsewhere (Varmus et al., 1973a, b) and was designed to avoid loss of nonintegrated viral DNA and preserve high molecular-weight DNA. Briefly, cells were lysed with 0.5% SDS and digested with pronase (500 μg/ml). After extractions with phenol, the nucleic acids were precipitated with ethanol, treated with pancreatic ribonuclease, reextracted with phenol, dialyzed extensively against 15 mM NaCl; 1.5 mM Na Citrate, and sheared at 50,000 psi in a pressure cell designed by the American Instrument Company.

II.3. Fractionation of DNA.

Table 3. Designation and Description of Cell Lines

Designation	Description	Infecting virus
BHK-21	Baby hamster kidney-21 cell line	-
MSV-BHK	Harvey strain murine sarcoma virus (H-MSV) transformed BHK-21 cell line	H-MSV
B-BHK	Bryan strain Rous sarcoma virus (B-RSV) stably transformed BHK-21 cell line	B-RSV
SR-3	Schmidt-Ruppin strain Rous sarcoma virus (SR-RSV) transformed BHK-21 cell line with high frequency of reversion	SR-RSV
SR-7	Schmidt-Ruppin strain Rous sarcoma virus (SR-RSV) transformed BHK-21 cell line with low frequency of reversion	SR-RSV
SR-3/1a	Transformed subclone of SR-3	SR-RSV
SR-3/4	Reverted subclone of SR-3	SR-RSV
SR-3/4a	Reverted subclone of SR-3	SR-RSV
SR-3/5	Reverted subclone of SR-3	SR-RSV
SR-3/11R	Reverted subclone of SR-3	SR-RSV
XC	Cells from rat tumor induced by Prague strain of Rous sarcoma virus (Pr-RSV) (Svoboda et al., 1963)	Pr-RSV
B77-3T3	B77 strain avian sarcoma virus (B77) transformed BALB/c-3T3 cell line (Varmus et al., 1973b)	B77
SR-Vole	SR-RSV transformed European field vole cells (from Dr. P.K.Vogt)	SR-RSV
SR-3T3	SR-RSV transformed BALB/c-3T3 cell line (Varmus et al., 1973b)	SR-RSV
SR-NRK	SR-RSV transformed normal rat kidney cells (Varmus et al., 1973b)	SR-RSV

DNA covalently linked to reiterated cellular sequences and, therefore, capable of forming precipitable "networks" was isolated according to Varmus et al. (1973a). Unsheared cellular DNA in 15 mM NaCl; 1.5 mM Na Citrate was denatured by heating at 100° for 3 min, then incubated at 100-200 μg/ml in a 68° bath in the presence of 0.6 M NaCl for 1 hr. The solutions were chilled to 4° and centrifuged for 15 min at 40,000 rpm in a Spinco fixed-angle 40 rotor. The supernatant fraction was removed, and the visible pellet was vigorously suspended in 15 mM NaCl; 1.5 mM Na Citrate. The amount of DNA in each fraction was determined by reading absorbance at 260 nm. These fractions were then sheared at 50,000 psi, precipitated with ethanol, and assayed for ASV-specific sequences.

II.4. Preparation of virus-specific slowly reassociating DNA.

The technique used has been published (Varmus et al., 1973b, 1971). A double-stranded DNA polymerase transcript of 70S viral RNA was prepared with Nonidet-P40 treated purified virus in the presence of 8 mM $MgCl_2$, 0.1 M Tris-HCl, pH 8.1, 2% β-mercaptoethanol according to Garapin et al. (1970), ^{3}H-TTP (17 Ci/mmole, Schwarz-Mann,

3.2×10^{-5} M), and unlabeled dGTP, dCTP, and dATP (8×10^{-5} M each);/ The specific activity of the product was about dTTP (1.5×10^{-5} M),

5×10^{3} cpm/ng. After 18-hr incubations at 37°, the reactions were terminated with 0.5% SDS and the DNA products prepared by pronase digestion (500 μg/ml), two phenol extractions, ethanol precipitation, and pancreatic RNase digestion. Double-stranded DNA was separated by batch elution from hydroxyapatite (DNA grade, BioRad) chromatography. Slowly reassociating DNA was separated from the bulk of the rapidly reassociating DNA on the basis of its reassociation kinetics (Varmus et al., 1971). Slowly reassociating DNA molecules have a $Cot_{1/2}$ of 1.1 to 1.3×10^{-2} mole-sec/liter corresponding to unique sequences with total molecular weight of 6×10^{6}. This determination is based upon the demonstrated relation between sequence complexity and reassociation kinetics (Britten & Kohne, 1968). Slowly reassociating DNA hybridizes with at least 30% of the ASV genome (Bishop et al., 1973).

II.5. <u>DNA reassociation</u>.

DNA reassociation was studied by incubating mixtures of labeled virus-specific, slowly reassociating DNA and unlabeled cell DNA at 68° in 0.4 M phosphate buffer

(equimolar NaH_2PO_4 and Na_2HPO_4) after denaturation of nucleic acid at 100° in 0.003 M EDTA. Samples were assayed by fractionation on hydroxyapatite; unlabeled DNA in eluates was measured spectrophotometrically and labeled DNA by trichloroacetic acid precipitation and counting in a Beckman scintillation counter with toluene-Liquifluor (New England Nuclear Co.). Results were plotted against the product of initial concentration and time (Cot), with correction for Na^+ concentration in the annealing mixture (Britten & Smith, 1970), according to the convention established by Britten and Kohne(1968). The assay was monitored routinely with DNA's of known secondary structure, and minor corrections of the raw data were made in accordance with these standards.

Computations of copy numbers were based on the estimated molecular complexity of slowly reassociating DNA (6×10^6), reported values for the molecular weights of diploid complements of mammalian cell DNA (approximately 4×10^{12}), or chick cell DNA (approximately 1.6×10^{12}) (Shapiro, 1968), and the arithmetic outlined by Gelb et al. (1971a). The ratio of viral sequences to diploid complements of cell DNA in a reassociation experiment was determined from the cpm/ml of the labeled virus-specific DNA and

from the optical density of the cell DNA. This ratio was used to compute the number of copies of virus-specific DNA in each diploid cell required to produce an observed accelerated reassociation of the labeled DNA. For example, a reaction usually contained one set of SR-RSV ^{3}H-labeled slowly reassociating DNA sequences for each diploid complement of BHK DNA; a 2-fold reduction in the $Cot_{1/2}$ of the ^{3}H-labeled slowly reassociating DNA (in comparison with its $Cot_{1/2}$ in the presence of an equivalent concentration of salmon sperm DNA) would indicate that one copy of viral sequences in each diploid genome caused a 2-fold increase in the effective concentration of slowly reassociating DNA.

II.6. 5-BUdR treatment.

5-BUdR stock solution (1 mg/ml in H_2O) was added to growth media to a final concentration of 0.025 mg/ml. Cellular RNA was extracted from treated cells after 24- or 42-hr incubation with 5-BUdR-containing media.

II.7. Preparation of virus-specific single-stranded DNA (ASV cDNA).

The preparation and characterization of labeled ASV cDNA has been described (Garapin et al., 1973). Endogenous RNA-directed DNA polymerase activity was stimulated

by addition of 0.025% Nonidet P-40 to purified SR-RSV or B77 virus in the presence of 0.1 M Tris-HCl, pH 8.1, one or two α-^{32}P-labeled deoxyribonucleotides (New England Nuclear, about 100 Ci/mmole) to a minimum concentration of 2×10^{-6} M each, and two or three remaining unlabeled deoxyribonucleotides to the concentration of 10^{-4} M each. Reaction mixtures also contained 8 mM $MgCl_2$, 1% β-mercaptoethanol and 100 μg/ml actinomycin D (Calbiochem). Reactions were incubated at 37° for about 4 hr and terminated by addition of 0.5% SDS. The DNA was purified by pronase treatment (500 μg/ml, 37°, 45 min), phenol extraction at room temperature, precipitated with ethanol and digested with pancreatic RNase (100 μg/ml, 37°, 1 hr in 3 mM EDTA). The DNA was then fractionated on hydroxyapatite to remove double-stranded product and the single-stranded DNA (cDNA) was treated with 0.3 N NaOH overnight at 37° to inactivate RNase (Garapin et al., 1973). The solution was neutralized, precipitated with ethanol and resuspended in a small volume of 0.02 M Tris-HCl, pH 7.4; 0.01 M EDTA for use in hybridization experiments. The specific activity (sp act) of fresh ^{32}P-labeled ASV cDNA is about 1.5 and 3×10^5 cpm/ng. ^{32}P-labeled ASV cDNA was made monthly to provide

the desirable high specific activity of this probe. Variation in the rate and extent of hybridization with the same viral RNA between different batches of those probes was occasionally observed.

II.8. Preparation of whole-cell RNA.

Confluent tissue culture cells were incubated in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.01 M EDTA containing pronase (500 μg/ml) and SDS (0.5%) for 1 hr at 37°. The mixtures were extracted two times at room temperature with phenol equilibrated with 0.02 M Tris-HCl, pH 8.1. After ethanol precipitation, the preparation was treated with 20 μg/ml of RNase-free DNase (Worthington) in 0.01 M $MgCl_2$, 0.02 M Tris-HCl, pH 7.4 for 2 hr at room temperature. The RNA was reextracted with phenol, precipitated with ethanol and resuspended in a small volume of 0.02 M Tris-HCl, pH 7.4. The concentration of RNA was determined from the absorbance at 260 nm in a Gilford spectrophotometer. The concentration of the residual DNA was determined by colorimetric reaction of a portion of the RNA sample mixed with equal volume of diphenylamine reagent (1 gm of diphenylamine, 100 ml of glacial acetic acid and 2.75 ml of H_2SO_4) and heated at 100° for 10 min (Shatkin,

1969). The intensity of the color was measured by the absorbance at 595 nm and converted to DNA concentration.

II.9. Preparations of cytoplasmic and nuclear RNA.

Tissue culture cells were scraped off the confluent petri dishes, centrifuged for 5 min at 2,000 rpm, resuspended at 2-5 x 10^7 cells/ml in ice-cold 1 mM NaCl, 0.15 mM $MgCl_2$ and 1 mM Tris-HCl, pH 7.4 (0.1X RSB) for 20 min. Sodium deoxycholate (DOC) was added to 0.1% and Nonidet-P40 (NP40) to 0.2% final concentration and the cells were broken by 10 strokes in a Dounce homogenizer (Penman, 1966). Cell homogenates were centrifuged at 2,000 rpm for 10 min to sediment nuclei and debris. The cytoplasmic supernatant was then adjusted to 0.01 M EDTA, 0.05 M Tris-HCl, pH 7.4, containing pronase (500 μg/ml) and SDS (0.5%). After 1 hr at 37° the mixtures were extracted two times with phenol equilibrated with 0.02 M Tris-HCl, pH 8.1. The cytoplasmic RNA was precipitated with ethanol and resuspended in a small volume of 0.02 M Tris-HCl, pH 7.4. The concentration was determined by reading the absorbance at 260 nm.

The sedimented nuclei were resuspended in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, and 0.01 M EDTA containing

pronase (500 μg/ml) and SDS (0.5%). After 1 hr at 37° the mixtures were extracted two times with phenol equilibrated with 0.02 M Tris-HCl, pH 8.1. The preparation was precipitated with ethanol and treated with 20 μg/ml of RNase-free DNase (Worthington) in 0.01 M $MgCl_2$ and 0.02 M Tris-HCl, pH 7.4 for 2 hr at room temperature, adjusted to 0.01 M EDTA and reextracted with phenol. The nuclear RNA was precipitated with ethanol and resuspended in a small volume of 0.02 M Tris-HCl, pH 7.4. The concentration of the residual DNA was determined by the diphenylamine method as described above.

II.10. RNA:DNA hybridization.

In general, hybridization mixtures contained 700 cpm of one or two labeled cDNAs, variable amounts of unlabeled RNA, and 0.6 M NaCl with 0.025 M Tris-HCl, pH 7.4, and 0.001 M EDTA in 40-50 μl reaction volumes. The mixtures were incubated in plastic tubes under mineral oil at 68° for several days. RNA:DNA hybrids were measured by the percentage of input cpm resistant to digestion with single-strand specific nuclease S1 from Aspergillus oryzae (Leong et al., 1972) in 0.3 M NaCl, 0.03 M Na acetate, 0.003 M $ZnCl_2$ containing 10 μg/ml denatured calf thymus

DNA for 2 hr at 50°.

II.11. Isolation of polyadenylated RNA by adsorption to oligo(dT)-cellulose.

About 0.5 gram of oligo(dT)-cellulose (T-3 grade, Collaborative Research, Inc.) was soaked in 0.02 M Tris-HCl, pH 7.4 containing 0.1% diethyl pyrocarbonate for several hours at room temperature to incativate any contaminating ribonuclease. The oligo(dT)-cellulose was then packed into a 1 cm by 15 cm column and washed with 20 ml of buffer I (0.5 M KCl, 0.02 M Tris-HCl, pH 7.4). Cellular RNA (up to 4 mg in 3 ml of buffer I) was applied to the top of the column (the capacity of the column was determined to be adequate for this procedure by the equal binding of labeled 70S RNA of ASV in the presence and absence of 4 mg of cellular RNA). Unbound RNA was washed through the column with buffer I and collected as 1 ml fractions. RNA concentration in each fraction was monitored by reading the absorbance at 260 nm. Poly(A)-containing RNA bound to the column was then similarly eluted with buffer II (0.02 M Tris-HCl, pH 7.4). Unbound and bound RNA was pooled separately and precipitated with ethanol. The RNA was resuspended in a small volume of

0.02 M Tris-HCl, pH 7.4; 0.01 M EDTA. The concentration of RNA was determined by reading the absorbance at 260 nm. To regenerate the column, oligo(dT)-cellulose column was washed with 10 ml of 0.1 N NaOH and subsequently washed with buffer I until the pH was neutral.

II.12. Polyacrylamide gel electrophoresis for determination of sizes of virus-specific RNA.

Polyacrylamide gel electrophoresis was carried out by adaptions of published methods (Bishop et al., 1967; Tsuchida & Green, 1974). Gels (0.6 cm x 9 cm) contained 2.3% acrylamide, 0.23% ethylene diacrylate, 0.04 M Tris-acetate, pH 7.2, 0.03 M Na acetate, 0.001 M EDTA, 0.08% N, N, N', N'-tetramethylethylenediamine, 0.08% ammonium persulfate. The electrophoresis buffer contained 0.04 M Tris-acetate, pH 7.2, 0.03 M Na acetate, 0.001 M EDTA, 0.1% SDS. Unlabeled RNA (100 μg or less) in 0.02 M Tris-HCl, pH 7.4; 0.01 M EDTA containing about 15% sucrose and 0.005% bromophenol blue was electrophoresed for 4 hr at 5 mA per gel. After electrophoresis, each gel was transferred to a rectangular quartz cell (9.5 cm long) and the absorbance at 260 nm was scanned and recorded by a Gilford spectrophotometer. The gel was then frozen and sliced

into 2 mm segments and collected into plastic tubes. After adding 40 µl of a hybridization mixture containing about 700 cpm of labeled ASV cDNA, 100 µg yeast carrier RNA, 0.6 M NaCl, 0.001 M EDTA and 0.025 M Tris-HCl, pH 7.4, RNA:DNA hybridization was carried out as described above (see II.10). The size classes of virus-specific RNA were then determined from their relative mobilities in the gel compared to 28S and 18S rRNAs as internal markers.

II.13. Rate-zonal centrifugation for determination of sizes of virus-specific RNA.

About 2 mg of whole-cell RNA extracted from hamster cells was disaggregated by heating to 100° in 0.02 M Tris-HCl; 0.01 M EDTA for 2 min and layered on the top of a 15%-30% sucrose gradient containing 0.01 M NaCl, 0.01 M EDTA, 0.02 M Tris-HCl, pH 7.4, and 0.5% SDS in a SW 27 rotor and centrifuged at 24,000 rpm for 18 hr at 20°. Gradients were collected from the bottom and 260 nm absorbance of ribosomal RNAs was continuously recorded by a Gilford spectrophotometer. RNA in each fraction was precipitated with ethanol after adding 100 µg/ml of yeast RNA as carrier. RNA was pelleted by centrifugation at 5,000 rpm for 20 min at 4° and dissolved in 50 µl 0.02 M Tris-

HCl, pH 7.4. A constant portion of RNA from each fraction was hybridized with ^{32}P-labeled ASV cDNA as described above (see II.10).

II.14. Isolation of polyribosomes.

To obtain maximum yields of polyribosomes, confluent tissue culture cells were fed 4 hr before harvesting (Van Venrooij et al., 1970). To avoid "run-off" of polyribosomes, 200 μg/ml of cycloheximide was added to cultured cells immediately before harvesting and during subsequent manipulations of the cells. Confluent tissue culture cells were washed and scraped off the petri dishes in Tris-glucose solution (0.127 M NaCl, 5.1 mM KCl, 5.5 mM glucose, and 25 mM Tris-HCl, pH 7) containing 200 μg/ml cycloheximide, centrifuged at 2,000 rpm for 5 min at 4°, resuspended at 2-5 x 10^7 cells/ml in ice-cold M-buffer (0.25 M KCl, 0.005 M $MgCl_2$, and 0.025 M Tris-HCl, pH 7.4) containing 200 μg/ml heparin. Triton X-100 was added to 1% final concentration and the cells were broken by 10 strokes in a Dounce homogenizer (Penman, 1966). The cell homogenates were centrifuged at 2,000 rpm for 10 min at 4° to sediment nuclei and debris. The supernatant was layered on the top of a stepwise sucrose gradient of 2 ml

of 2 M and 2 ml of 0.7 M sucrose in M-buffer containing 100 μg/ml heparin in a SW 41 centrifuge tube. The polyribosomes were pelleted by centrifugation at 32,000 rpm in a SW 41 rotor for 15 hr at 4°, and the pellet was resuspended in P-buffer (25 mM NaCl, 25 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$).

II.15. Detection of virus-specific RNA in polyribosomes.

Pelleted polyribosomes were divided into two equal portions and one was treated with 25 mM EDTA to dissociate polyribosomes and release mRNA. Each portion was then layered on the top of a 10%-40% sucrose gradient in P-buffer containing 100 μg/ml heparin in a SW 41 centrifuge tube. The polyribosomes were centrifuged at 36,000 rpm in a SW 41 rotor for 75 min at 4°. Gradients were collected from the bottom and 260 nm absorbance of polyribosomes was continuously recorded by a Gilford spectrophotometer. Pronase (500 μg/ml), SDS (0.5%) and 100 μg/ml yeast RNA were added to each fraction. After 1 hr incubation at 37°, RNA was extracted from each fraction with phenol, precipitated with ethanol and resuspended in a small volume of 0.02 M Tris-HCl, pH 7.4. A constant portion of RNA from each fraction was hybridized with

^{32}P-labeled ASV cDNA as described above (see II.10).

II.16. Preparation of labeled $cDNA_{sarc}$.

The isolation and characterization of $cDNA_{sarc}$ has been described (Stehelin et al., 1976). In principle, $cDNA_{sarc}$ was selected from ASV cDNA which failed to anneal with viral RNA from transformation-defective deletion mutants of ASV but hybridized with ASV RNA. The $cDNA_{sarc}$ made from PrC ASV hybridized completely with genome RNAs from six strains of ASV (PrC, B77, Carr-Zilber, Schmidt-Ruppin, Bryan and Fujinami), whereas the cDNA failed to hybridize with RNA from transformation-defective variants of ASV, avian leukosis virus, and Rous associated virus-0. In addition, there was no detectable hybridization between $cDNA_{sarc}$ and RNAs of tumor viruses from other species (murine sarcoma-leukosis virus, feline sarcoma-leukosis virus and murine mammary tumor virus). (Stehelin et al., 1976).

II.17. Preparation of labeled representative cDNA ($cDNA_{rep}$).

$cDNA_{rep}$ was selected from ASV cDNA by hybridization of a large quantity of the cDNA with a limited amount of ASV 70S RNA. To make a large quantity of ^{3}H-labeled

ASV cDNA, the 10 ml reaction mixtures contained 10 mCi ^{3}H-TTP (47 Ci/mmole) in a final concentration of 2.1 x 10^{-5} M, unlabeled dATP, dCTP, and dGTP to the concentration of 2 x 10^{-4} M each, and 2 ml of a 5-fold concentrated purified PrC ASV. The reaction mixtures also contained 100 μg/ml actinomycin D, 8 mM $MgCl_2$, 0.75% β-mercaptoethanol and 0.5% NP40. ^{3}H-labeled ASV cDNA was then purified from the bulk of DNA products of the incubated reaction mixtures as described above (II.7). The yield of ^{3}H-labeled ASV cDNA was about 200 x 10^{6} cpm. Since the specific activity of the cDNA was 2 x 10^{4} cpm/ng, the total amount of the cDNA was about 1 μg. Tests of hybridization of ^{3}H-labeled ASV cDNA have shown that 80% of ^{32}P-labeled ASV 70S RNA annealed to the cDNA at a DNA to RNA mass ratio of 100. Therefore, the amount of ASV 70S RNA employed for the selection of $cDNA_{rep}$ should be within 100 ng (an 100-fold less of 1 μg as the total yield of ASV cDNA) in order to obtain a $cDNA_{rep}$ uniformly representing a major portion of the viral genome. A mixture of 30 ng of unlabeled PrC 70S RNA and 14 ng of ^{32}P-labeled B77 70S RNA (180,000 cpm) as internal monitor for measuring the extent of hybridization of 70S RNA was used in annealing

reaction with the cDNA. (Since the ratio of transformation-defective (td) RNA to nondefective ASV RNA was 1:1 in PrC 70S RNA and 10:1 in B77 70S RNA, the 70S RNA used for the selection of $cDNA_{rep}$ contained 2-fold more td viral genomes than nondefective ASV genomes. Thus, $cDNA_{rep}$ presumably was about 3-fold defecient in cDNA complementary to the Sarc sequences.) The annealing mixtures were incubated in a 30 µl volume containing 0.6 M NaCl, 0.001 M EDTA, 0.025 M Tris-HCl, pH 7.4, and 0.7% SDS at 68° for 4 hr to a Cot of 73 and Crt of 0.36 (sufficient for complete hybridization of 70S RNA). The RNA:DNA hybrid was separated from the bulk of single-stranded DNA by hydroxyapatite chromatography. To remove RNA from RNA:DNA hybrid, the nucleic acids were treated with 0.3 N NaOH at 37° overnight, neutralized with HCl, precipitated with ethanol and resuspended in a small volume of 0.02 M Tris-HCl, pH 7.4; 0.01 M EDTA.

If there was any /duplex DNA contaminant in the ASV cDNA from which $cDNA_{rep}$ was selected, the concentration of duplex DNA would have been enriched in the preparation of $cDNA_{rep}$, since $cDNA_{rep}$ was isolated from 0.44% of ASV cDNA as RNA:DNA hybrid which eluted similarly as the duplex DNA from hydroxyapatite chroma-

tography. To test the possibility of duplex DNA contamination in the preparation of $cDNA_{rep}$, $cDNA_{rep}$ was incubated to a sufficient Cot of 0.23 to allow the formation of DNA duplex. The S1 background of incubated $cDNA_{rep}$ was then compared with that of the preparation before incubation. A 10% increment of S1 resistance after incubation suggested the contamination of DNA duplex in $cDNA_{rep}$. Therefore, the incubated $cDNA_{rep}$ preparation was fractionated by hydroxyapatite chromatography to remove the duplex DNA contamination. The characteristics of the $cDNA_{rep}$ was described below (see III.3.1).

II.18. Preparation of labeled $cDNA_{3'}$.

Labeled $cDNA_{3'}$ has been prepared and described by Tal et al. (manuscript in preparation). $cDNA_{3'}$ contained sequences complementary to 100-200 nucleotides adjacent to the poly(A) sequences at the 3' end of ASV genome. In principle, $cDNA_{3'}$ was made from a reconstructed polymerase reaction containing 35S ASV genome and purified DNA polymerase primed with oligo(dT). $cDNA_{3'}$ was then selected from the overall polymerase products by virtue of its covalently linked oligo(dT) sequence. To

select $cDNA_3$,, the preparation from the polymerase reaction was incubated with poly(A) nucleotides to allow the formation of oligo(dT):poly(A) hybrid for $cDNA_3$,. The $cDNA_3$,, in a form of partial hybrid, was fractionated by hydroxyapatite chromatography to remove nonspecific cDNA.

III. Results

Chapter 1. The Persistence and Expression of Virus-Specific DNA in Revertants of Avian Sarcoma Virus-Transformed BHK-21 Cells.

III.1.1. ASV DNA in transformed and reverted hamster cells.

To determine whether virus-specific DNA persists in various lines of reverted cells, I measured the influence of unlabeled DNA from normal, ASV-transformed and reverted hamster cells upon the reassociation kinetics of labeled ASV polymerase product (slowly reassociating DNA). As shown in Fig. 2, DNA from uninfected BHK-21 cells did not affect the rate of reassociation, but DNA from ASV-transformed clones (SR-3, SR-7, and B-BHK) and reverted clones (SR-3/4, SR-3/5, and SR-3/11R) doubled the rate, indicating about one copy of viral sequences per diploid cell. No ASV-specific DNA was detected in BHK cells transformed by a mammalian sarcoma virus (MSV-BHK). (Under the same conditions, I measured four copies per cell in an ASV-induced chicken tumor.) These results indicate that equal amounts of ASV-specific DNA homologous to the slowly reassociating polymerase product are present in transformed and reverted cells. These results do not exclude the

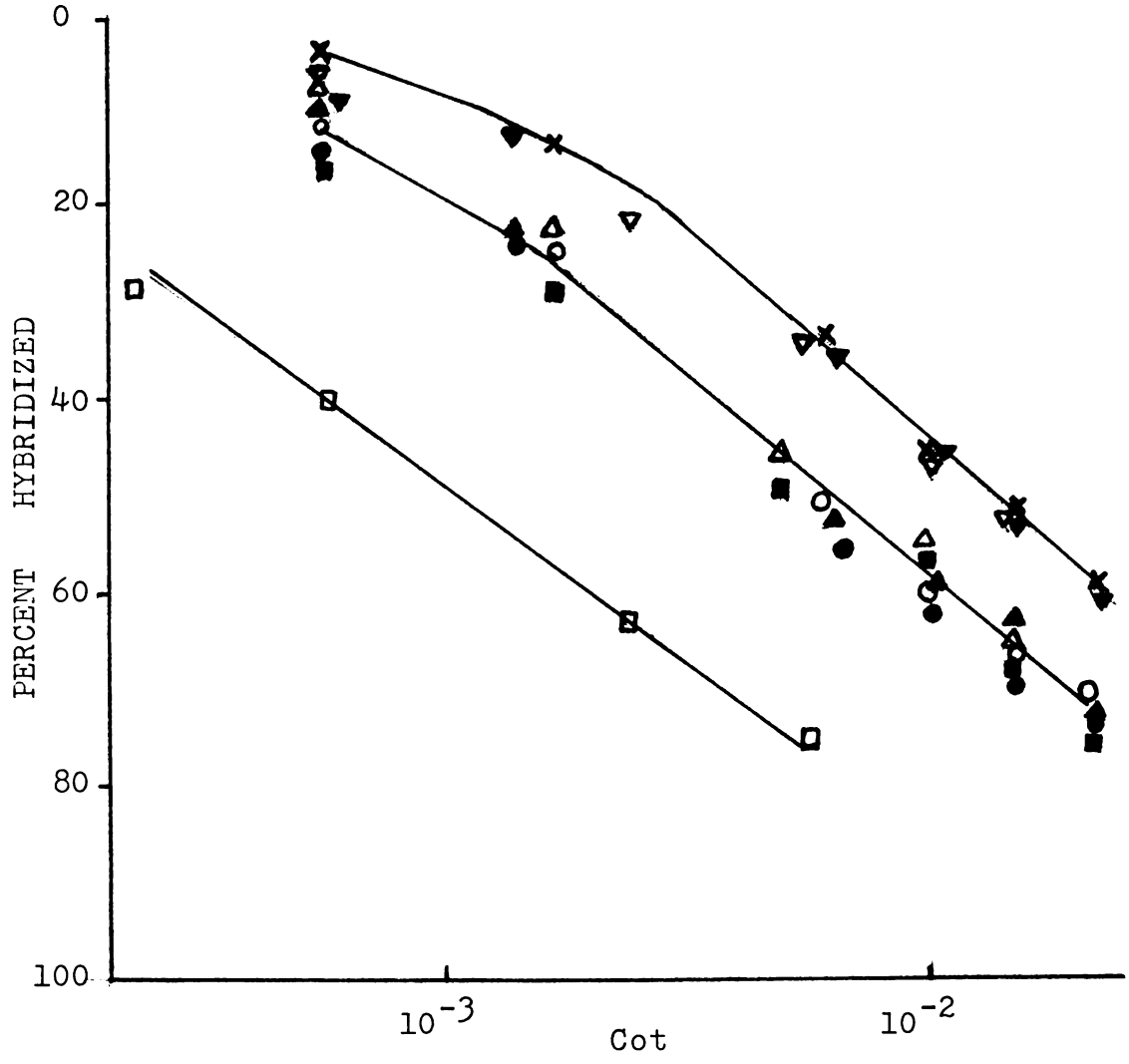

Fig.2. Detection of slowly reassociating virus-specific sequences in cellular DNA. Slowly reassociating ^{3}H-labeled ASV-specific DNA(5.2 ng/ml) was reannealed in the presence of 4 mg/ml of salmon sperm DNA(▽), BHK-21 DNA(×), MSV-BHK DNA(▼), B-BHK DNA(▲), SR-7 DNA(■), SR-3/4 DNA(△), SR-3/5 DNA(●), SR-3/11R DNA(○), or ASV-induced sarcomas DNA(□). Reassociation was studied as outlined in the text and the percentage of labeled DNA reassociated was plotted logarithmically against Cot (concentration of DNA x time of incubation).

possibility that some portion of the viral genome is deleted from the DNA of the reverted cells, since the in vitro double-stranded polymerase product may not represent the entire genome (Varmus et al., 1971; Bishop et al., 1973), and since the annealing technique may fail to demonstrate that a fraction of labeled viral DNA is not present in cell DNA. On the other hand, fully infectious virus can be rescued from the reverted cells, suggesting that the complete viral genome is present (Boettiger, 1974). The copy number estimates have been made per weight of DNA in a diploid cell; since karyotypic analysis of these clones has not been done, it is possible that some clones contain more (or, less likely, less) viral DNA per cell than per weight of diploid DNA.

III.1.2. <u>The integration of the ASV DNA in transformed and reverted hamster cells</u>.

When denatured cell DNA reanneals to relatively low Cot values at which repeated sequences (but not unique sequences) reassociate, the widespread presence of reiterated DNA (Britten & Kohne, 1968) permits the formation of DNA "networks" which can be centrifuged out of solution (Britten , 1965). Testing "network" DNA for viral

sequences constitutes a test for integration of viral DNA into high molecular-weight cell DNA strands which contain reiterated sequences (Varmus et al., 1973a). If the viral DNA is not integrated, it will be absent from networks, and the supernatant fraction will be proportionately enriched for viral sequences. I employed this assay to ask whether ASV-specific DNA was integrated into the DNA from transformed and reverted cells; 66% of SR-3 (transformed clone) and 59% of SR-3/11R (reverted clone) DNA formed networks. Network DNA from both clones accelerated the reassociation of labeled ASV slowly reassociating DNA to the same degree as unfractionated DNA (Fig. 3). As expected, the DNA remaining in the supernatant was not enriched for ASV DNA (data not shown). The results indicate that the ASV DNA in both transformed and reverted hamster cells is integrated into high molecular weight cell DNA.

III.1.3. ASV RNA in transformed and reverted hamster cells.

Since ASV-specific DNA is present in reverted as well as transformed BHK cells, I asked whether the reversion phenomenon might be dependent upon transcriptional controls. To measure viral RNA in those cells, highly

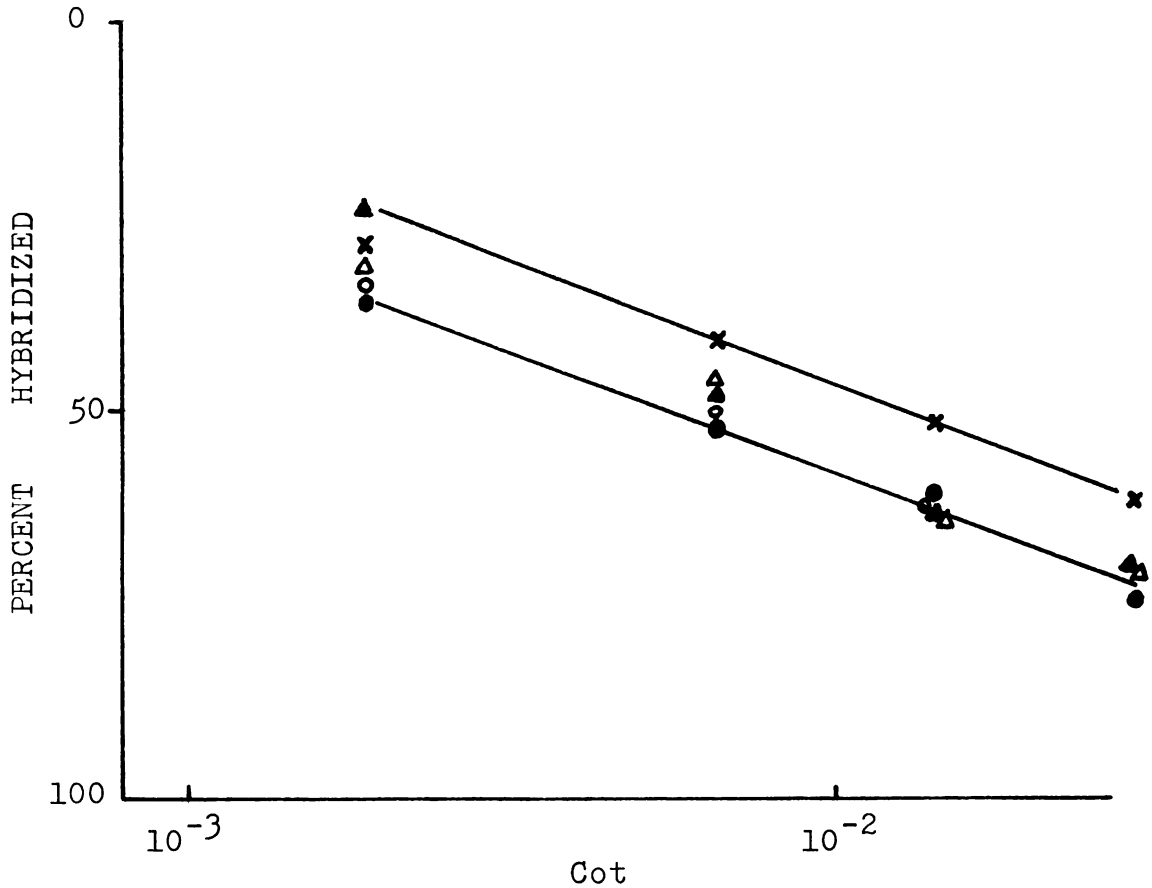

Fig.3. Detection of ASV-specific DNA in network fractions of cell DNA. ^{3}H-slowly reassociating DNA (5.2 ng/ml) was reassociated in the presence of 4 mg/ml of unfractionated DNA from uninfected BHK-21 cells (×), unfractionated (▲) and network (△) DNA from transformed cells (SR-3); and unfractionated (●) and network (O) DNA from reverted cells (SR-3/11R). Unfractionated and network DNA's from transformed and reverted cells reduce the $Cot_{1/2}$ from 1.3×10^{-2} mole-sec/liter to 6.5×10^{-3} mole-sec/liter.

labeled, single-stranded DNA synthesized by the DNA polymerase of avian sarcoma viruses (ASV cDNA) was annealed to increasing quantities of RNA extracted from several clones of transformed and reverted hamster cells (Fig. 4, Table 4). The $Crt_{1/2}$ was determined for RNA from each clone and the quantity of virus-specific RNA per cell was computed in relation to the $Crt_{1/2}$ for ASV 70S RNA (Bishop et al., 1973a; Varmus et al., 1973c; Leong et al., 1972). ASV-specific RNA was observed in all clones examined, but the concentration of viral RNA was from 2- to 85-fold greater in the transformed than in the reverted cells.

As shown in Table 4, decreasing amounts of virus-specific RNA per cell were accompanied by decreasing frequencies of recovery of infectious virus after Sendai-mediated fusion with chick cells. These results suggest that the concentration of viral RNA is important in determining the expression of viral function in infected BHK cells.

The experiments shown in Fig. 4 and Table 4 were performed with a small amount of highly ^{32}P-labeled ASV cDNA (sp act 3 x 10^5 cpm/ng) and with relatively large quantities of RNA (up to 650 μg/sample for each clone).

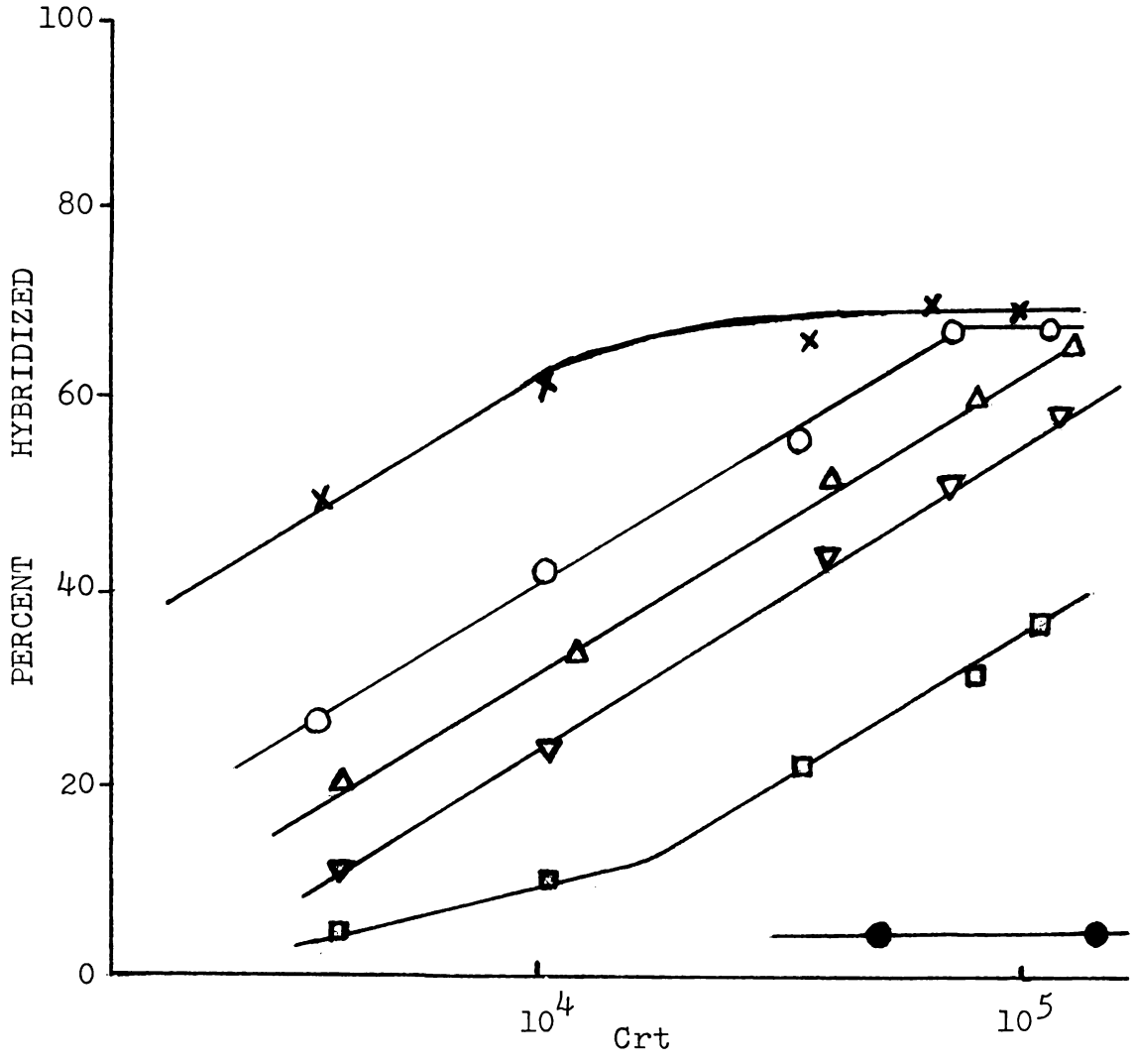

Fig.4. ASV-specific RNA in transformed and reverted hamster cells. RNA was extracted from unfractionated cells of several hamster clones. ^{32}P-labeled ASV cDNA (700 cpm/sample, sp act 3 x 10^5 cpm/ng) was incubated with increasing quantities of RNAs in a 40 µl volume containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, and 0.001 M EDTA at 68° for 148 hr. Hybridization was measured as described in methods. Symbols for curves (from left to right): X SR-3/1a; O SR-3; Δ SR-3/5; ∇ SR-3/4a; □ SR-3/11R; ● HeLa. The $Crt_{1/2}$'s for these curves are listed in Table 4.

Table 4. Correlation of $Crt_{1/2}$ with rescue index of transformed and reverted ASV-infected BHK-21 cells.

Cell line	Morphology	Rescue index[a]	$Crt_{1/2}$[b]	Viral 35S RNA equiv/cell[c]
SR-3/1a	Transformed	3	1.2×10^3	37.5
SR-3	Transformed	1	6.4×10^3	7
SR-3/5	Reverted	0.15	1.4×10^4	3.5
SR-3/4a	Reverted	0.1	2.2×10^4	2
SR-3/11R	Reverted	0.01	10^5	0.45

[a]Frequency of virus production after Sendai-mediated fusion with chick embryo fibroblasts was determined by Boettiger (1974) and is expressed in relation to the frequency of virus rescue from SR-3.

[b]Derived from data presented in Fig. 4, assuming that the hybridization of revertant RNA to labeled cDNA is complete when 70% of input DNA cpm is resistant to S1 nuclease.

[c]Determined in relation to $Crt_{1/2}$ for ASV RNA of 1.5×10^{-2} mole-sec/liter as described previously (Bishop et al., 1973a; Varmus et al., 1973c);

Viral 35S RNA equivalent/cell:

$$\frac{1.5 \times 10^{-2} \text{ mole-sec/liter } (Crt_{1/2} \text{ of ASV RNA})}{Crt_{1/2} \text{ of hamster cell RNA}} \times$$

$$\frac{1.5 \times 10^{-8} \text{ mg (weight of RNA per hamster cell)}}{5 \times 10^{-15} \text{ mg (weight of 1 molecule of ASV 35S RNA)}}$$

The resulting mass ratio of virus-specific RNA to DNA in the hybridization mixtures was, therefore, at least 31-fold with RNA from the most reverted subclone SR-3/11R at the point of maximum hybridization observed. As reported by others (Hayward & Hanafusa, 1973) the RNA-driven annealing reactions proceed at a somewhat slower rate when performed with low ratios of viral RNA to DNA. In several experiments using ^{3}H-labeled virus-specific DNA of lower specific activity than that of the ^{32}P-labeled hybridization reagent employed in the experiments shown above, I consistently observed the correlation between $Crt_{1/2}$ and rescue index shown in Table 4 in spite of a 6-fold absolute increase in the $Crt_{1/2}$'s. The effect of the RNA/DNA ratio upon the $Crt_{1/2}$'s complicates comparison of these results with those obtained with RNA from other ASV-transformed mammalian cells. Table 5 gives representative values for $Crt_{1/2}$'s determined with the hamster clones and other mammalian cells using similar hybridization conditions. The transformed hamster clones have levels of ASV-specific RNA comparable to those observed in ASV-transformed rat, mouse, and vole cells, whereas levels of RNA in the reverted clones are several-fold lower. As noted previously (Bishop et al., 1973a), nonpermissive

Table 5. Comparison of $Crt_{1/2}$ of Infected BHK-21 Cells with Other ASV-Infected Mammalian Cells Using Similar Hybridization Conditions[a].

Cell line	Morphology	$Crt_{1/2}$	Max. hybridization observed
SR-3/1a	Transformed	7×10^3	85%
SR-3	Transformed	3.5×10^4	92%
SR-3/5	Reverted	9×10^4	58%[b]
SR-3/4a	Reverted	1.4×10^5	68%[b]
SR-3/11R	Reverted	7×10^5	37%[b]
XC[c]	Transformed	3×10^3	78%
SR-NRK[d]	Transformed	3.6×10^3	93%
B77-3T3[e]	Transformed	$7\text{-}8 \times 10^3$	95%
SR-Vole[d]	Transformed	1.4×10^4	70%
SR-3T3[d]	Transformed	2.4×10^4	85%
CEF(SR)[f]	Transformed	5	95%

[a] ^3H-labeled ASV cDNA made from ^3H-TTP (17 Ci/mmole, New England Nuclear), sp act 7×10^3 cpm/ng.

[b] In these cases, the annealing did not proceed to completion (a plateau was not observed) and the $Crt_{1/2}$'s were determined upon the assumption that maximal hybridization would be the same as with the transformed lines, as in Fig. 4.

[c] D. Stehelin, unpublished observation.

[d] N. Jackson, unpublished observation.

[e] N. Quintrell, unpublished observation.

[f] SR-RSV-infected permissive chick embryo fibroblasts. Data from Bishop et al., 1973a.

cells transformed by ASV have 2-4 logs less viral RNA than do transformed permissive cells.

III. 1.4. Enhancing ASV RNA by 5BUdR treatment.

Boettiger (1974) has shown that treatment of revertant subclones with 5BUdR before cell fusion and virus rescue causes a 10-fold increase in the observed rescue frequency. I asked whether the increased rescue frequency after 5BUdR treatment was accompanied by an increased amount of ASV-specific RNA in the treated revertant subclones. Cells from the reverted subclone SR-3/11R were exposed to 5BUdR (0.025 mg/ml) for 24 or 42 hr. Cultures treated for 24 hr were not significantly different from the untreated cultures with respect to cell morphology and cell number; after 42 hr, however, I found widespread morphological changes, cell killing, and loss of about 80% of the cells from the monolayer. RNA from the cells remaining after either 24- or 42-hr exposure to 5BUdR contains about 5-fold more virus-specific sequences than RNA from control cultures (Table 6). Treatment with 5BUdR enhanced the ASV RNA concentration in SR-3/11R subclone to the level of another reverted subclone SR-3/4a which has a 10-fold higher rescue frequency than the untreated SR-3/11R subclone. The result

Table 6. Enhancing ASV RNA by 5BUdR Treatment[a]

Subclone	$Crt_{1/2}$	Rescue frequency[b]
SR-3/11R (24 hr)	2.2×10^4	-
SR-3/11R (42 hr)	2×10^4	0.1
SR-3/11R (untreated)	1×10^5	0.01
SR-3/4a (untreated)	2.2×10^4	0.1

[a] RNA from SR-3/11R cells exposed to 5BUdR (0.025 mg/ml) for 24 or 42 hr was annealed to ^{32}P-labeled ASV cDNA. RNA from untreated SR-3/11R and SR-3/4a cells was also tested.

[b] Boettiger, 1974.

also supports the argument that the amount of virus-specific RNA per cell is related to the frequency of recovery of infectious virus after Sendai-mediated fusion with chick cells.

Chapter 2. Characteristics of Virus-Specific RNA in Avian Sarcoma Virus-Transformed BHK-21 Cells and Revertants.

III.2.1. Virus-specific RNA in transformed and reverted hamster cells is polyadenylated.

Poly(A) sequences have been found in the RNA of ASV (Lai & Duesberg, 1972); two-thirds of the 35S subunits of 70S ASV RNA contain a poly(A) stretch consisting of about 200 nucleotides at or near the 3' end of the RNA, and over 90% of the 70S RNA contains poly(A) sequences (Wang & Duesberg, 1974).

To ask whether virus-specific RNA in hamster cells contains poly(A) sequences, polyadenylated RNA from transformed and reverted hamster cells was selected by affinity chromatography on oligo(dT)-cellulose column and annealed to ^{32}P-labeled ASV cDNA at increasing quantities of selected RNA. If the viral RNA in hamster cells contains poly(A) sequences, the RNA selected by affinity chromatography on oligo(dT)-cellulose should be significantly

enriched for viral RNA. After the fractionation by oligo-(dT)-cellulose column, the recovery of bound and unbound RNA amounted to 86% of RNA applied to the column. About 2-3% of the recovered hamster cellular RNA bound to the oligo(dT)-cellulose. Hybridization analysis of fractionated RNA from hamster cells showed that the concentration of viral RNA in polyadenylated RNA was 10- to 20-fold greater than in unfractionated RNA from the same clone (Fig. 5). Therefore, about 37.5% of viral RNA in hamster cells had been selected by affinity chromatography on oligo(dT)-cellulose. The proportions of polyadenylated RNA in total virus-specific RNA in both hamster clones represented minimum estimates, since only 50% (instead of 100%) of labeled polyadenylated ASV 70S RNA was recovered as polyadenylated RNA after similar extraction procedure for the isolation of polyadenylated hamster RNA. It is not known if all the ASV RNA in both hamster clones contains poly(A) sequences. However, the reversion phenomenon is unlikely to arise from the absence of poly(A) sequences in virus-specific RNA in reverted hamster cells.

III.2.2. Intracellular distribution of virus-specific RNA in transformed and reverted hamster cells.

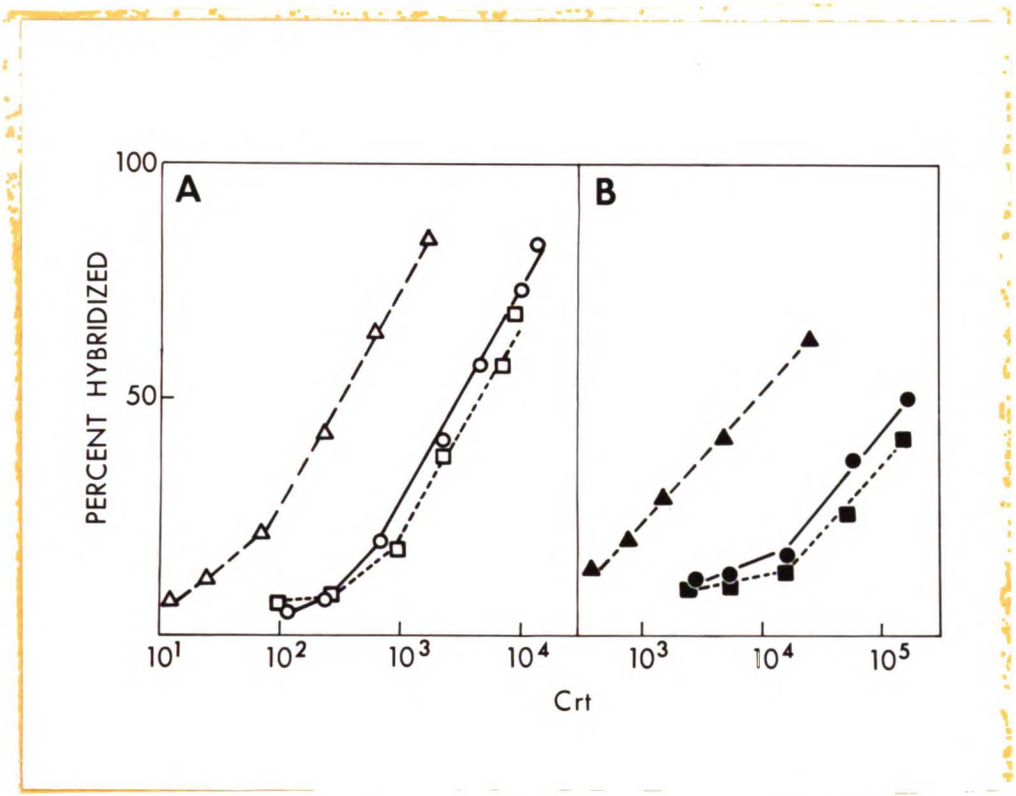

Fig.5. Detection of polyadenylated ASV-specific RNA in (A) transformed (SR-3/1a) and in (B) reverted (SR-3/4a) hamster cells. RNA was extracted from unfractionated cells and polyadenylated RNA was selected by affinity chromatography on oligo(dT)-cellulose. ^{32}P-labeled ASV cDNA (700 cpm/sample, sp act 6.7 x 10^4 cpm/ng) was incubated with increasing quantities of RNAs in a 40 µl volume containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, and 0.001 M EDTA at 68° for 138 hr, as described in methods. Hybridization was measured by resistance of the cDNA to hydrolysis with S1 nuclease. The percentage of labeled cDNA hybridized was plotted logarithmically against Crt (concentration of RNA x time of incubation). Polyadenylated RNA(Δ , ▲); unfractionated RNA(O , ●); and unbound RNA(□ , ■).

In order to investigate whether the regulation of reversion operating at the level of transport of virus-specific RNA from nuclei into the cytoplasm of reverted clones, the intracellular distribution of ASV RNA in hamster cells was measured. Hamster cells were fractionated into nuclei and cytoplasm by Dounce homogenization (Penman, 1966) in the presence of detergents (0.1% DOC and 0.2% NP40) and the RNA was extracted from each fraction separately. The nuclear preparation was treated with RNase-free DNase and the residual DNA content was determined. After correction for DNA contamination, nuclear RNA constituted about 7% of the total RNA in both transformed and reverted clones. ASV RNA concentration in nuclei and cytoplasm of transformed and reverted hamster clones was measured by hybridization analysis with ^{32}P-labeled ASV cDNA. As shown in Fig. 6, the nuclear RNA of transformed (SR-3/1a) and reverted (SR-3/4a) hamster clones was about 5-fold enriched for ASV RNA sequences compared with the cytoplasmic RNA of the same clone. The calculated ratio of viral RNA in nuclei/viral RNA in cytoplasm is about 0.38 (see legend of Fig. 6 for the calculation) for both clones. The results rule out the possibility

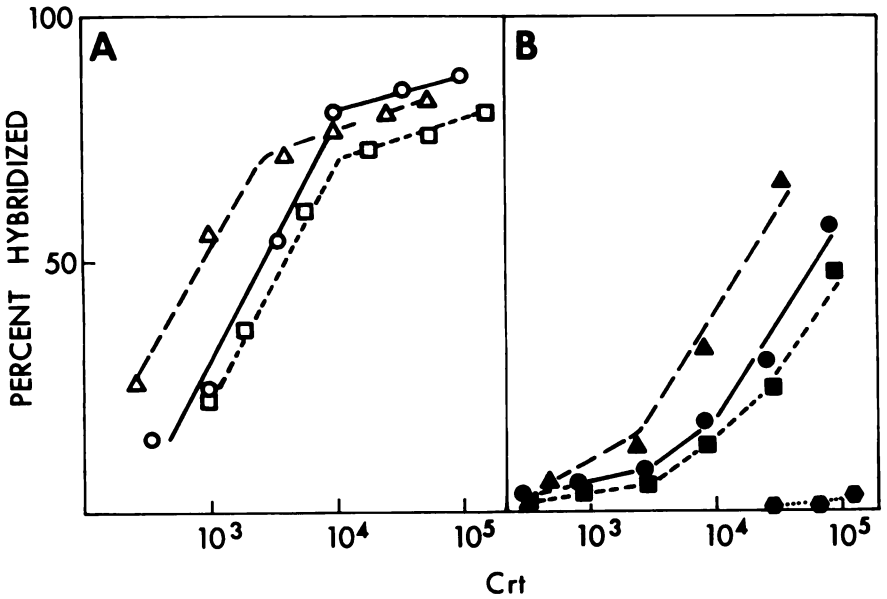

Fig.6. Intracellular distribution of ASV-specific RNA in (A) transformed (SR-3/1a) and in (B) reverted (SR-3/4a) hamster cells. Cells were fractionated into nuclei and cytoplasm by Dounce homogenization in the presence of detergents (0.1% DOC and 0.2% NP40) as described in methods. RNA was extracted from both fractions and from unfractionated cells. ^{32}P-labeled ASV cDNA (700 cpm/sample, sp act 6.3 x 10^4 cpm/ng) was incubated with increasing quantities of RNAs in a 40 µl volume containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, and 0.001 M EDTA at 68° for 87 hr. Hybridization was measured as described in the legend for Fig.5. Nuclear RNA (△ , ▲); whole-cell RNA (○ , ●); cytoplasmic RNA (□ , ■); and yeast RNA (⬢).

Viral RNA in nuclei/viral RNA in cytoplasm =

$$\frac{Crt_{1/2} \text{ of cytoplasmic RNA}}{Crt_{1/2} \text{ of nuclear RNA}} \times \frac{\text{Fraction of total RNA in nuclei}}{\text{Fraction of total RNA in cyto.}}$$

$$= 5 \times (0.07/0.93) = 0.38$$

that the reversion phenomenon is caused by the restriction of the bulk of ASV RNA to the nuclei of reverted hamster cells. However, the results do not exclude the possible selective restriction of some specific ASV sequences to the nuclei of reverted cells.

III.2.3. Sizes of virus-specific RNAs in transformed and reverted hamster cells.

To determine sizes of virus-specific RNAs in transformed and reverted hamster cells, intracellular RNAs isolated from confluent cultures were fractionated either by electrophoresis on polyacrylamide gels or by rate-zonal centrifugation. To identify virus-specific RNA, each fraction was annealed with a constant amount of labeled ASV cDNA and the percentage of cDNA hybridized was measured.

As shown in Fig. 7, whole-cell RNA from a clone of transformed hamster cells (SR-3/1a) contains two distinct virus-specific RNA species resolved by polyacrylamide gel electrophoresis. These viral RNAs migrated as 35S and 24S species as compared with the mobilities of internal 28S and 18S ribosomal RNA markers electrophoresed in the same gel; and with the mobility of the labeled 35S viral RNA marker electrophoresed in a separate gel. The 35S and 24S

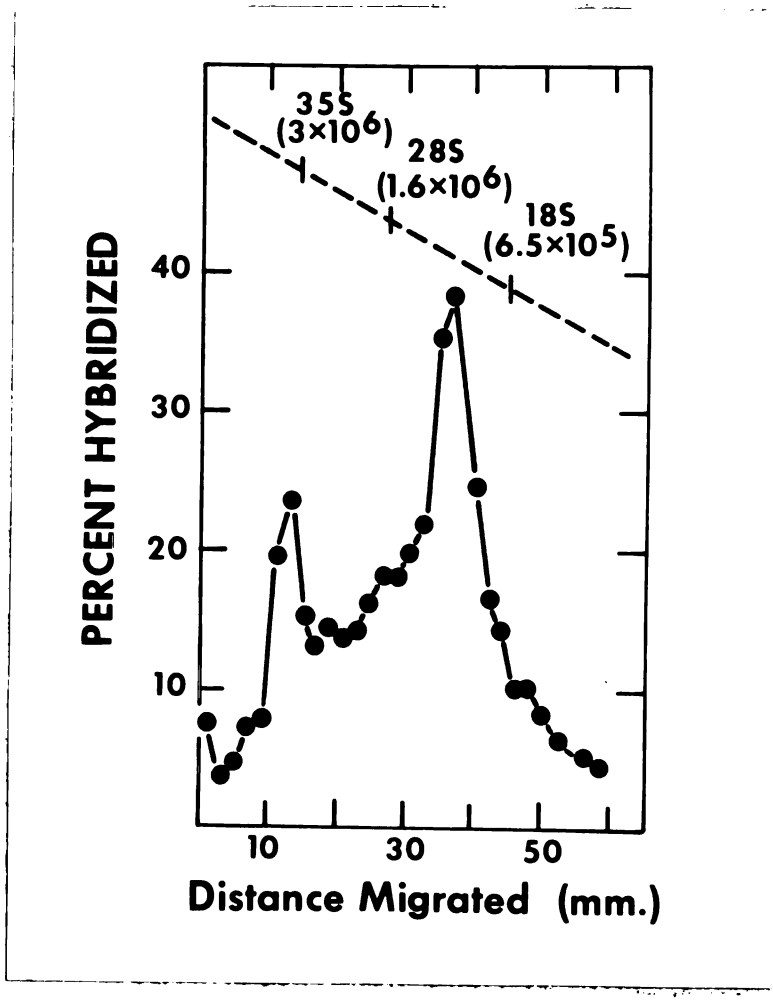

Fig.7. Electrophoretic mobility of ASV-specific RNA from transformed hamster cells (SR-3/1a). RNA was prepared from unfractionated cells and 101 μg was electrophoresed in a 2.3% polyacrylamide gel containing 0.23% ethylene diacrylate for 4 hr at 5 mA per gel. After electrophoresis, internal rRNA markers were detected by scanning the gel at 260 nm absorbance. The labeled 35S RNA prepared from ASV and unlabeled hamster rRNAs were electrophoresed in a separate gel to serve particularly as a 35S marker. ASV-specific RNA was detected by incubation of ^{32}P-labeled ASV cDNA (390 cpm/point, sp act 7.5 x 10^4 cpm /ng) with each gel slice in a 100 μl volume containing 0.3 M NaCl, 12.5 mM Tris-HCl, pH 7.4, and 0.5 mM EDTA at 68° for 88.5 hr. Hybridization was measured by resistance of the cDNA to hydrolysis with S1 nuclease.

species of viral RNA are unlikely to be aggregates of smaller RNA species since heat denatured (100° for 2 min) whole-cell RNA from transformed hamster cells (SR-3/1a) also contained 35S and 24S species of ASV-specific RNA (Fig. 8). However, the "24S" RNA species sedimented slightly slower in sucrose gradients in relative to ribosomal RNA markers than expected from electrophoretic mobility of this species in polyacrylamide gels.

Heat denatured (100° for 2 min) whole-cell RNA from a reverted hamster clone (SR-3/5) contains 24S ASV RNA and probably 35S ASV RNA as determined by centrifugation in sucrose gradient (Fig. 9). Two factors make the detection of 35S ASV RNA in the reverted cells difficult:(1) the 10-fold reduction of the amount of virus-specific RNA in reverted cells and (2) the 2-fold reduced hybridization of labeled ASV cDNA with 35S RNA as compared with 24S RNA in hamster cells.

To determine the size of virus-specific RNA in cytoplasm of infected hamster cells, cytoplasmic RNA was isolated from confluent hamster cultures. To minimize the nuclease activity during the preparation of cytoplasmic RNA, cells were fractionated into cytoplasm and nuclei

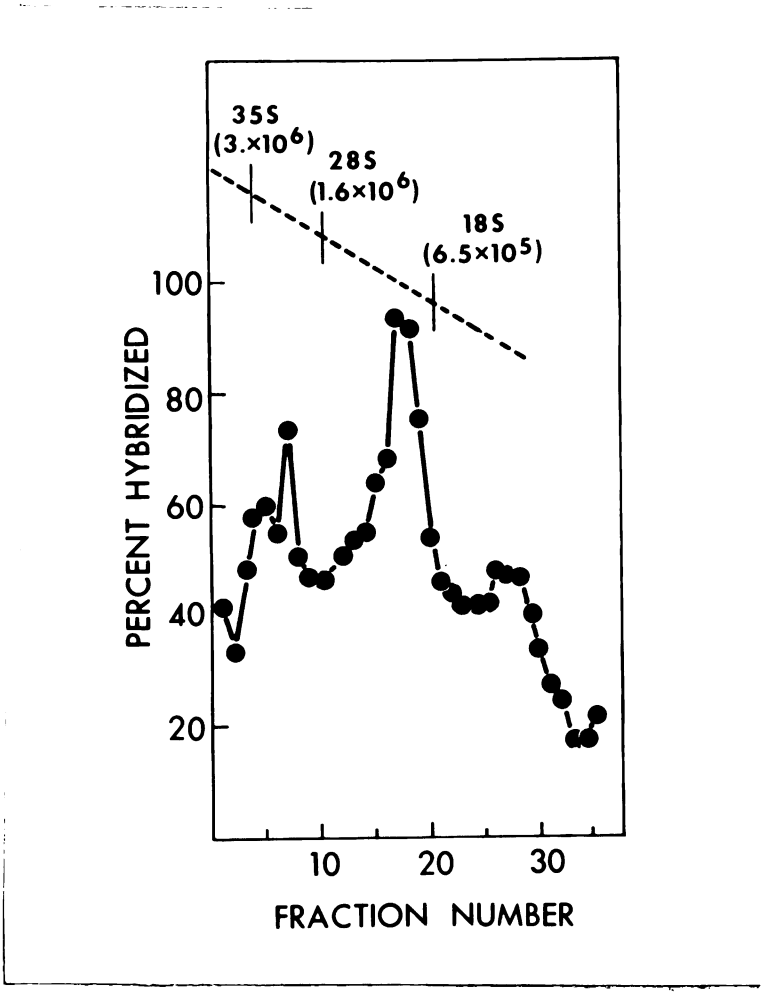

Fig.8. Rate-zonal sedimentation of ASV-specific RNA from transformed hamster cells (SR-3/1a). 2.1 mg of heat denatured (100°, 2 min) whole-cell RNA from SR-3/1a cells was centrifuged in 15%-30% sucrose gradients containing 0.01 M NaCl, 0.01 M EDTA, 0.02 M Tris-HCl, pH 7.4, and 0.5% SDS in a SW 27 rotor at 24,000 rpm for 18 hr at 20°. Fractions were collected from the bottom and the absorbance of internal rRNA markers at 260 nm was continuously recorded. ^{32}P-labeled ASV 35S RNA and unlabeled hamster rRNAs were centrifuged in a separate gradient to serve particularly as a 35S marker. ASV-specific RNA in hamster RNA was detected by incubation of 10% of RNA from each fraction with ^{32}P-labeled ASV cDNA (580 cpm/point, sp act 7.5 x 10^4 cpm/ng) in a 40 µl volume containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, and 0.001 M EDTA at 68° for 40 hr. Hybridization was measured by resistance of the cDNA to hydrolysis with S1 nuclease.

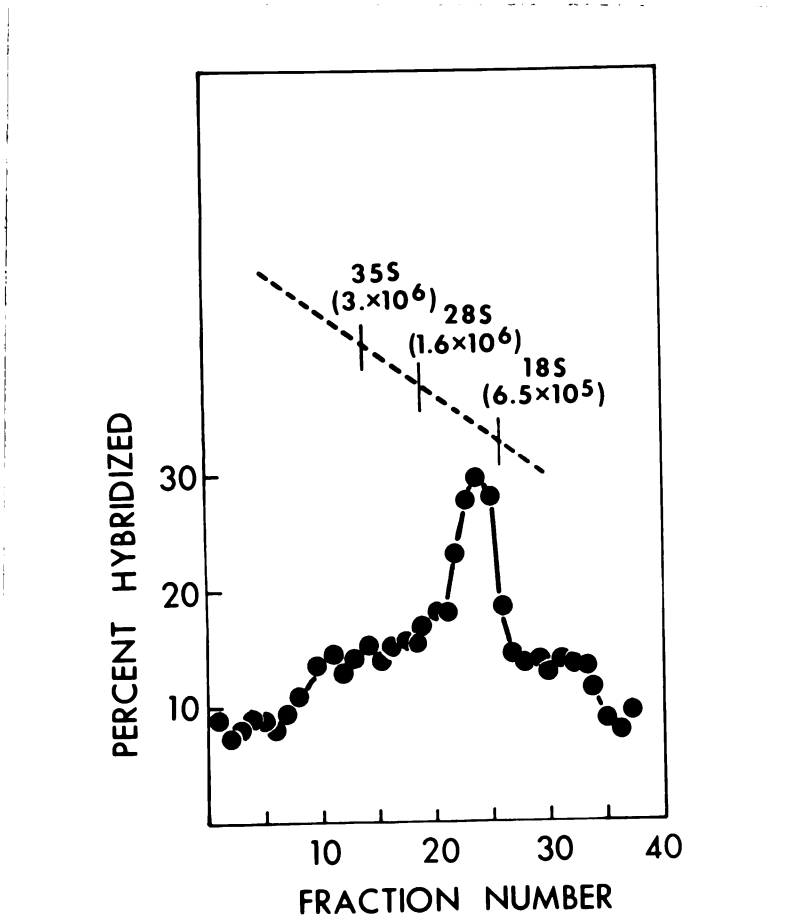

Fig.9. Rate-zonal sedimentation of ASV-specific RNA from reverted hamster cells (SR-3/5). 1.8 mg of heat denatured (100°, 2 min) whole-cell RNA from SR-3/5 cells was centrifuged in 15%-30% sucrose gradients containing 0.01 M NaCl, 0.01 M EDTA, and 0.02 M Tris-HCl, pH 7.4 in a SW 27 rotor at 19,000 rpm for 43 hr at 4°. Fractions were collected from the bottom and the absorbance of internal rRNA markers at 260 nm was continuously recorded. ASV-specific RNA in hamster RNA was detected by incubation of RNA from each fraction with ^{32}P-labeled ASV cDNA (700 cpm/point, sp act 4.4 x 10^4 cpm/ng) in a 40 µl volume containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, and 1 mM EDTA at 68° for 115 hr. Hybridization was measured as described in the legend for Fig. 8.

without any added detergents (Penman, 1969). In order to monitor the integrity of cytoplasmic virus-specific RNA in transformed hamster cells after the entire procedure for preparing cytoplasmic RNA, labeled 35S subunits of ASV RNA were added to a preparation of transformed hamster culture before the fractionation of the cells and subjected to the entire procedure. The RNAs thus obtained were then resolved by rate-zonal centrifugation in sucrose gradients. A comparison of Fig.10A & 10B shows that the integrity of added 35S viral RNA was not compromised by the procedure presumably. Cytoplasmic RNA from a transformed hamster clone (SR-3/1a) isolated in the same way as for the monitoring labeled 35S viral RNA contained only a 24S species of virus-specific RNA by polyacrylamide gel electrophoresis (Fig. 11). This 24S species of ASV RNA in the cytoplasm of transformed hamster cells is not an aggregate of smaller RNA species since heat denatured (80^{o} for 2 min) cytoplasmic RNA of SR-3/1a cells also demonstrated the presence of a 24S ASV RNA (Fig. 12). The heterogeneous virus-specific RNAs smaller than the 24S species in this experiment presumably represent the breakdown products of 24S RNA. Polyadenylated RNA selected by affinity chromatography on

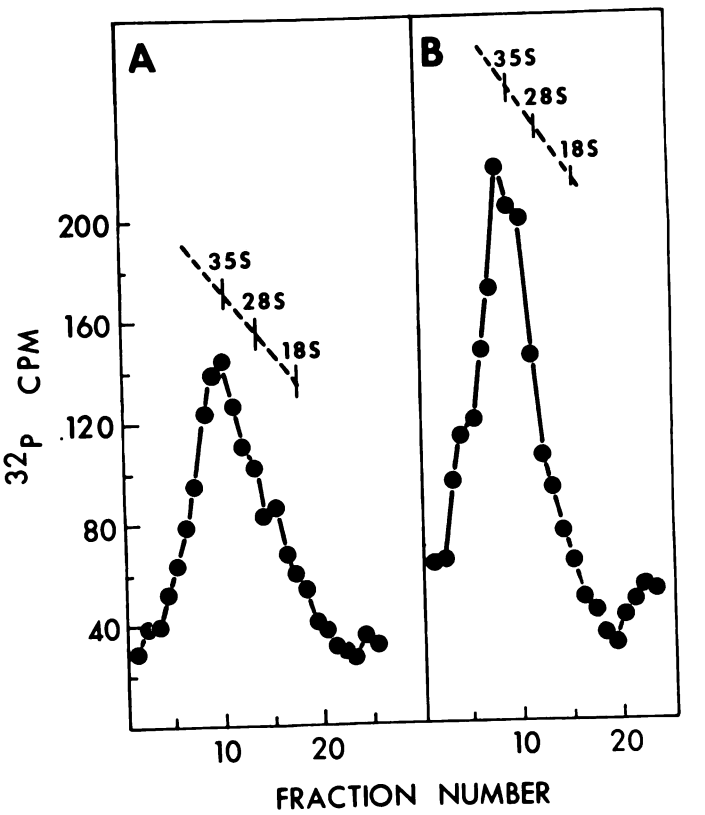

Fig.10. Effect of extraction procedure upon viral RNA. ^{32}P-labeled ASV RNA was prepared from the fresh harvest of labeled B77 strain of ASV. The RNA was heat dissociated and fractionated in a 15%-30% sucrose gradient. The 35S RNA was recovered from the gradient and precipitated with ethanol. 2,000 cpm of ^{32}P-labeled ASV 35S RNA was added to: (A) 5.3 x 10^7 hamster cells to detect the effect of the extraction procedure for cytoplasmic hamster RNA upon the labeled viral 35S RNA as described in methods; (B) 96 μg of unlabeled hamster rRNAs as the control for A. The RNAs were centrifuged in 15%-30% sucrose gradients containing 0.15 M NaCl, 0.01 M EDTA, and 0.02 M Tris-HCl, pH 7.4 in a SW 65 rotor at 64,000 rpm for 2.5 hr at 4°. Internal rRNA markers were detected as described in the legend for Fig. 8. ^{32}P-labeled 35S RNA was detected by cpm.

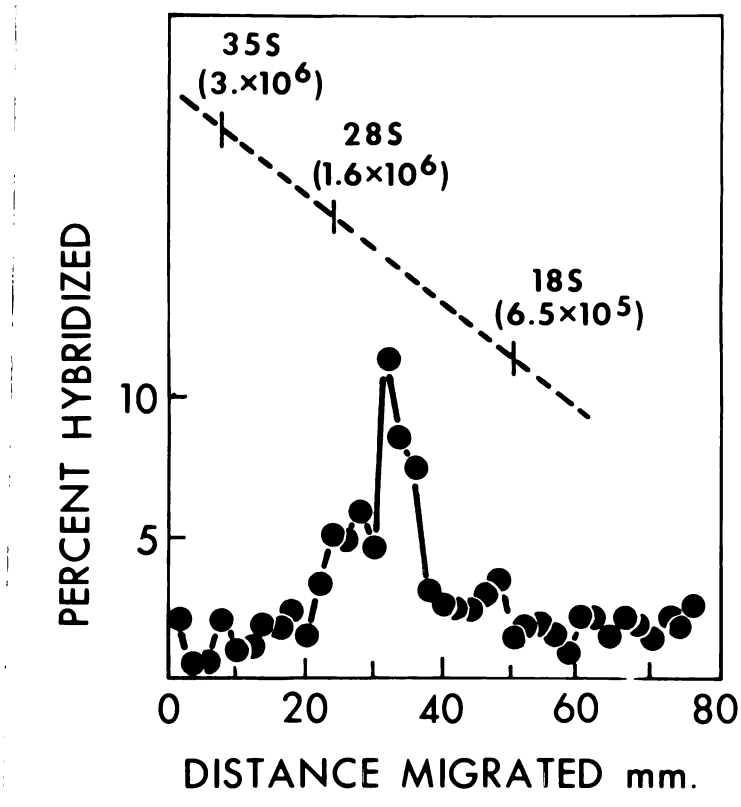

Fig.11. Electrophoretic mobility of ASV-specific RNA from the cytoplasm of transformed hamster cells (SR-3/1a). RNA was prepared from the cytoplasm of SR-3/1a cells and 96 μg was electrophoresed in a 2.3% polyacrylamide gel containing 0.23% ethylene diacrylate for 4 hr at 6 mA per gel. Internal rRNA markers were detected as described in the legend for Fig. 7. ASV-specific RNA was detected by incubation of ^{32}P-labeled ASV cDNA (600 cpm/point, sp act 6 x 10^4 cpm/ng) with each gel slice in a 150 μl volume containing 0.3 M NaCl, 12.5 mM Tris-HCl, pH 7.4, and 0.5 mM EDTA at 68° for 115 hr. Hybridization was measured by resistance of the cDNA to hydrolysis with S1 nuclease.

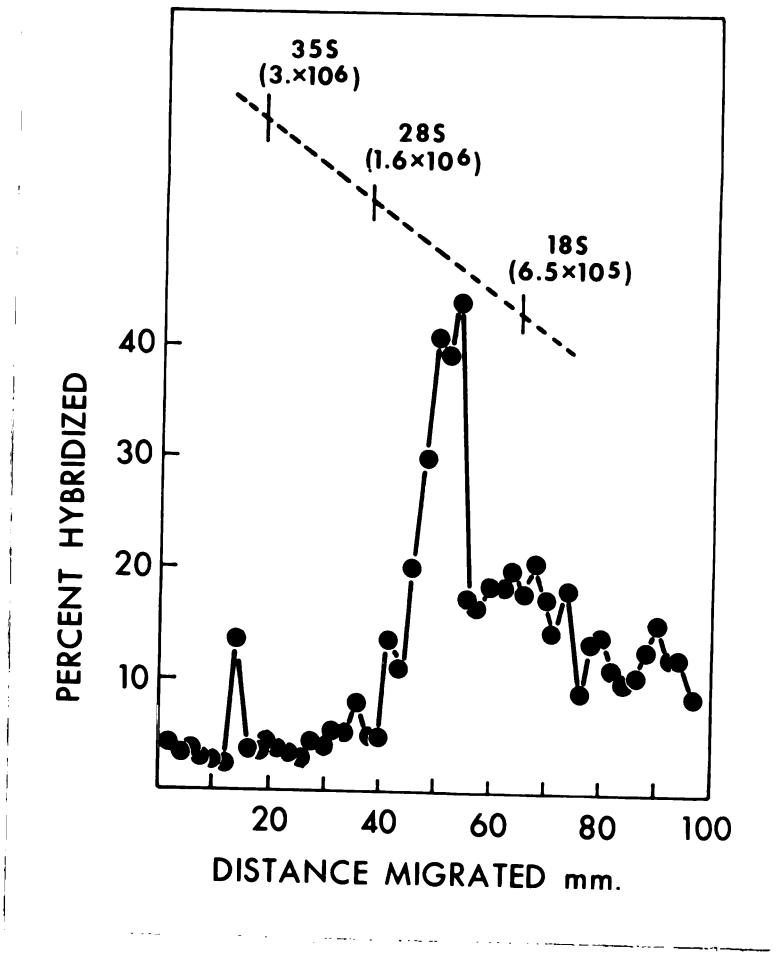

Fig.12. Electrophoretic mobility of ASV-specific RNA from the cytoplasm of transformed hamster cells (SR-3/1a). RNA was prepared from the cytoplasm of SR-3/1a cells and 96 μg was denatured (80°, 2 min) and electrophoresed in a 2.3% polyacrylamide gel containing 0.23% ethylene diacrylate for 4 hr at 6 mA per gel. Internal rRNA markers were detected as described in the legend for Fig. 7. ASV-specific RNA was detected by incubation of ^{32}P-labeled ASV cDNA (700 cpm/point, sp act 8.9 x 10^4 cpm/ng) with each gel slice in a 120 μl volume containing 0.3 M NaCl, 12.5 mM Tris-HCl, pH 7.4, and 0.5 mM EDTA at 68° for 161 hr. Hybridization was measured by resistance of the cDNA to hydrolysis with S1 nuclease.

oligo(dT)-cellulose column from the cytoplasm of transformed and reverted cells also included virus-specific 24S RNA (Fig. 13 & 14).

III.2.4. Association of virus-specific RNA with polyribosomes in transformed and reverted hamster cells.

It has been reported that virus-specific RNA is present in polyribosomes of infected permissive cells replicating RNA tumor viruses where it serves as messenger RNA for the synthesis of virus-coded proteins (Vecchio et al., 1973; Fan & Baltimore, 1973; Schincariol & Joklik, 1973).

Experiments were carried out to ask whether virus-specific RNA in nonpermissive hamster cells associates with polyribosomes. Polyribosomes isolated from transformed hamster cells (SR-3/1a) were fractionated by rate-zonal centrifugation in sucrose gradients. RNA was then extracted from each polyribosome fraction and annealed to a constant amount of ^{32}P-labeled ASV cDNA. ASV RNA was detected in all size classes of polyribosomes (Fig. 15). To eliminate the possibility that ribonucleoprotein aggregates contained the viral RNA detected in the polyribosome regions of these gradients, polyribosomes were also treated

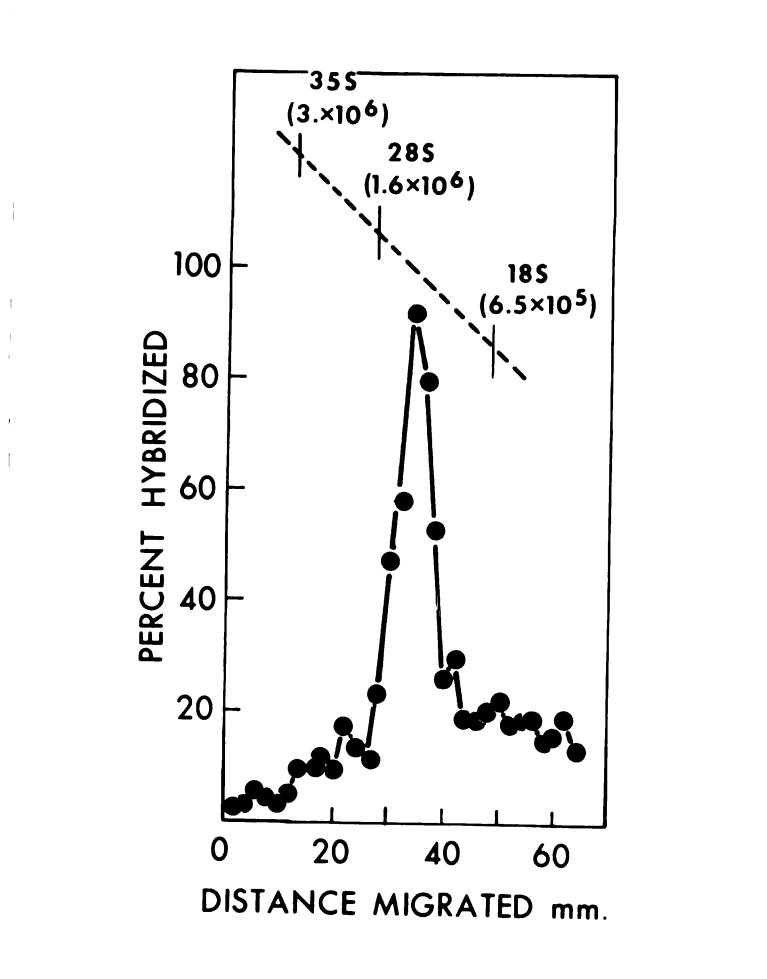

Fig.13. Electrophoretic mobility of polyadenylated ASV-specific RNA from the cytoplasm of transformed hamster cells. Polyadenylated cytoplasmic RNA from SR-3/1a cells was selected by affinity chromatography on oligo-(dT)-cellulose. The selected RNA (42 μg) was electrophoresed in a 2.3% polyacrylamide gel containing 0.23% ethylene diacrylate for 4 hr at 6 mA per gel. The residual rRNAs (as contaminants in polyadenylated RNA) were detected as described in the legend for Fig. 7. ASV-specific RNA was detected by incubation of each gel slice with ^{32}P-labeled ASV cDNA (600 cpm/point, sp act 6 x 10^4 cpm/ng) in a 100 μl volume containing 0.15 M NaCl, 12.5 mM Tris-HCl, pH 7.4, and 0.5 mM EDTA at 68° for 16 hr. Hybridization was measured by resistance of the cDNA to hydrolysis with S1 nuclease.

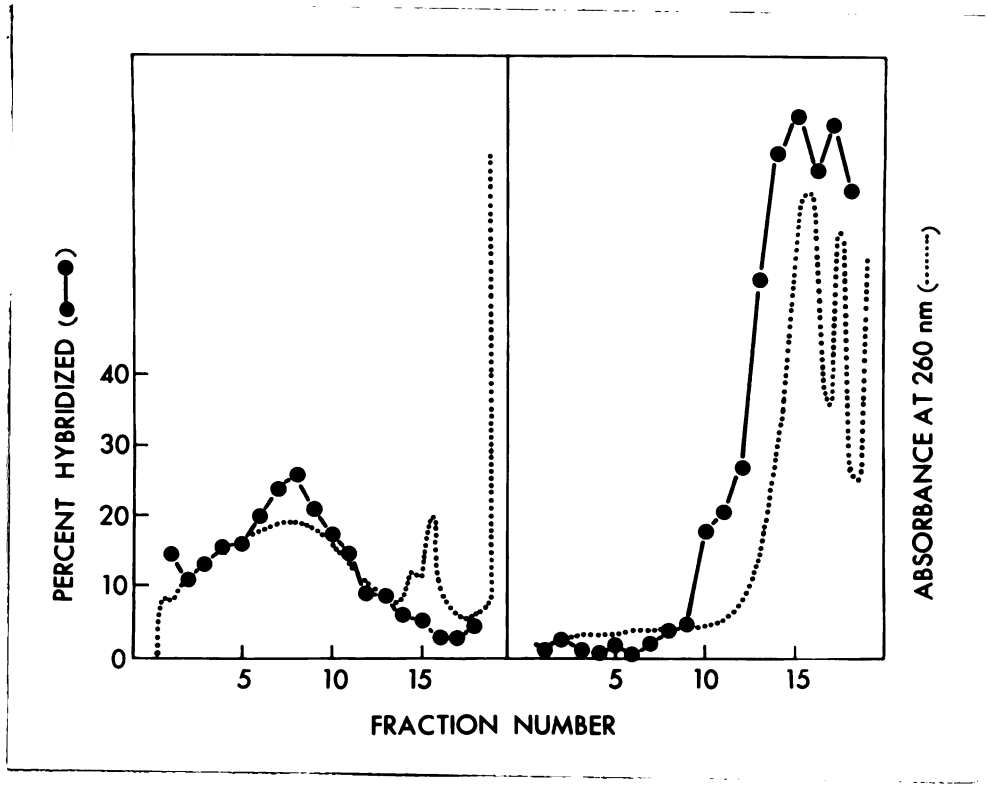

Fig.15. Detection of ASV-specific RNA associated with polyribosomes in transformed hamster cells. Pelleted polyribosomes from SR-3/1a cells were prepared as described in methods. A volume of 0.45 ml polyribosomes containing 44 units of absorbance at 260 nm in 25 mM NaCl, 25 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$ was divided into two equal portions: one portion with no EDTA treatment (left panel); and the other portion adjusting to 25 mM EDTA (right panel). The samples were sedimented in 10%-40% sucrose gradients containing 25 mM NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 100 μg/ml heparin in a SW 41 rotor at 36,000 rpm for 75 min at 4°. Fractions were collected from the bottom and recorded continuously for absorbance at 260 nm. ASV-specific RNA was detected by incubation of 50% RNA from each fraction with ^{32}P-labeled ASV cDNA (500 cpm/point, sp act 5.4×10^4 cpm/ng) in a 50 μl volume containing 0.6 M NaCl, 25 mM Tris-HCl, pH 7.4, and 1 mM EDTA at 68° for 90 hr. Hybridization was measured by resistance of the cDNA to hydrolysis with S1 nuclease.

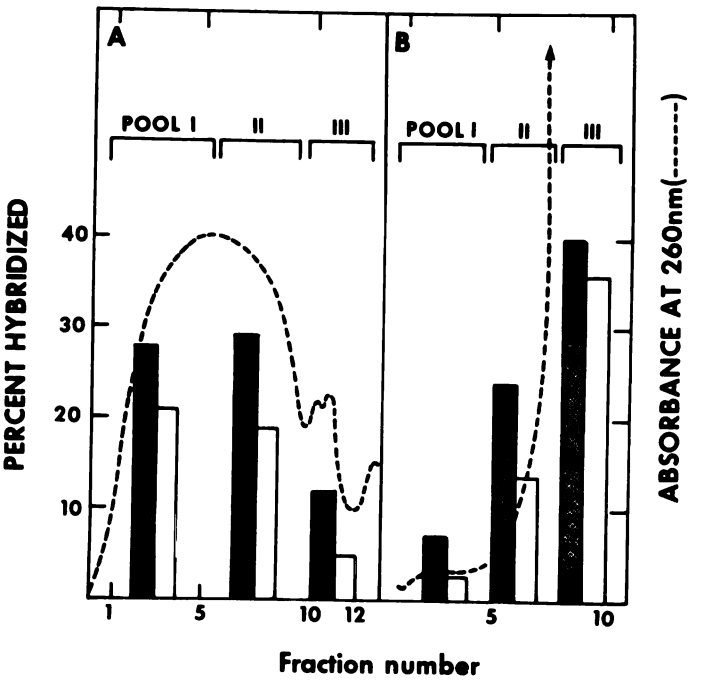

Fig.16. Detection of the Sarc transcript and of ASV-specific RNA associated with polyribosomes in reverted hamster cells. Pelleted polyribosomes from SR-3/5 cells were prepared as described in methods. A volume of 0.75 ml polyribosomes containing 160 units of absorbance at 260 nm in 25 mM NaCl, 25 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$ was divided into two equal portions: one portion with no EDTA treatment (A); and the other portion adjusting to 25 mM EDTA (B). The samples were sedimented in 10%-40% sucrose gradients containing 25 mM NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 100 μg/ml heparin in a SW 41 rotor at 36,000 rpm for 75 min at 4°. Fractions were collected as described in the legend for Fig. 15. RNA was extracted separately from the pooled fractions as indicated. 50% of the RNA from each pool was incubated with ^{3}H-labeled $cDNA_{sarc}$ (solid bar, 750 cpm/sample, sp act 2×10^4 cpm/ng) and ^{32}P-labeled ASV cDNA (open bar, 700 cpm/sample, sp act 9×10^4 cpm/ng) in a 15 μl volume containing 0.6 M NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM EDTA at 68° for 141 hr. Hybridizations were measured by resistance of the cDNAs to hydrolysis with S1 nuclease.

with 0.025 M EDTA, a procedure that dissociates polyribosomes and releases messenger RNA (Perry & Kelley, 1968), before sucrose gradient centrifugation. EDTA treatment released most of the viral RNA from polyribosomes, so that it migrated slowly in the gradients (Fig. 15).

To determine whether the reversion phenomenon could be ascribed to the absence of viral messenger RNA in reverted hamster cells, polyribosomes isolated from a reverted hamster clone (SR-3/5) were subjected to the same procedure for detection of virus-specific messenger RNA. As shown in Fig. 16, ASV RNA was again detected in polyribosome regions and was released by EDTA treatment.

III.2.5. The transcription of the viral transforming gene in transformed and reverted hamster cells.

"Sarc" nucleotide sequences represent the portion of the ASV genome present in the RNA of avian sarcoma viruses but absent from the RNA of transformation-defective (td) deletion mutants of ASV (Duesberg & Vogt, 1970; Vogt, 1971; Martin & Duesberg, 1972; Duesberg & Vogt, 1973a, b). The amount of the deletion in td ASV is 10-20% of the genetic information in the parental transforming ASV (Duesberg & Vogt, 1973b; Lai et al., 1973; Neiman et al.,

1974). Radioactive single-stranded DNA complementary to Sarc sequences has been selected and denoted as $cDNA_{sarc}$ by Stehelin et al. (1976). $cDNA_{sarc}$ is a relatively uniform transcript from 16% of the nucleotide sequences in the genome of ASV and thus represents the entire deletion (Stehelin et al., 1976). Consequently $cDNA_{sarc}$ can be used as annealing probe to detect the expression of most or all of the ASV transforming gene.

In order to ask whether RNA from ASV-transformed hamster cells contains Sarc sequences, $cDNA_{sarc}$ was employed in the hybridization analysis. As shown in Fig. 17, polyadenylated 24S ASV RNA from the cytoplasm of a transformed clone (SR-3/1a) hybridized with ^{3}H-labeled $cDNA_{sarc}$ and ^{32}P-labeled ASV cDNA.

It is possible that the reversion phenomenon correlates with the failure to express Sarc sequences. To test this possibility, RNA was extracted from polyribosomes of reverted hamster cells (SR-3/5) and hybridized with $cDNA_{sarc}$. As shown in Fig. 16A, RNA extracted from polyribosome regions (pool I and pool II) hybridized with ^{3}H-labeled $cDNA_{sarc}$ and with ^{32}P-labeled ASV cDNA to a greater extent than RNA extracted from the region where ribosomal

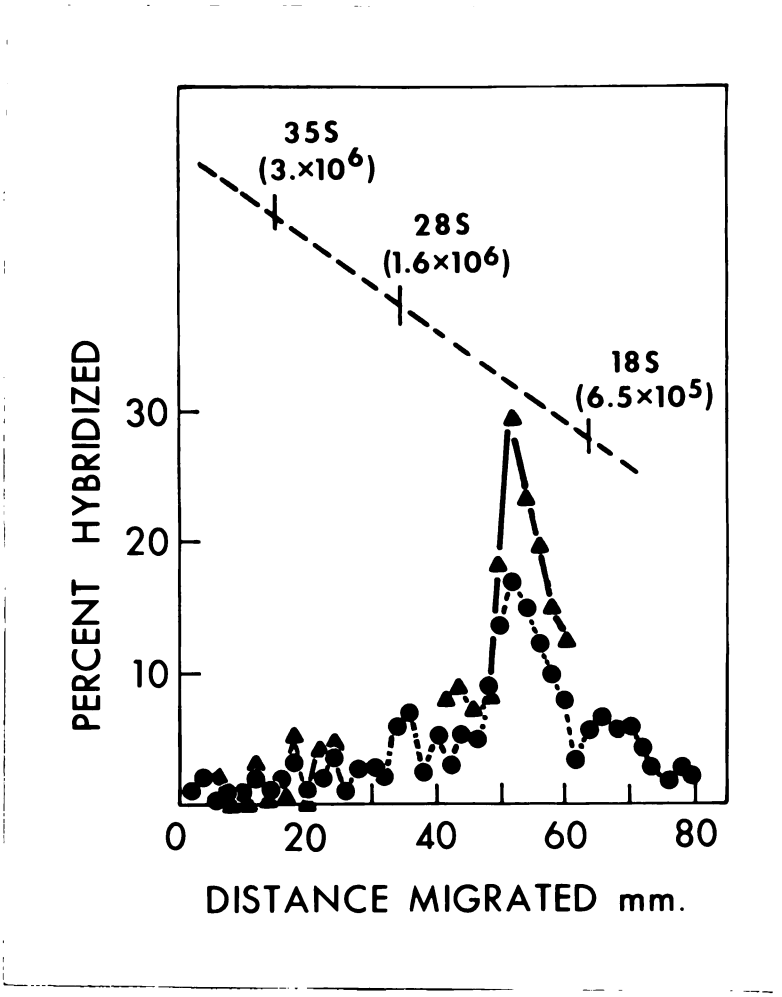

Fig.17. Electrophoretic mobility of RNA containing Sarc sequences from the cytoplasm of transformed hamster cells. Polyadenylated cytoplasmic RNA from SR-3/1a cells was selected by affinity chromatography on oligo-(dT)-cellulose. 28 μg of the polyadenylated RNA was electrophoresed in a 2.3% polyacrylamide gel containing 0.23% ethylene diacrylate for 4 hr at 6 mA per gel. The residual rRNAs (as contaminants in polyadenylated RNA) were detected as described in the legend for Fig. 7. The Sarc-containing and ASV-specific RNA was detected by incubation of each gel slice with ^{3}H-labeled $cDNA_{sarc}$ (▲ , 300 cpm/point, sp act 2 x 10^4 cpm/ng) and ^{32}P-labeled ASV cDNA (● , 500 cpm/point, sp act 6.9 x 10^4 cpm/ng) in a 100 μl volume containing 0.3 M NaCl, 12.5 mM Tris-HCl, pH 7.4, and 0.5 mM EDTA at 68° for 162 hr. Hybridizations were measured by resistance of the cDNAs to hydrolysis with S1 nuclease.

subunits sediment (pool III). To verify the fact that the RNA which hybridized with $cDNA_{sarc}$ was actually messenger RNA, RNA released from polyribosomes with EDTA was also hybridized with ^{3}H-labeled $cDNA_{sarc}$ and ^{32}P-labeled ASV cDNA. As shown in Fig. 16B, RNA released from polyribosomes annealed with $cDNA_{sarc}$ and ASV cDNA. Furthermore, polyribosomal RNA of reverted hamster cells (SR-3/5) hybridized with at least 60% of $cDNA_{sarc}$ as compared with at least 80% hybridization of $cDNA_{sarc}$ with polyribosomal RNA of transformed hamster cells (SR-3/1a) (Fig. 18A). These results render unlikely the possibility that the reversion phenomenon is the consequence of a failure to express Sarc sequences in reverted hamster cells.

However, the gene dosage effect may be important in determining the phenotype of infected hamster cells as shown in Fig. 18; the concentration of Sarc transcript in polyribosome-associated RNA was about 10-fold greater in SR-3/1a (transformed clone) than in SR-3/5 (reverted clone). This is suggested by the 10-fold difference in $Crt_{1/2}$'s of hybridization of the RNA with ^{3}H-labeled $cDNA_{sarc}$ (Fig.18A). Similarly, polyribosomal ASV RNA detected by hybridization with ^{32}P-labeled ASV cDNA was 10-fold greater in SR-3/1a

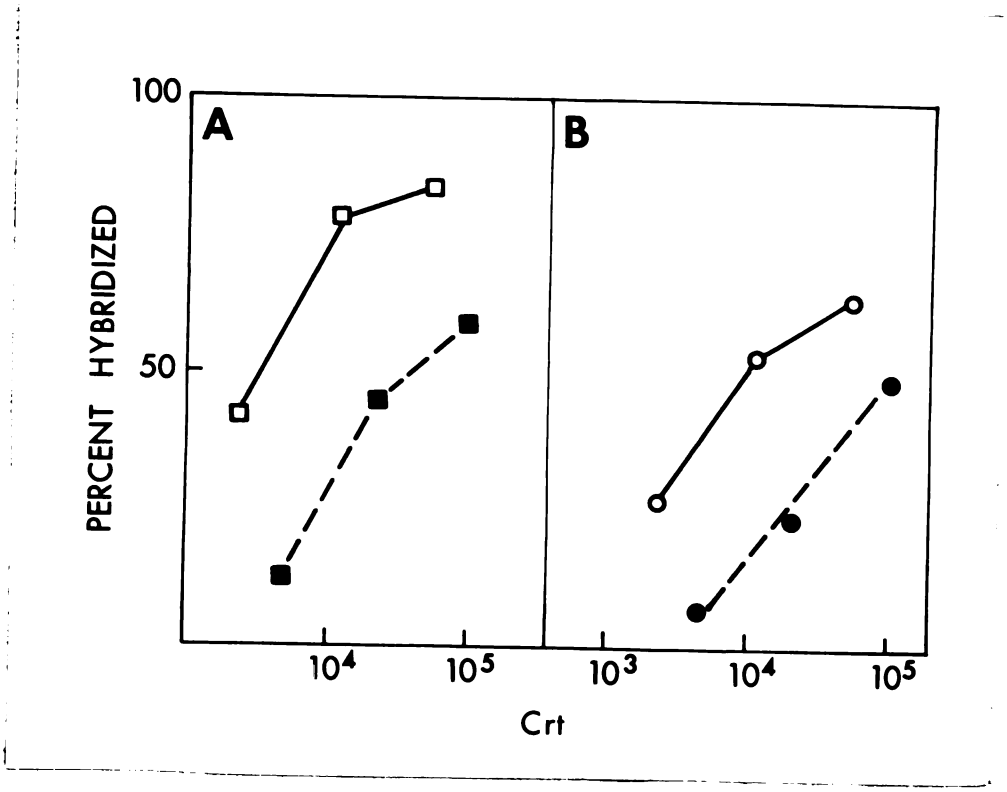

Fig.18. Concentration of Sarc-containing RNA (A) and ASV-specific RNA (B) in polyribosomal RNA from transformed (□ , ○) and reverted (■ , ●) hamster cells. RNA was extracted from pelleted polyribosomes of SR-3/1a and SR-3/5 cells as described in methods. ^{3}H-labeled cDNA$_{sarc}$ (□ , ■ ; 455 cpm/sample, sp act 2 x 10^4 cpm/ng) and ^{32}P-labeled ASV cDNA (○ , ● ; 530 cpm/sample, sp act 6.3 x 10^4 cpm/ng) were incubated with increasing quantities of polyribosomal RNA in a 40 µl volume containing 0.6 M NaCl, 25 mM Tris-HCl, pH 7.4, and 1 mM EDTA at 68° for 138 hr. Hybridizations were measured by resistance of the cDNAs to hydrolysis with S1 nuclease. The percentage of labeled cDNA hybridized was plotted logarithmically against Crt (concentration of RNA x time of incubation).

(transformed clone) than in SR-3/5 (reverted clone) (Fig. 18B).

Chapter 3. Complexity of Virus-Specific RNA in Avian Sarcoma Virus-Transformed BHK-21 Cells and Revertants.

III.3.1. Characteristics of labeled representative cDNA ($cDNA_{rep}$).

Single-stranded DNA synthesized in vitro by avian sarcoma viruses in the presence of actinomycin D (ASV cDNA) is not uniformly representative of the viral genome. This is indicated by the limited hybridization of ^{32}P-labeled ASV 70S RNA with ^{3}H-labeled ASV cDNA at unit mass ratio of cDNA to RNA; however, viral 70S RNA completely hybridizes with ASV cDNA at a greater than 10:1 sequence excess of cDNA over RNA (Garapin et al., 1973). Therefore, to determine the complexities of virus-specific RNAs in hamster cells, a population of cDNA's uniformly representing the viral genome ($cDNA_{rep}$) was prepared. $cDNA_{rep}$ was selected from a large quantity of ^{3}H-labeled ASV cDNA by hybridization with a limited amount of ASV 70S RNA. Since ASV 70S RNA employed for the selection contained 2-fold more transformation-defective (td) viral genomes than non-defective avian sarcoma virus genomes, $cDNA_{rep}$ presumably

was about 3-fold deficient in cDNA complementary to the region deleted in the formation of td mutants ($cDNA_{sarc}$). However, I have independently tested RNA in these cells for hybridization with $cDNA_{sarc}$ (see III.2.5). To test the quality of $cDNA_{rep}$, ^{32}P-labeled B77 70S RNA (containing a 10:1 ratio of td B77 to B77 RNA) was hybridized with ^{3}H-labeled $cDNA_{rep}$ at various mass ratio of DNA:RNA. As shown in Fig. 19, 80% of ^{32}P-labeled 70S RNA was rendered resistant to pancreatic and T1 RNase digestion after hybridization with $cDNA_{rep}$ at DNA:RNA ratio of 1.2; the resistance increased to 93% at DNA:RNA ratio of 9. (Prior to the selection of $cDNA_{rep}$, the same preparation of ASV cDNA hybridized only 40% of labeled 70S RNA at a DNA:RNA ratio of 10.) In order to test whether $cDNA_{rep}$ can detect the reduced complexity of viral RNA in cells infected by a deletion mutant, $cDNA_{rep}$ was hybridized with the excess amount of R(-)Q RNA isolated from quail cells infected with Bryan strain of RSV and producing noninfectious RSV(-) virus. This virus does not contain envelope glycoproteins due to a 20% deletion of the viral genome affecting the <u>env</u> gene (Kawai & Hanafusa, 1973; Duesberg et al., 1975). Only 80% of $cDNA_{rep}$ hybridized with R(-)Q

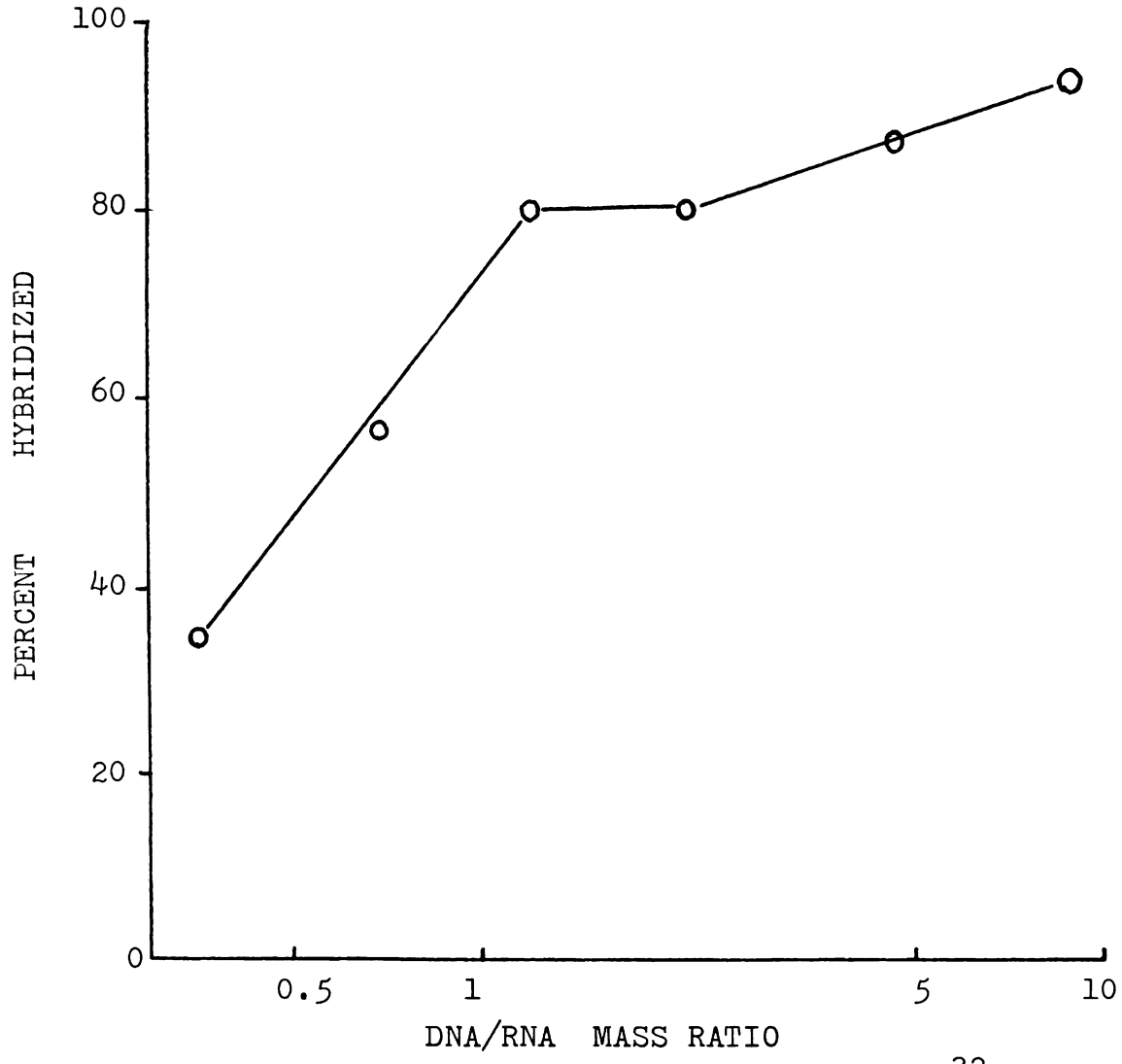

Fig.19 Detection of the extent of hybridization of ^{32}P-labeled ASV 70S RNA with ^{3}H-labeled cDNA$_{rep}$. cDNA$_{rep}$ was selected from ^{3}H-labeled ASV cDNA (sp act 2 x 10^{4} cpm/ng) by hybridization with a 200-fold less amount of ASV 70S RNA as described in methods. 3,500 cpm of ^{32}P-labeled B77 70S RNA (sp act 8,750 cpm/ng) was incubated with increasing quantities of cDNA$_{rep}$ in a 5 µl volume containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, and 0.001 M EDTA in a sealed capillary pipette at 68° for 88 hr. After incubation, the mixture was adjusted to 0.5 M NaCl, 0.01 M EDTA and 0.025 M Tris-HCl, and incubated with 50 µg/ml pancreatic and 10 µg/ml T1 RNase at 37° for 30 min. The percentage of RNA resistant to RNase digestion was plotted logarithmically against the mass ratio of DNA to RNA.

RNA whereas 99% of $cDNA_{rep}$ hybridized with cytoplasmic RNA of ASV-transformed chick cells (Fig. 20) and 100% with ASV 70S RNA (Fig. 21). The results suggest that $cDNA_{rep}$ is a reliable hybridization reagent for measuring the complexity of ASV-specific RNA in hamster cells.

III.3.2. The extent of the expression of proviral DNA in ASV-transformed hamster cells and revertant.

I have used $cDNA_{rep}$ to attempt to answer two questions about the extent of the expression of proviral DNA in hamster cells: (1) is the sequence content of viral RNA important in determining the phenotype of ASV-infected mammalian cells? and (2) is the nonpermissiveness of mammalian cells for replication of ASV based upon incomplete expression of the viral genome as RNA? Since $cDNA_{rep}$ seems to be a reliable hybridization reagent to measure the complexity of viral RNA (see III.3.1), it was employed to measure the extent of expression of proviral DNA in reverted and transformed hamster cells. About 95% of ^{3}H-labeled $cDNA_{rep}$ and of ^{32}P-labeled ASV cDNA hybridized with whole-cell RNA from a transformed hamster clone (SR-3/1a) (Fig. 22); a similar but incomplete analysis of whole-cell RNA from a reverted hamster clone (SR-3/5)

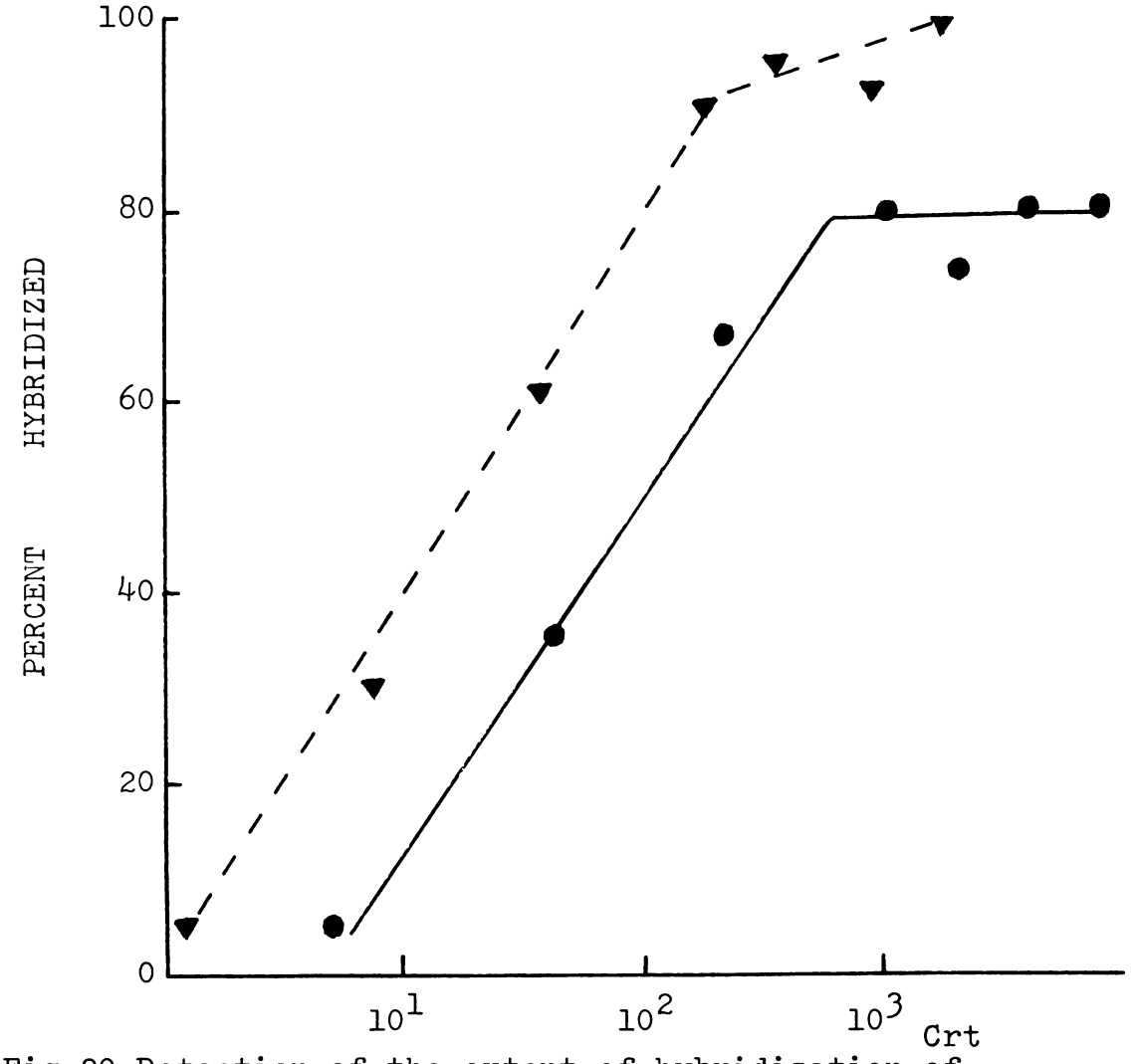

Fig.20 Detection of the extent of hybridization of ^{3}H-labeled $cDNA_{rep}$ with RNA from R(-)Q cells. RNA was extracted from unfractionated quail cells producing replication-defective RSV(-) virus (●) and from cytoplasmic RNA of SR-ASV infected chick cells (▼). ^{3}H-labeled $cDNA_{rep}$ (700 cpm/sample, sp act 2 x 10^4 cpm/ng) was incubated with increasing quantities of RNA in a 40 µl volume containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, and 0.001 M EDTA at 68° for 137 hr. Hybridization was measured by resistance of the cDNA to hydrolysis with S1 nuclease. The percentage of labeled cDNA hybridized was plotted logarithmically against Crt (concentration of RNA x time of incubation).

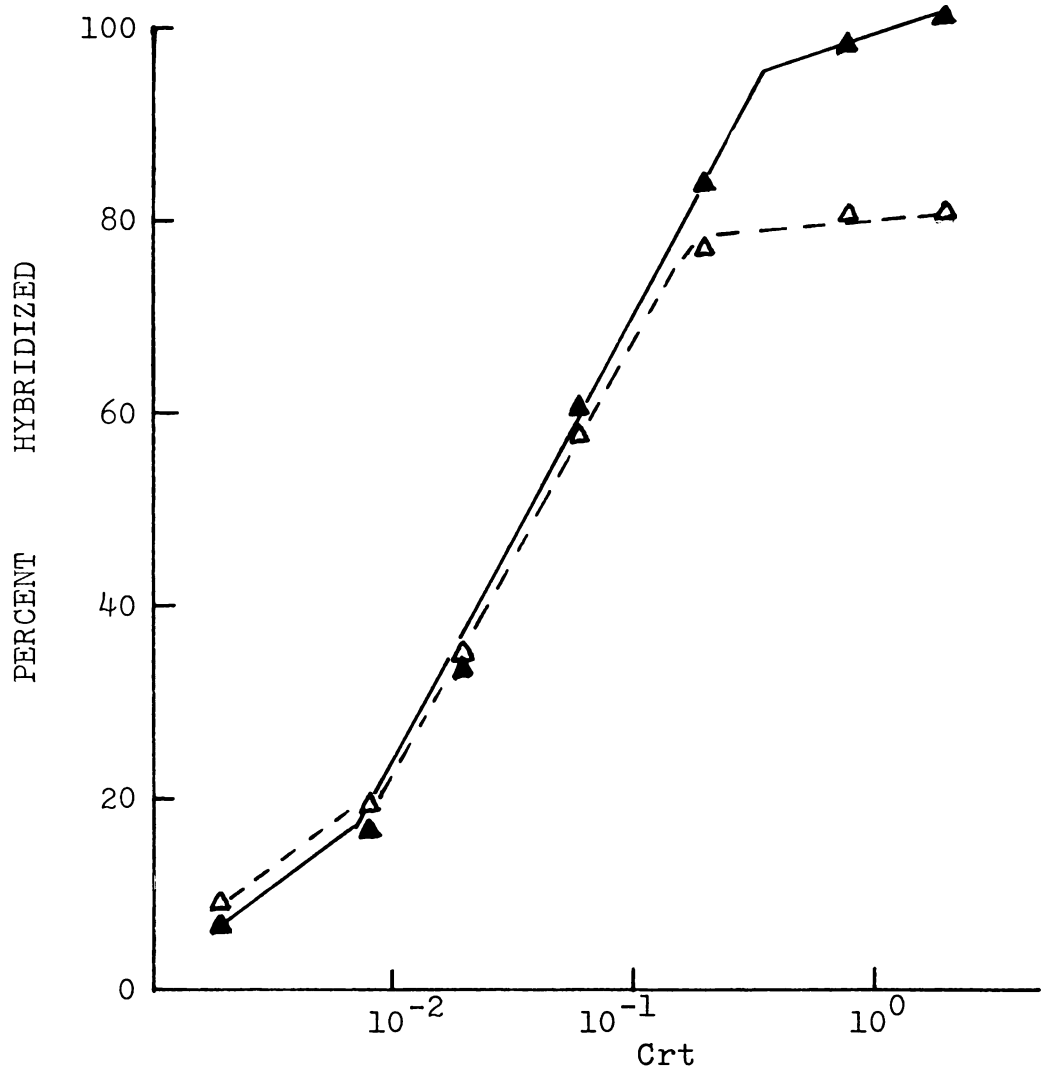

Fig.21 Complete hybridization of ^{3}H-labeled cDNA$_{rep}$ with homologous ASV 70S RNA. RNA was extracted from the same preparation of PrC ASV used for the in vitro synthesis and selection for cDNA$_{rep}$ as described in methods. 550 cpm of ^{3}H-labeled cDNA$_{rep}$ (▲) and 500 cpm of ^{32}P-labeled ASV cDNA (Δ , sp act 8.7 x 10^{4} cpm/ng) were incubated with increasing quantities of PrC 70S RNA in a 45 µl volume containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, 0.001 M EDTA at 68° for 19 hr. Hybridizations were measured as described in the legend for Fig. 19.

complexity of viral RNA is affected in reverted cells. However, restricted expression of the Sarc nucleotide sequences would be most likely to produce the reversion phenomenon, and I have shown directly (with $cDNA_{sarc}$) that those sequences are present in RNA from reverted cells (see III.2.5).

III.3.3. Complexities of 35S and 24S virus-specific RNA in ASV-transformed hamster cells.

Since proviral DNA appears to be completely transcribed into RNA in ASV-transformed hamster cells, I attempted to determine whether some viral sequences are confined to nuclear RNA. ^{3}H-labeled $cDNA_{rep}$ and ^{32}P-labeled ASV cDNA were hybridized with increasing quantities of cytoplasmic or nuclear RNA from a transformed hamster clone (SR-3/1a) (Fig. 24). ^{3}H-labeled $cDNA_{rep}$ hybridized 4-fold slower than ^{32}P-labeled ASV cDNA with the cytoplasmic RNA; whereas both cDNA's hybridized at about equal rates with the nuclear RNA. Furthermore, 90-100% of both cDNA's hybridized with the cytoplasmic or nuclear RNA. Whole-cell RNA contains 35S and 24S ASV RNA, whereas the cytoplasmic RNA contains only the 24S species (see III.2.3); to analyze the significance of the annealing reactions in

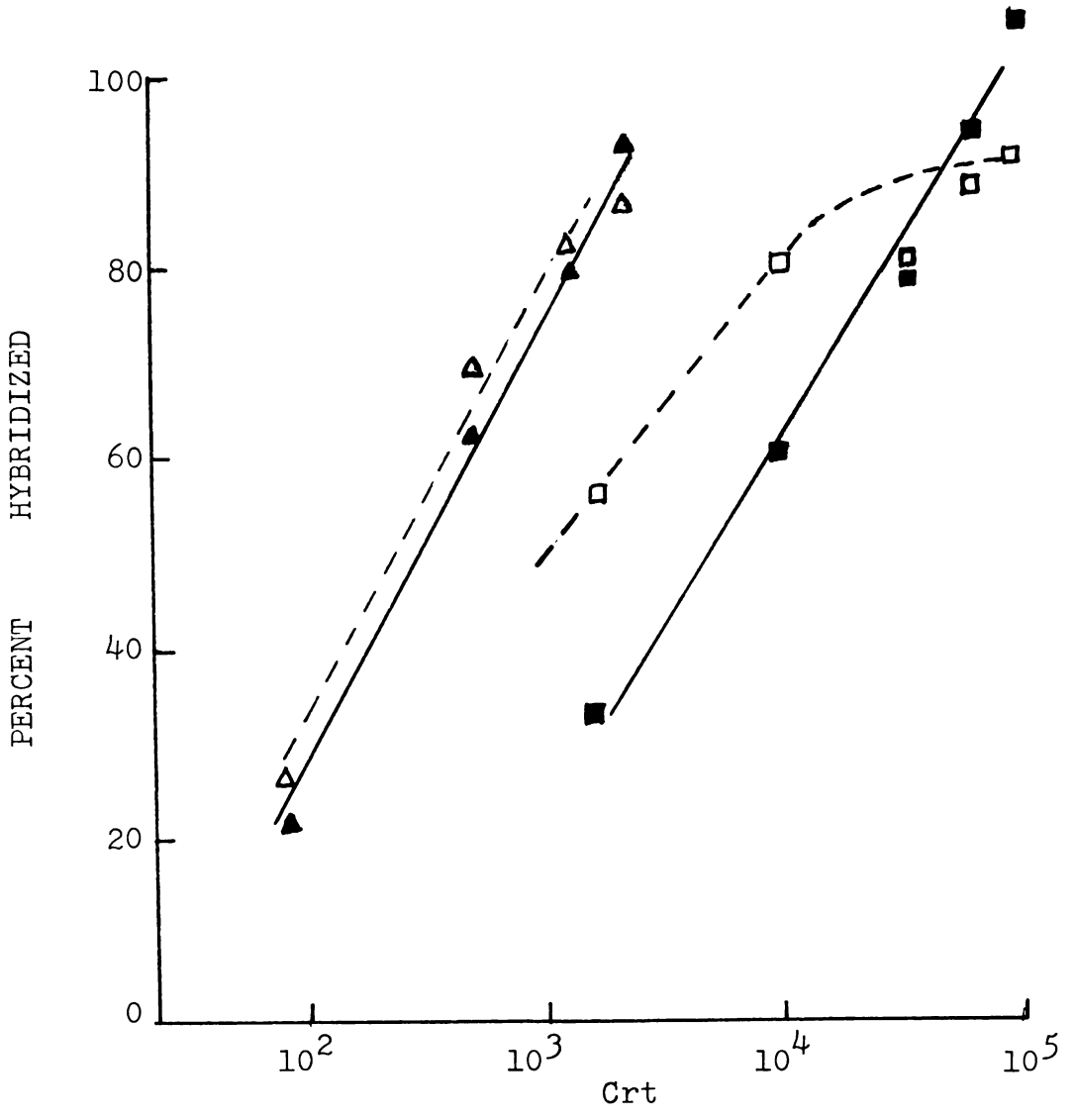

Fig.24. Comparison of the complexity of viral RNA from nuclei with that from cytoplasm of transformed hamster cells by hybridization analysis with ^{3}H-labeled cDNA$_{rep}$ and ^{32}P-labeled ASV cDNA. SR-3/1a cells were fractionated into nuclei and cytoplasm by Dounce homogenization as described in methods. RNA was extracted from both fractions. 600 cpm of ^{3}H-labeled cDNA$_{rep}$ (▲ , ■) and of ^{32}P-labeled ASV cDNA (Δ , □ ; sp act 8 x 10^4 cpm/ng) were incubated with increasing quantities of RNA in a 40 µl reaction containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, 0.001 M EDTA at 68° for 91 hr. Hybridizations were measured as described in the legend for Fig. 19. Nuclear RNA (Δ , ▲); cytoplasmic RNA (□ , ■).

showed that at least 40% of ^{3}H-labeled $cDNA_{rep}$ was hybridized (Fig. 23A). Since the amount of viral RNA in reverted hamster cells is small, the failure to anneal $cDNA_{rep}$ completely may have been due to an inadequate ratio of RNA:DNA in the annealing reaction; to enrich the viral RNA in the RNA from reverted cells, polyadenylated RNA was selected by affinity chromatography with oligo(dT)-cellulose as described above (see III.2.1). Polyadenylated RNA was enriched 10-fold for viral RNA as shown by a 10-fold decrease in Crt values required for annealing with $cDNA_{rep}$ (Fig. 23), nevertheless, the amount of polyadenylated viral RNA was still inadequate to reach a plateau hybridization with $cDNA_{rep}$. Therefore, it is not clear whether the entire proviral DNA is transcribed into viral RNA in reverted hamster cells. On the other hand, proviral DNA in a transformed hamster clone (SR-3/1a) seems fully transcribed into viral RNA (Fig. 22) suggesting that, within the limits of the assay, the restriction of the replication of ASV in hamster cells appears not to be at the level of the transcriptional control of proviral DNA. In view of the inconclusive results with $cDNA_{rep}$ and RNA from reverted cells, I have not been able to determine whether the

Fig. 24, I performed similar analyses with 35S and 24S viral RNA. These species of viral RNA were pooled from rate-zonal sucrose gradients of whole-cell RNA from a transformed hamster clone (SR-3/1a). $cDNA_{rep}$ and ASV cDNA had the same pattern of hybridization with the 24S viral RNA (Fig. 25B) from whole cells as observed with the cytoplasmic RNA (Fig. 24); in addition, both cDNA's hybridized at equal rates with the 35S viral RNA (Fig. 25A) as they did with nuclear RNA (Fig. 24) and with PrC 70S RNA (Fig. 21).

Since ASV cDNA is undefined with respect to the portion of the viral RNA it detects, experiments similar to those described above were carried out with a hybridization reagent specific for the 100-200 nucleotides adjacent to the poly(A) sequences at the 3' end of the viral genome ($cDNA_{3'}$) (Tal et al., manuscript in preparation). ^{32}P-labeled $cDNA_{3'}$ hybridized 2- to 3-fold faster than ^{3}H-labeled $cDNA_{rep}$ with the 24S viral RNA (Fig. 26), whereas both cDNA's hybridized at about the same rates with the 35S viral RNA (Fig. 27). Previously I showed that $cDNA_{sarc}$ hybridized slightly faster than ASV cDNA with cytoplasmic RNA from ASV-transformed hamster cells

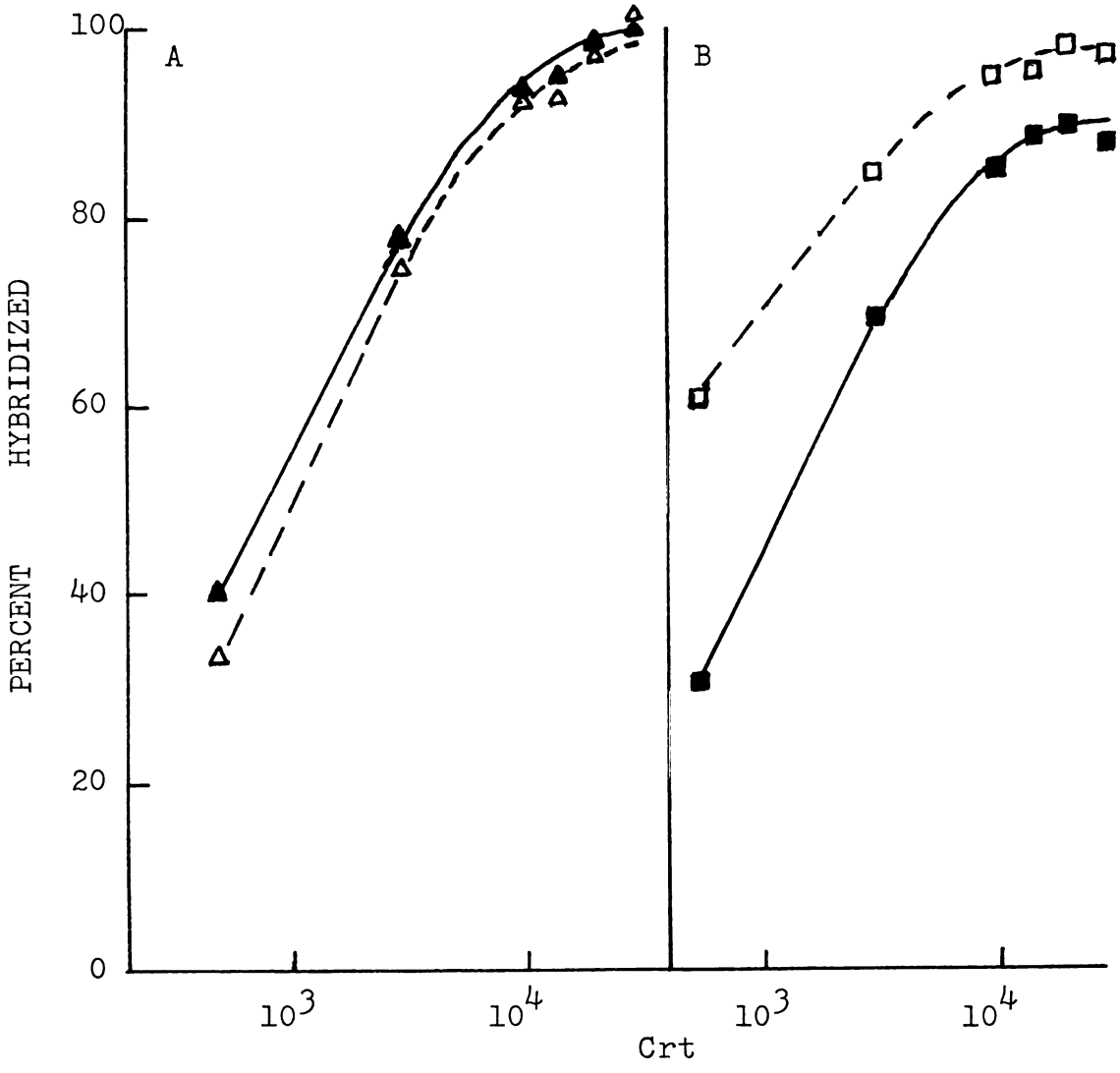

Fig.25. Comparison of the complexity of 35S ASV RNA (A) with that of 24S ASV RNA (B) from whole-cell RNA of transformed hamster cells by hybridization analysis with ^{3}H-labeled cDNA$_{rep}$ and ^{32}P-labeled ASV cDNA. RNA was extracted from unfractionated SR-3/1a cells and sedimented in rate-zonal sucrose gradients (as in Fig. 8). The 35S and 24S species of viral RNA were pooled from the gradients and precipitated with ethanol. ^{3}H-labeled cDNA$_{rep}$ (▲ , ■) and ^{32}P-labeled ASV cDNA (Δ , □) were incubated with increasing quantities of RNA as described in the legend for Fig. 23.

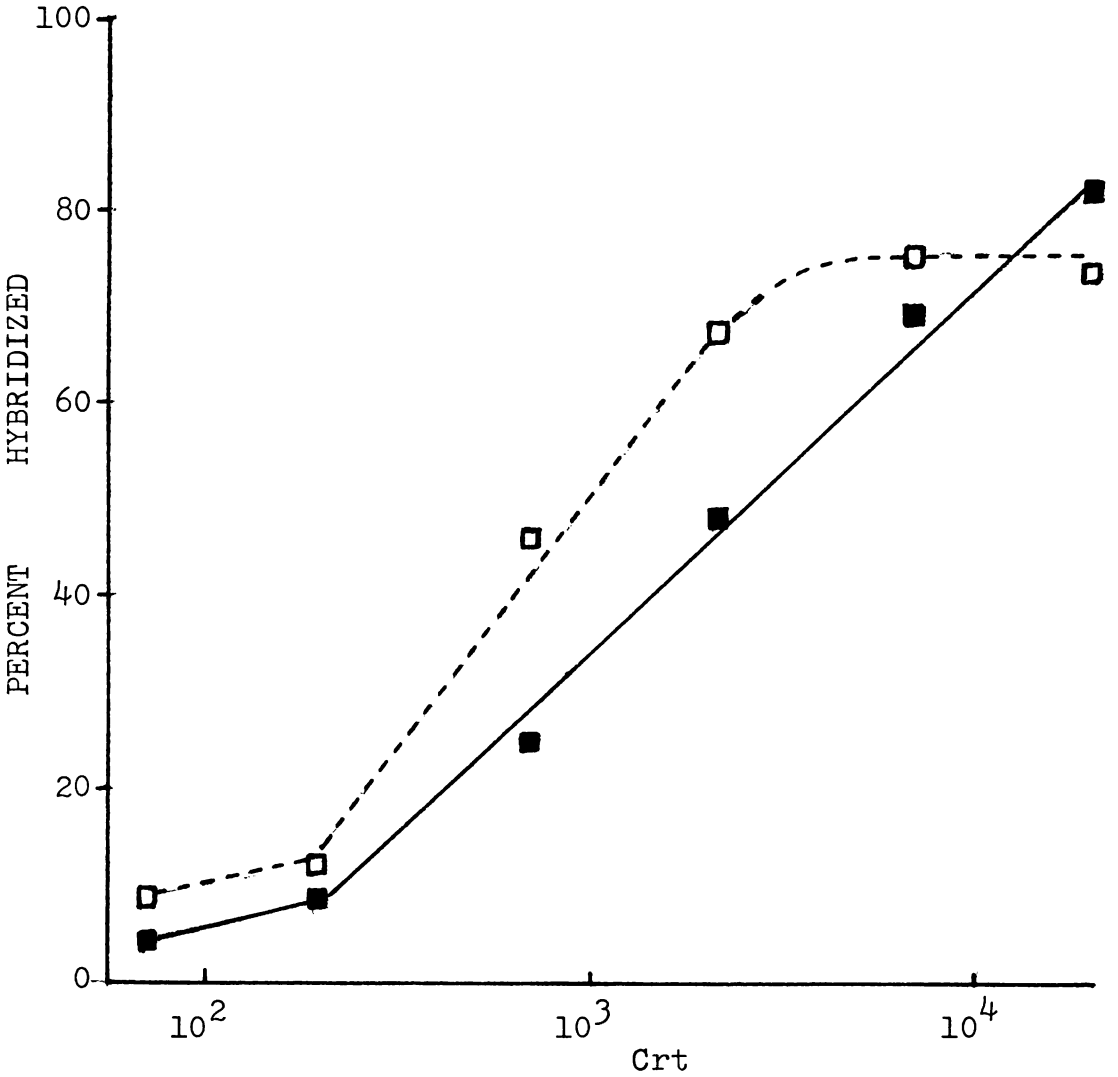

Fig.26. Kinetics of hybridization of ^{32}P-labeled cDNA$_3$, and ^{3}H-labeled cDNA$_{rep}$ with 24S ASV RNA from transformed hamster cells. 24S ASV RNA was prepared as described in the legend for Fig. 24. 430 cpm of ^{32}P-labeled cDNA$_3$, (□, sp act 7 x 10^4 cpm/ng) and 640 cpm of ^{3}H-labeled cDNA$_{rep}$ (■) were hybridized with increasing quantities of RNA in a 40 µl volume containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, 0.001 M EDTA at 68° for 64 hr. Hybridizations were measured as described in the legend for Fig. 19.

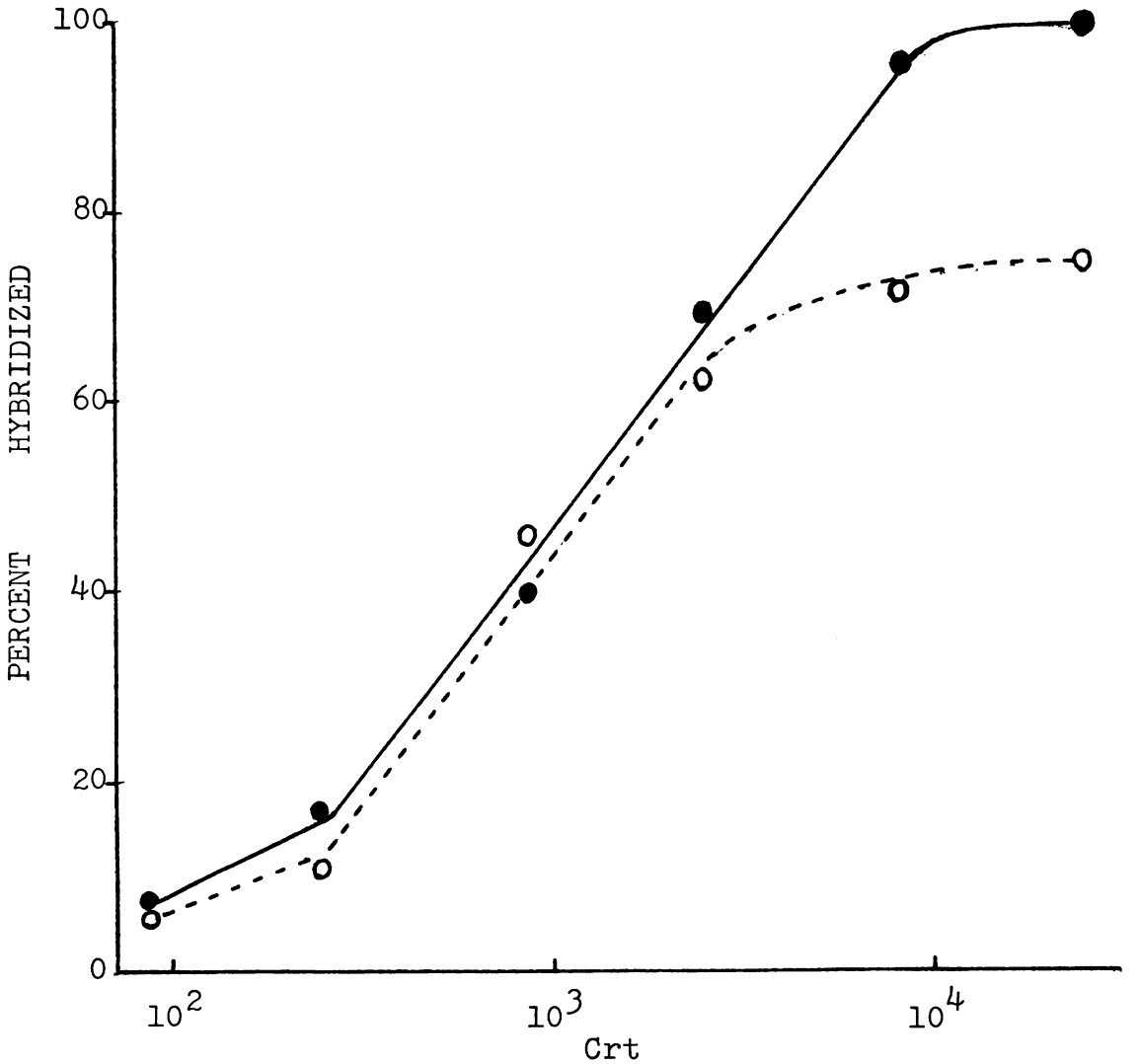

Fig.27. Kinetics of hybridization of ^{32}P-labeled cDNA$_3$, and ^{3}H-labeled cDNA$_{rep}$ with 35S ASV RNA from transformed hamster cells. 35S ASV RNA was prepared as described in the legend for Fig. 24. ^{32}P-labeled cDNA$_3$, (O) and ^{3}H-labeled cDNA$_{rep}$ (●) were hybridized with increasing quantities of RNA as described in the legend for Fig. 25.

(Fig. 17) and with polyribosomal RNA from reverted hamster cells (Fig. 16). Since ASV cDNA hybridized faster than the $cDNA_{rep}$ with the 24S viral RNA (Fig. 25B), $cDNA_{sarc}$ must also hybridize faster than the $cDNA_{rep}$ with 24S viral RNA from whole-cell RNA or with cytoplasmic RNA.

I offer the following interpretation for this peculiar hybridization pattern of the 24S (cytoplasmic) viral RNA. The 24S viral RNA contains both a major and a minor species: the more abundant species contains poly(A), the "C" region (see Fig. 1) (detected by $cDNA_{3'}$), and the Sarc sequences; whereas the minor species contains some or all of the remainder of the viral genomic sequences. The major and minor species together account for the hybridization of over 90% of $cDNA_{rep}$; their differences account for the faster annealing rates of $cDNA_{3'}$ and $cDNA_{sarc}$ than of $cDNA_{rep}$. There are two likely explanations for the presence of the minor species in 24S viral RNA: (1) nicked 35S viral RNA sedimented in the region pooled to obtain the 24S viral RNA; and (2) a bona fide minor species sedimented similarly to the major 24S species. In the later case, the minor species might be polyadenylated. To test this possibility, ^{3}H-labeled $cDNA_{rep}$ and ^{32}P-labeled ASV

cDNA were hybridized with the increasing quantities of polyadenylated RNA from the cytoplasm of a transformed hamster clone (SR-3/1a). As shown in Fig. 28, at least 70% of $cDNA_{rep}$ was hybridized with the polyadenylated RNA (which migrates as 24S species, see Fig. 13) and the ratio of rates of annealing of $cDNA_{rep}$ and ASV cDNA was the same as with total cytoplasmic or 24S RNA. Assuming this preparation of cytoplasmic RNA was free from contamination with intact polyadenylated 35S viral RNA from the nuclei (as was previous preparation shown in Fig. 13), the extensive hybridization with $cDNA_{rep}$ may indicate the presence of a polyadenylated bona fide minor species of viral RNA in the cytoplasm. The comparative properties of the heterogeneous 24S viral RNAs from cytoplasm of ASV-transformed hamster cells are summarized in Table 7.

These results suggest that the entire viral transcript (as 35S RNA) from the integrated provirus in ASV-transformed hamster cells is present in the nucleus. Although the 35S viral RNA is confined to the nucleus, probably more than one species of 24S viral RNA is present in the cytoplasm. The 24S viral RNAs may be generated as partial transcripts of proviral DNA or as post-transcrip-

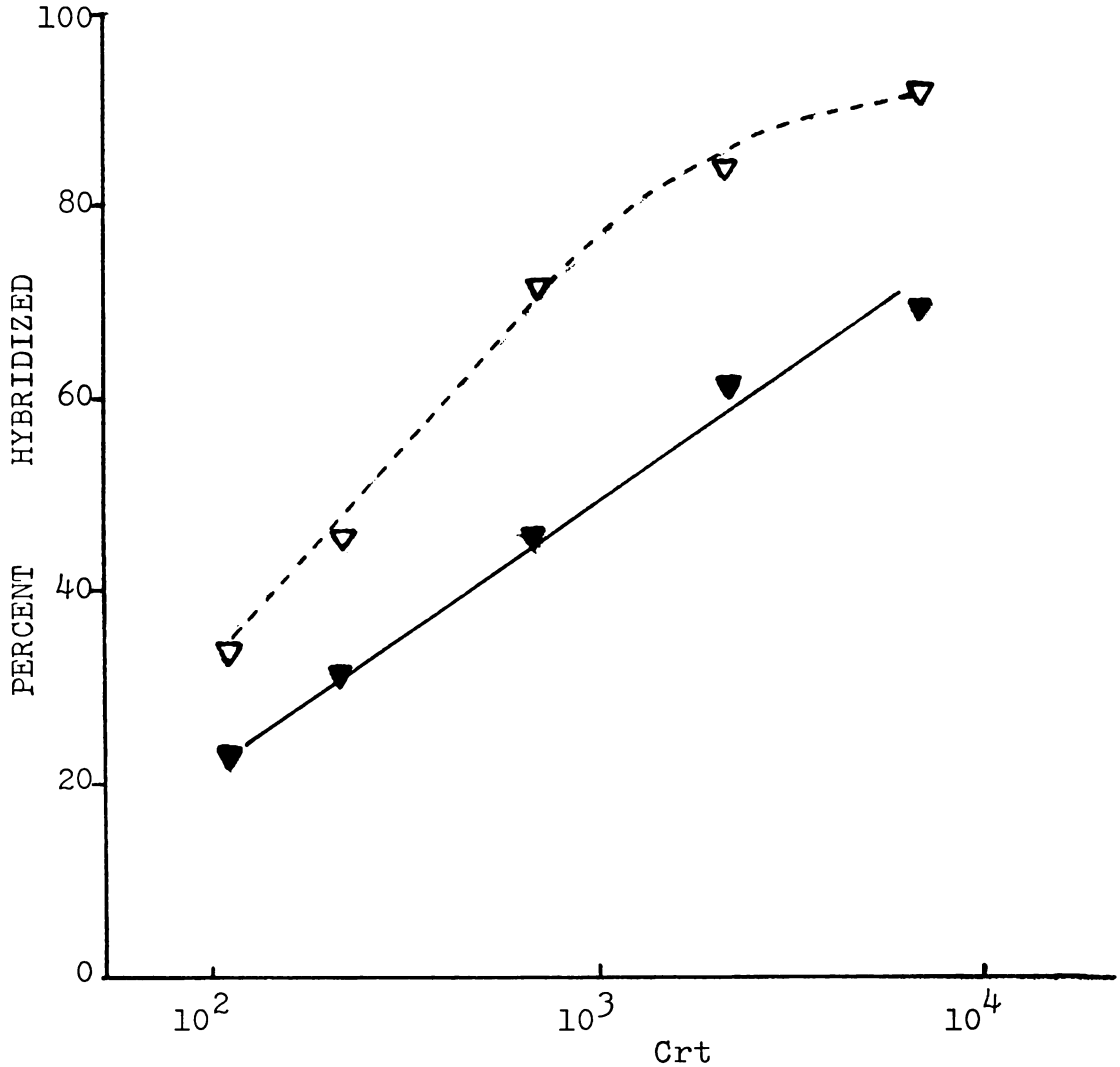

Fig.28. The complexity of polyadenylated viral RNA from cytoplasm of transformed hamster cells as determined by hybridization analysis with ^{3}H-labeled cDNA$_{rep}$. RNA was extracted from cytoplasm of SR-3/1a cells and selected for polyadenylated RNA by affinity chromatography with oligo-(dT)-cellulose. 700 cpm of ^{3}H-labeled cDNA$_{rep}$ (▼) and 600 cpm of ^{32}P-labeled ASV cDNA (▽ , sp act 6 x 10^4 cpm /ng) were incubated with increasing quantities of RNA in a 40 µl reaction containing 0.6 M NaCl, 0.025 M Tris-HCl, pH 7.4, 0.001 M EDTA at 68° for 141 hr. Hybridizations were measured as described in the legend for Fig. 19.

Table 7. The properties of the heterogeneous viral RNAs from cytoplasm of ASV-transformed hamster cells.

	Major species	Minor species
Location	cytoplasm	cytoplasm
Size	24S[a]	about 24S(?)
Polyadenylation	yes	yes
Genomic sequences		
(1) detected by hybridization with specific cDNA's	"C" and Sarc[b]	rest of ASV genome
(2) other	gag(?)[c]	
Possible origions	(i) partial transcript of proviral DNA	(i) contamination
	(ii) post-transcriptional product of 35S ASV RNA	(A) of cytoplasmic RNA by nuclear RNA
		(B) of 24S RNA by nicked 35S RNA
		(ii) bona fide species generated by either mechanism proposed for major species

[a]The molocular weight of 24S RNA is about 1.2×10^6 representing 40% of viral 35S RNA (3×10^6 daltons).

[b]"C" and Sarc sequences together constitute only 20% of ASV 35S genome (see Fig. 1).

[c]The gene coding for group-specific (gs) antigens. Avian gs antigens are detectable in ASV-transformed hamster cells (Macpherson, 1966; Fleissner, 1970; L. Levintow personal communication).

tional products of the 35S viral RNA; it is not known whether 24S RNA is present in the nucleus. The restriction of the 35S viral RNA to the nucleus is likely to be peculiar to ASV-infected nonpermissive cells, but it is not clear whether smaller (24S) species of viral RNA is also observed in the cytoplasm of permissive cells infected with ASV.

IV. Discussion

IV.1. The mechanism of reversion in ASV-transformed hamster cells.

The discovery that revertants of ASV-transformed hamster cells contain the viral genome persisting as an integrated DNA provirus raised the question of how the expression of viral genes, particularly the transforming gene, is governed in these cells. I found that the concentration of virus-specific RNA is higher in transformed clones than in reverted clones (see Talbe 8) and that the amount of viral RNA in these clones correlates with the frequency with which ASV can be rescued after fusion with chick embryo cells. Furthermore, treatment of a reverted clone with 5-bromodeoxyuridine increased the concentration of viral RNA and the frequency of rescue (Boettiger, 1974). Therefore, it appeared from those studies that viral gene expression in reverted cells is at least partially regulated by the concentration of virus-specific RNA, which in turn could be regulated by the rates of synthesis and/or degradation of viral RNA. However, since some viral RNA was observed in all of the revertants studied, I have asked whether additional qualitative differences in the

Table 8. Comparisons of ASV-transformed and reverted hamster cells.

Phenotype	Transformed		Reverted		
Clones	SR-3/1a	SR-3	SR-3/5	SR-3/4a	SR-3/11R
Provirus per diploid cell	-	1 copy	1 copy	1 copy	1 copy
Integration of provirus	-	1 copy	-	-	1 copy
Viral 35S RNA equivalent per cell	37.5	7	3.5	2	0.45
Polyadenylation of ASV RNA	yes	-	-	yes	-
ASV RNA in nuclei	26%	-	-	28%	-
in cytoplasm	74%	-	-	72%	-
Size of viral RNA in whole cell	35S+24S	-	35S(?)+24S	-	-
in cytoplasm	24S	-	24S	-	-
Polyadenylation of 24S ASV RNA	yes	-	yes	-	-
Association of ASV RNA with polyribosomes	yes	-	yes	-	-
ASV RNA released from polyribosomes with EDTA	yes	-	yes	-	-
^{3}H-cDNA$_{sarc}$ hybridized with polyribosomal RNA	at least 80%	-	at least 60%	-	-
Relative concentration of Sarc sequences in polyribosomes	10	-	1	-	-
Relative concentration of ASV RNA in polyribosomes	10	-	1	-	-
Polyadenylated 24S ASV RNA contains Sarc sequences	yes	-	-	-	-
Sarc sequences released from polyribosomes with EDTA	-	-	yes	-	-

- Not tested.

metabolism of viral RNA serve to differentiate transformed and reverted cells.

The scheme currently advanced to describe processing of eukaryotic mRNAs offers a possible mechanism for post-transcriptional regulation: the addition of poly(A) sequences to the 3' end of heterogeneous nuclear RNA (HnRNA) appears to be an essential part of conserving and processing of polyadenylated mRNA into the cytoplasm, whereas the 5' end of HnRNA is removed and rapidly destroyed (Darnell et al., 1971; Mendecki et al., 1972; Weinberg, 1973; Brawerman, 1974). I therefore compared several characteristics of virus-specific RNA in transformed and reverted cells to see whether the post-transcriptional metabolism of viral RNA could produce qualitative differences in the expression of viral genes in hamster cells with differing phenotypes. In brief, I found no qualitative differences in the metabolism of viral RNA to correlate with the different phenotypes (see Table 8).

Since the ASV cDNA employed to detect viral RNA in hamster cells did not equally represent the entire ASV genome (Garapin et al., 1973), the expression of some

viral sequences in hamster cells may not be detected if these sequences are poorly transcribed into DNA during the in vitro synthesis of ASV cDNA. The reverted phenotype raises the possibility that the viral transforming gene, in particular, might not be present in ASV RNA in those cells. Therefore, I tested RNA from reverted cells with a cDNA shown to be specific for the region of the ASV genome deleted in the formation of transformation-defective mutants (Stehelin et al., 1976; Duesberg & Vogt, 1973a, b). Hybridization analysis suggests that this reagent, called $cDNA_{sarc}$, represents most or all of the viral transforming gene (Stehelin et al., 1976; Martin, 1970; Kawai & Hanafusa, 1971; Bader, 1972). Using $cDNA_{sarc}$, I have shown that polyribosomal RNA in both transformed and reverted cells contains transformation-specific sequences; however, the concentration of those sequences reflects the overall concentration of viral RNA in these cells. I have also shown that the predominant, if not exclusive species of viral RNA in the cytoplasm of both reverted and transformed cells has a sedimentation coefficient of 24S and that, in both types of cells, essentially all of the polyribosome-associated viral RNA is released from polyribosomes after

treatment with EDTA. This argues strongly that, in both cases, the Sarc sequences are present in a 24S species of mRNA. Since I can find no apparent qualitative differences in the pattern of viral gene expression in these phenotypically varied cells (see Table 8), I conclude that the quantitative differences in concentration of viral RNA (particularly the Sarc sequences) are sufficient to produce the marked phenotypic variations among them.

Another approach to study the mechanism of reversion is to measure the complexity of viral RNA in reverted hamster cells as compared with that in transformed cells. Due to the insufficient concentration of viral RNA in reverted hamster cells, my attempt to measure the complexity of viral RNA in reverted cells is unsuccessful. Although I have not been able to determine whether the complexity of viral RNA is affected in reverted cells, at least I have shown that the Sarc nucleotide sequences, which would have been restricted most likely to generate reversion phenomenon, are present in RNA from reverted cells.

IV.2. Reported mechanisms of reversion of other RNA and DNA tumor viruses transformed cells.

Since revertants from other RNA and DNA tumor viruses transformed cells have been reported(Fischinger et al., 1972; Rabinowitz & Sachs, 1968), it is interesting to compare the mechanisms of reversion in other systems with what I found in ASV-transformed hamster cells.

The properties of revertants from RNA tumor viruses transformed cells are summarized in Table 9 and described as follows. Cloned, spontaneous reverted cell lines derived from clones of Moloney murine sarcoma virus transformed cat cells had no evidence of murine sarcoma virus-specific RNA and DNA as judged by RNA:cDNA or DNA: cDNA hybridization, whereas parental transformed cat cells contain virus-specific sequences in their DNA and RNA (Frankel et al., 1976). This appears to be a clear example of loss of provirus to account for the reversion phenomenon.

Revertants from Kirsten murine sarcoma virus (Ki-MSV) transformed cells (nonproductive) following mitomycin C mutagenization contain levels of sarcoma viral RNA quantitatively and qualitatively indistinguishable from that present in the parental transformed clone. Following rescue with helper leukemia virus, the revertants release low levels of wild-type transforming virus and a large

Table 9. Properties of Revertants of RNA Tumor Viruses-Transformed Cells.

Reversion System			Evidence of Presence of Transforming Virus				Retransformed (by)			REFERENCE
Induced mutagen	Transformed cells	Transforming virus	Virus rescuable	DNA	RNA	gs Antigens	Spontaneously	Same virus	Other virus	
-	ASV-BHK	ASV	+	+	+	-	-	-	ND	1
-	MSV-cat cell	MSV(FeLV)	-	-	-	-	ND	ND	ND	2
Mitomycin C	MSV-Balb/3T3	Kirsten-MSV(MuLV)	+ defective	ND	+	ND	+	+	ND	3
-			+	ND	ND	+	+	+	ND	
FdU	S+L-	Moloney-MSV(MuLV)	+	ND	ND	+	+	+	ND	4
Colcemid			-	ND	ND	+	-	+	ND	
FdU	MSV-Balb/3T3	Kirsten-MSV(MuLV)	-	ND	-	ND	ND	ND	ND	5
BrdU	MSV-NRK	Kirsten-MSV(MuLV)	+	ND	ND	-	ND	*	ND	
BrdU	SR-NRK	SR-ASV	ND	ND	ND	+	ND	ND	+	6
?	B77-Balb/3T3	B77	ND	ND	ND	+	ND	ND	+	

ND = not determined. *low efficiency

1. Macpherson, 1965, 1966.
2. Frankel et al., 1976.
3. Greenberger et al., 1974.
4. Nomura et al., 1973;Fischinger et al., 1972.
5. Ozanne & Vogel, 1974; Goldberg et al., 1976.
6. Stephenson et al., 1972, 1973.

excess of transformation-defective sarcoma virus. The defective viruses can be transmitted to new cells in the absence of morphologic alteration. These results suggest that the revertants contain mutant viruses defective in transforming functions. The release of wild-type sarcoma virus by cells in a revertant culture appears to occur concomitantly with the spontaneous appearance of re-transformed cells. This suggests that the reversion of mutant virus to wild-type within the cell occurs as a result of reversion of a point mutation in the integrated sarcoma viral genome (Greenberger et al., 1974).

MSV-transformed 3T3FL cells (S+L- cells, containing no infectious murine leukemia virus) produced spontaneously and at variable rates flat variants with some properties of nontransformed cells (revertants). The frequency of occurrence of such variant cells increased after treatment of S+L- cells with fluorodeoxyuridine (FdU) or Colcemid. MSV could be rescued from the transformed S+L- cells by superinfection with murine leukemia virus (MuLV). On the other hand, MSV was no longer rescuable from revertants by superinfection with MuLV or by cell fusion with 3T3FL, Balb/3T3 or normal rat kidney (NRK) cells. However,

some revertants spontaneously underwent re-transformation during extended cultivation and the MSV genome could again be rescued. These studies suggest that these revertants of S+L- cells occurred by different mechanisms apparently involving either the viral or cellular genes or both, and that some flat variant sublines had retained at least one complete MSV genome in a non-rescuable form for some period of time (Nomura et al., 1973).

Revertants of Ki-MSV transformed nonproducer Balb/3T3 cells (KA31 cells) have been selected from the transformed cultures following treatment of the suspended cultures in methylcellulose with 5-fluorodeoxyuridine. The FdU treatment killed the dividing transformed cells but not the nondividing reverted cells of suspended cultures in methylcellulose (Ozanne & Vogel, 1974). One such revertant from which biological transforming activity could not be rescued contains no Kirsten sarcoma virus-specific RNA as judged by the failure to hybridize with the Kirsten sarcoma virus-specific cDNA (Goldberg et al., 1976). The absence of viral RNA in the revertant may indicate the loss or complete repression of provirus.

Morphologic revertants which contain avian or

murine sarcoma viruses have been isolated at low frequency from clonal lines of transformed mammalian cells (Stephenson et al., 1972). The susceptibility of the revertants from Ki-MSV-transformed cells to transformation by the same virus Ki-MSV(MuLV) was strikingly reduced to a level of 10^4-fold less than that of control NRK cells. The block to re-transformation of the revertant from Ki-MSV-transformed NRK cells by Ki-MSV was specific. This was suggested by the fact that revertant from ASV-transformed NRK, which contained the ASV genome, was as susceptible to focus formation by Ki-MSV(MuLV) as were NRK cells. In other studies, it was found that the revertant of B77-transformed Balb/3T3 cells was as sensitive as normal Balb/3T3 to transformation by SV40. These experiments all suggest that cellular factors which may block expression of transformation by one tumor virus do not necessarily influence the action of other RNA or DNA tumor viruses (Stephenson et al., 1973).

The properties of revertants from DNA tumor viruses transformed cells are summarized in Table 10 and described as follows. When cultures of transformed cells growing at a high cell density are exposed to 5-fluoro-

Table 10. Properties of Revertants of SV40- and Polyoma Virus-Transformed Cells.

Reversion System			Evidence of Presence of Transforming Virus				Retransformed by		REFERENCE(S)
Property selected against	Transformed line	Killing agent	Virus rescuable	DNA	RNA	T antigen	Same virus	Other virus	
Growth to high density	SV3T3	FdU	10^{-6}*	+	+	+	-	MSV	1,2
	Py3T3	FdU	NP	ND	ND	+	-	ND	
	SV3T3	Colchicine	10^{-3}	ND	+	+	-	ND	3
	PyBHK	Glutaraldehyde	NP	ND	ND	+	-	ND	6
Growth in agar suspension	PyBHK	BrdU-light	NP	ND	ND	+	-	MSV	5
Growth in 1% serum	SV3T3	BrdU-light	10^{-6}	ND	+	+	-	ND	2
Exposure of lectin sites	SV3T3	Con A	10^{-6}	+	+	+	-	-	2,3,4
Growth at 39°	SV3T3	FdU	10^{-3}	ND	ND	+	-	MSV	7
Chromosomes carrying viral genome	PyBHK	Thioguanine	NP	ND	ND	-	+	ND	8,9

NP= not possible with transformed parent. ND= not determined.
*Fraction of revertant cells yielding virus on fusion with permissive cells.
1. Pollack et al., 1968; 2. Ozanne et al., 1973; 3. Culp & Black, 1972; 4. Ozanne & Sambrook, 1971; 5. Wyke, 1971a, b; 6. Rabinowitz & Sachs, 1968; 7. Renger & Basilico, 1972; 8. Marin & Littlefield, 1968; 9. Marin & Macpherson, 1969.

deoxyuridine, all the cells which are dividing are killed. The survivors are the cells which had stopped growing before the FdU treatment, presumably because they maintained partial growth control and susceptibility to contact inhibition despite being transformed (Pollack et al., 1968; Culp & Bladk, 1972). The "flat" revertants from SV40-transformed 3T3 cells (SV3T3) contain the same amount of SV40 DNA per diploid quantity of cell DNA as the parental SV3T3 cells; although these revertants contain some SV40 RNA, it is present in lower concentrations than in SV3T3 cells; and because of the difficulties in obtaining useful amounts of SV40-specific RNA from the flat revertants, the pattern of transcription of the viral DNA has yet to be compared with that in SV3T3 cells (Ozanne et al., 1973).

Reverted cells which grow to low saturation densities have also been isolated using colchicine as a killing agent (Vogel & Pollack, unpublished data) and by seeding transformed cells on monolayers of untransformed cells fixed with glutaraldehyde (Rabinowitz & Sachs, 1968). The rationale of this last selection procedure is obscure. Dividing cells incorporate the thymidine analog 5-bromodeoxyuridine and can be killed by irradiation with blue

light. This method was used to kill polyoma-transformed BHK cells in suspension culture and hence to recover variants of transformed cells which do not grow in suspension (Wyke, 1971a, b).

When populations of SV3T3 cells are cultured for a few generations in the presence of concanavalin A (Con A), most cells are killed. The survivors have the following properties: (1) They are neither killed nor agglutinated by concentrations of Con A which kill or agglutinate SV3T3 cells; (2) the survivors grow to low saturation densities in media containing 1% serum and in media containing 10% serum; (3) the survivors contain the same amount of SV40 DNA per diploid quantity of cell DNA as SV3T3 cells, and (4) the extent and pattern of transcription of the viral DNA is the same in the survivors as in SV3T3 cells (Ozanne & Sambrook, 1971; Ozanne et al., 1973; Culp & Black, 1972).

Complete SV40 can be rescued from some revertants of SV40-transformed cells and the rescued virus has always proved to be wild-type in its transforming ability and infectivity (Pollack et al., 1968; Ozanne & Sambrook, 1971; Culp et al., 1971; Culp & Black, 1972). This suggests that at least in these revertants from which SV40 can be rescued,

reversion is the result of some change in the cell genome rather than some change in the viral genome. However, interpretation of the rescue experiments cannot be rigorous because the revertants, as well as the parental transformants, contain more than one genome equivalent of SV40 DNA. It is possible, therefore, that the genome which is rescued is not the genome controlling transformation and reversion.

Using FdU as a killing agent for dividing cell, Renger and Basilico (1972) selected from populations of SV3T3 cells those which grow at 39° to the low saturation density of untransformed 3T3 cells. Like SV3T3 cells these temperature-sensitive (ts) revertant/transformants form colonies when plated on top of monolayers of 3T3 cells at 32°, but at 39° the temperature-sensitive cells, but not the SV3T3 cells, lose this ability. The ts cells contain T antigen at both temperatures and they require less serum than 3T3 at both 32° and 39°. SV40 rescued from these cells appears to be wild-type. The simplest interpretation of these data is that these cells carry a ts mutation in a cell gene, the product of which is essential for the maintenance of transformation.

Marin and Littlefield (1968) fused cells of two strains of BHK-21, one of which was resistant to 5-bromodeoxyuridine while the other was resistant to 6-thioguanine. The resulting hybrids, which were able to incorporate both drugs and were therefore sensitive to both, were transformed with polyoma virus. The transformed and sensitive hybrids were then selected for resistance to 6-thioguanine. The development of such resistance implies the loss, through aberrant mitoses, of the chromosome(s) specifying the pathway which determines sensitivity to thioguanine. Marin and Littlefield argued that by selecting for the loss of chromosomes in this way, the chromosomes carrying the polyoma genomes might be lost concomitant with the loss of the chromosomes determining sensitivity to the drug. Two of Marin and Littlefield's surviving clones proved to be revertants (Marin & Macpherson, 1969) that grew to low saturation densities and could not grow suspended in Methocel. Since these revertants do not contain T antigen, it is possible that they have lost the viral genomes along with some cell chromosomes, although this has not been established by molecular hybridization.

IV.3. Transcripts of provirus.

Little is known about viral RNA transcribed from the integrated provirus. ASV-transformed mammalian cells (nonpermissive) offer a suitable system for studying the viral RNA in the absence of synthesis of genomic RNA to be packaged into progeny virus. I have shown here that viral RNA transcripts in ASV-transformed hamster cells include both 35S and 24S species; however, only the 24S species is detectable in the cytoplasm of these cells. Since I have shown that virus-specific RNA in ASV-transformed hamster cells is associated with polyribosomes and released with EDTA treatment, the 24S viral RNA species is likely to be viral mRNA. There are precedents for the detection of viral RNA of subgenome size in RNA tumor viruses infected cells. It has been reported that in addition to 35S viral RNA, 20S viral RNA and 10-30S viral RNA were detected as viral mRNA in permissive cells infected with MSV(MuLV) and ASV, respectively (Shanmugan et al., 1974; Schincariol and Joklik, 1973). The molecular weight estimate of 24S viral RNA is 1.2×10^6 daltons (Spirin, 1963) representing approximately 40% of 35S ASV genome with molecular weight of 3×10^6 daltons (Billeter et al., 1974; Beemon et al.,

1974). Using $cDNA_{sarc}$ for the hybridization analysis, I have shown that 24S ASV RNA contains nucleotide sequences which encode the viral transforming gene (src gene) (Fig. 17). The 24S viral RNA is also likely to contain sequences specifying the avian group-specific antigen since these antigens have been detected in ASV-transformed hamster cells (Macpherson, 1966; Fleissner, 1970; L. Levintow personal communication). Two observations suggest that the 24S viral RNA may not include the sequences coding for the glycoprotein of ASV (env gene): (1) the 24S viral RNA from B77-transformed 3T3 cells did not hybridize with $cDNA_{gp}$, a specific hybridization reagent for detecting the sequences of env gene (N. Quintrell personal communication; Tal et al., manuscript in preparation); and (2) cytoplasmic RNA from hamster cells transformed by a Bryan strain of ASV (containing no env gene) (Sarma et al., 1966) still resolved to a 24S species of virus-specific RNA (author's unpublished observation). The proposed genetic map of ASV 35S RNA arranges genes for transformation (src), glycoprotein (env), polymerase (pol) & group-specific antigens (gag) sequentially away from the 3' end poly(A) segment (Wang et al., 1975, 1976; Duesberg et al., 1976)

(Fig. 1). Circular permutation of the provirus during integration may bring 5' and 3' ends adjacent, thereby generate a 24S viral RNA containing the noncontiguous src and gag genes. Alternatively, there may be more than one species of 24S viral RNA.

IV.4. Regulation of nonpermissive infection.

Mammalian cells (nonpermissive) may be transformed at a low frequency (about 10^{-4} to 10^{-6}) but cannot support ASV replication (Temin, 1971; Altaner & Temin, 1970). The restriction for viral replication in nonpermissive cells may depend on several factors. The first barrier of nonpermissive cells may be located at cell surface and may prevent the penetration of certain subgroups of ASV. It has been shown that, with the exception of B77 ASV, only subgroup D viruses infect mammals (Duff & Vogt, 1969). Other factors of restriction may be intracellular, as reported in Bryan ASV transformed hamster cells; in these cells, the precursor polypeptide of viral structure proteins is synthesized, but is not cleaved to generate viral structure proteins (Eisenman et al., 1974).

The quantitative reduction of viral RNA in transformed mammalian cells may also be important in restricting

the replication of ASV. The amount of viral RNA in transformed mammalian cells is about 100-fold less than in permissive cells (Bishop et al., 1973a). In addition, I have found only 24S viral RNA species present in the cytoplasm of ASV-transformed hamster cells, whereas in permissive cells, 35S viral RNA is present and serves as messenger (Fan & Baltimore, 1973; Schincariol & Joklik, 1973; Shanmugan et al., 1974). Complexity studies of 35S and 24S virus-specific RNA in ASV-transformed hamster cells suggest that the entire viral transcript (as 35S RNA) from the integrated provirus exists only in the nucleus; and the cytoplasmic viral RNA as 24S species may represent a heterogeneous population of RNA containing partial sequences of the 35S ASV RNA. The restriction of the 35S viral RNA to the nucleus appears to be another factor to prevent the viral replication in ASV-infected nonpermissive cells.

Recently, it has been shown that acute infection of nonpermissive cells by ASV leading to the synthesis and integration of provirus in host cells occurs more often than the actual transformation of infected cells (unpublished data of H. E. Varmus). This finding indicates that a relatively large number of mammalian cells infected with

ASV may contain integrated provirus with no phenotypic manifestation of transformation. Some of these cells generate infectious ASV after rescue with permissive cells (Boettiger, 1976). The infected mammalian cells with no phenotypic transformation but containing integrated provirus may be analogous to revertants of ASV-transformed hamster cells. Thus the regulatory mechanism which differentiates the revertants from the transformants, the quantitative reduction of functional viral RNA, may also operate to differentiate the nontransformed from the transformed phenotype in acute infection of nonpermissive cells by ASV.

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