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Size and Genetic Content of Viral RNAs in Avian Oncovirus-Infected Cells

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Viral complementary DNA (cDNA) sequences corresponding to the *gag*, *pol*, *env*, *src*, and *c* regions of the Rous sarcoma virus genome were selected by hybridizing viral cDNA to RNA from viruses that lack the *env* or *src* gene or to polyadenylic acid [poly(A)]-containing RNA fragments of different lengths and isolating either hybridized or unhybridized DNA. The specificities, genetic complexities, and map locations of the selected cDNA's were shown to be in good agreement with the size and map locations of the corresponding viral genes. Analyses of virus-specific RNA, using the specific cDNA's as molecular probes, demonstrated that oncovirus-infected cells contained genome-length (30–40S) RNA plus either one or two species of subgenome-length viral RNA. The size and genetic content of these RNAs varied, depending on the genetic makeup of the infecting virus, but in each case the smaller RNAs contained only sequences located near the 3′ end of the viral genome. Three RNA species were detected in Schmidt-Ruppin Rous sarcoma virus-infected cells: 39S (genome-length) RNA; 28S RNA, with an apparent sequence of *env-src-c*-poly(A); and 21S RNA, with an apparent sequence of *src-c*-poly(A). Cells infected with the Bryan high-titer strain of Rous sarcoma virus, which lacks the *env* gene, contained genome-length (35S) RNA and 21S *src*-specific RNA, but not the 28S RNA species. Leukosis virus-infected cells contained two detectable RNA species: 35S (genome-length) RNA and 21S RNA, with apparent sequence *env-c*-poly(A). Since *gag* and *pol* sequences were detected only in genome-length RNAs, it seems likely that the full-length transcripts function as mRNA for these two genes. The 28S and 21S RNAs could be the active messengers for the *env* and *src* genes. Analyses of sequence homologies among nucleic acids of different avian oncoviruses demonstrated substantial similarities within most of the genetic regions of these viruses. However, the "common" region of Rous-associated virus-0, an endogenous virus, was found to differ significantly from that of the other viruses tested.

The viral RNA synthesized in RNA tumor virus-infected cells must function both as mRNA and as the genome for progeny virus. The infected cell contains two major size classes of virus-specific RNA: genome-length (30–40S) RNA and smaller RNAs with sedimentation values ranging from roughly 15 to 30S (4, 7, 11, 14, 23, 49, 61). Only the 30–40S RNA is packaged in substantial amounts in newly assembled virus particles. However, both 30–40S and 15–30S RNA appear to function as mRNA. These two classes of RNA are found associated with polyribosomes in a form that is released by treatment with EDTA (11, 14, 49, 51), and both contain polyadenylate [poly(A)] tracts. The mechanism that allows the cell to distinguish between the genomic and messenger functions is not known. The polyribosome-associated viral RNA has the same polarity (positive strand) as the virion RNA (11, 49, 51); polyribosomal and virion RNAs are both polyadenylated (13, 44, 51, 64); and the sedimentation value of the virion RNA is the same as that of the higher-molecular-weight (30–40S) polyribosome-associated RNA. The cell apparently contains minute amounts of negative-strand viral RNA (56), but its function is unknown.

We have previously shown that Rous-associated virus-2 (RAV-2)-infected cells contain envelope glycoprotein gene (*env*)-specific sequences in a subgenome-length RNA, sedimenting at roughly 20 to 24S, as well as in 35S RNA (23). Using microinjection techniques, Stacey et al. (55) have shown that the 20–24S RNA from these cells can function as messenger for the envelope glycoprotein in recipient cells, complementing the *env* deletion in the Bryan high-titer strain of Rous sarcoma virus

(BH-RSV). In contrast, the 35S RNA was not active as messenger for the *env* gene. Thus, the functional mRNA for the envelope glycoprotein would appear to be the smaller RNA species. There is evidence that the genome-length viral RNA functions as mRNA for the group-specific antigen gene (*gag*). Mueller-Lantzsch and Fan (36) have shown that polyribosomes immunoprecipitated with anti-p30 serum contain only 35S viral RNA. Furthermore, several laboratories have demonstrated that the virion 35S RNA serves as a template for cell-free translation of *gag* proteins (32, 37, 40, 63). Gielkens et al. (13) have shown that 35S RNA from murine leukemia virus-infected cells can direct the cell-free synthesis of *gag*-related polypeptides and that 20–22S RNA from these cells can direct the synthesis of an additional polypeptide that appears to be virus specific. Deng et al. (7) have identified a 26S *src*-specific RNA in the cytoplasm of hamster cells nonproductively infected with B77. Thus, it seems likely that several different viral RNA species are present in infected cells and that these RNAs function as mRNA's for different viral genes.

In the present study we have further characterized the viral RNA in infected cells to determine the size and genetic content of each RNA species and to determine the relative abundance of viral RNAs transcribed from each viral gene. Five genetic regions have been identified in RSV: *gag* (coding for internal structural proteins), *pol* (RNA-dependent DNA polymerase), *env* (envelope glycoprotein), *src* (sarcomagenic transformation), and *c* (a "common" sequence, whose function is unknown, located adjacent to the 3′ termini in RNAs of both leukosis and sarcoma viruses) (1). These genes have been mapped by Wang et al. (65, 66) and Joho et al. (27, 28), using oligonucleotide analysis of various mutant virus RNAs. Based on this mapping data we have isolated five highly specific cDNA probes, corresponding to the five genetically defined regions, and have used these probes to identify and quantitate specific viral RNAs in infected cells. The data demonstrate that cells infected with avian leukosis or sarcoma viruses contain 30–40S viral RNA, plus one or more distinct species of subgenome-length viral RNA. The number of RNA species present in the cell and the size and genetic content of each RNA species are dependent on the genetic makeup of the infecting virus. However, in each case the subgenome-length RNAs contain only sequences located near the 3′ terminus of the viral genome.

MATERIALS AND METHODS

Cells and viruses. All chicken embryo fibroblasts (SPAFAS, Inc., Norwich, Conn.), except line 7 × 15, were negative for both chicken helper factor (*chf*) and viral group-specific (gs) antigen, as determined by complement fixation (18). Line 7 × 15 embryos, which spontaneously release Rous-associated virus-0 (RAV-0) (6), were provided by L. B. Crittenden. Viruses used in these studies were RAV-0; RAV-2; RAV-7; Schmidt-Ruppin strain of RSV, subgroup B (SR-RSV-B); SR-RSV-N8, a mutant of SR-RSV-A (31); Prague strain of RSV, subgroup B; BH-RSV. SR-RSV-B is a recombinant between SR-RSV-A and RAV-2 that has acquired the *src* gene of SR-RSV-A but has the subgroup B host range of RAV-2 (30). SR-RSV-B, provided by H. Hanafusa, had been recently cloned but was passaged four to five times after cloning. In one experiment, as indicated in the text, SR-RSV-B was recloned by colony isolation (31) to eliminate transformation-defective (td) segregants and passaged only once before analysis. BH-RSV and SR-RSV-N8 contain deletions within the *env* gene, representing approximately 20% of the viral genome (9, 31, 48). BH-RSV-infected cells were prepared by infecting gs^-chf^- cells with BH-RSV(−) in the presence of UV-inactivated Sendai virus (16); cells were transferred until cultures became fully transformed (four to five transfers). RAV-2- and SR-RSV-B-infected cells were infected at multiplicities of infection of 1 to 5. Cells were transferred once and were used for RNA extraction 3 days after transfer. Other aspects of tissue culture techniques and virus growth have been described (15). Viruses for RNA extraction or complementary DNA (cDNA) synthesis were harvested from roller bottles at 3-h intervals and purified as previously described (20), except that the hyaluronidase and Pronase treatments were omitted.

RNA extraction. The RNA extraction procedure was adapted, with minor modifications, from procedures previously described by Fan and Baltimore (11) and by Perry et al. (41). All steps were performed at room temperature, unless otherwise indicated. Phenol (Mallinckrodt Chemical Works, St. Louis, Mo.) was distilled under nitrogen at 22 mm of Hg and stored under nitrogen at −20°C.

Cell monolayers were treated with Pronase (50 μg/ml, 5 min at 37°C) to facilitate removal of cells and to release budding virus particles from the cell surface. Cells were pelleted by low-speed centrifugation, washed once with TEN buffer (0.01 M Tris-hydrochloride, pH 7.4; 0.001 M EDTA; 0.1 M NaCl), and resuspended in the same buffer (2 ml/10^7 cells). Freshly prepared ^{32}P-labeled 35S RAV-2 RNA (0.5 to 5 ng/mg of cell RNA, 5×10^6 to 10×10^6 cpm/μg) was added to the cell suspension to serve as a marker for evaluating recovery of viral RNA and for monitoring possible degradation of RNA during extraction. At this low concentration the marker RNA does not interfere with subsequent hybridization analyses of viral RNA in the cell. Proteinase K (EM Laboratories, Inc., E. Merck, Elmsford, N.Y.) was added to the cell suspension up to a concentration of 1.5 mg/ml, sodium dodecyl sulfate (SDS) was added up to 0.5%, and the lysate was allowed to stand for 30 to 60 min at room temperature. The crude extract was passed three to four times through a 20-gauge needle

to reduce viscosity caused by cell DNA. (In our hands, fragmentation of cell DNA by sonic treatment [11] caused some breakdown of RNA.) The nucleic acids were extracted three times with a mixture of phenol-chloroform-isoamyl alcohol (1:1:0.01 [vol/vol]) containing 0.05% (wt/vol) 8-hydroxyquinoline and once with chloroform containing 1% isoamyl alcohol. The aqueous phase was adjusted to 0.2 M NaCl, and RNA was precipitated by addition of 2 volumes of ice-cold ethanol. After storage at −20°C for at least 2 h the RNA was pelleted by centrifugation at 8,000 × *g* for 30 min. Recoveries of ^{32}P-labeled marker RNA were generally greater than 90%. RNA prepared in this way also contains cell DNA, but the DNA does not interfere with the hybridization analyses of virus-specific RNA, since the infected cells contain 10^3 to 10^4 times more viral RNA than viral DNA. Poly(A) RNA, which was used in many experiments, does not contain significant amounts of DNA, since the DNA does not bind to polyuridylic acid [poly(U)]-Sepharose.

For extraction of virion RNA, purified virus was incubated 30 min at 37°C in the presence of 200 μg of proteinase K per ml and 0.5% SDS and then extracted twice with the phenol-chloroform-isoamyl alcohol mixture and once with chloroform-isoamyl alcohol. RNA was precipitated with ethanol, using 50 μg of purified yeast tRNA (Schwarz/Mann, Orangeburg, N.Y.) as carrier. The precipitated RNA was dissolved in ETS buffer (0.01 M EDTA; 0.01 M Tris-hydrochloride, pH 7.4; 0.2% SDS), incubated for 90 s at 85°C to dissociate RNA subunits, and centrifuged on linear sucrose gradients as described below. Fractions containing 30–40S RNA were pooled, and RNA was precipitated with ethanol in the presence of 50 μg of yeast tRNA.

^{32}P-labeled viral RNA. Virus-infected cells were transferred 3 days after infection. At 24 h after transfer the growth medium was removed, and phosphate-free Temin-modified Eagle minimal essential medium containing 2% calf serum was added; 12 h later this was replaced with the same medium containing 2 mCi of carrier-free [^{32}P]phosphate (New England Nuclear, Boston, Mass.) per ml. After 12 to 18 h the ^{32}P medium was removed, the plates were washed, and a small volume (4 ml per 100-mm plate) of phosphate-free medium was added; supernatant fluids were harvested 3 h later and virus was purified. RNA was extracted, and 30–40S RNA was recovered from gradient fractions after rate zonal centrifugation, as described below. Purified ^{32}P-labeled viral RNA, used either as a gradient marker or for nucleic acid hybridization, was freshly prepared for each experiment. Specific activities of ^{32}P-labeled viral RNA, estimated from the specific activity of the cell RNA (assuming one round of cell division, during which 50% of the cellular RNA would be labeled), were 5×10^6 to 10×10^6 cpm/μg. When precise values were needed, specific activities of the [^{32}P]RNA were calculated by determining the amount of radiolabeled [^{3}H]DNA and [^{32}P]RNA in a symmetrical hybrid, as described in the section about nucleic acid hybridization.

Poly(U)-Sepharose fractionation of RNA. Poly(U)-Sepharose was obtained commercially (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) or prepared as described in the accompanying paper (67). RNA was dissolved in NETS buffer (0.2 M NaCl; 0.01 M EDTA; 0.01 M Tris, pH 7.4; 0.2% SDS) to give an RNA concentration of less than 2 mg/ml and was applied to a column containing poly(U)-Sepharose (approximately 1-ml packed volume for 1 mg of cell RNA or 50 μg of viral RNA). All steps were performed at room temperature. The column was washed successively with 5 to 10 column volumes of ETS buffer, 2 volumes of 50% (vol/vol) formamide in ETS, and 1 volume of 90% formamide in ETS. DNA and non-adenylated RNA appeared in the flow-through; poly(A)-containing RNA, which bound to the poly(U)-Sepharose, was eluted in the 50% formamide wash. Approximately 85% of the ^{32}P-labeled 35S viral RNA was recovered in the 50% formamide wash, whereas only 3 to 5% of the total cell RNA was present in this fraction. Bound and unbound RNA fractions were pooled separately, and RNA was concentrated by ethanol precipitation after adjusting the NaCl concentration to 0.2 M and, for the poly(A)-RNA fraction, after adding 50 μg of yeast tRNA as carrier.

Sucrose gradient centrifugation and molecular weight determinations. Total cell RNA or poly(A)-containing RNA (usually less than 50 μg per gradient; see figure legends) was dissolved in 0.4 ml of ETS buffer and heated for 1 min at 85°C to dissociate RNA aggregates. The sample was quickly cooled to room temperature and applied to a linear gradient of 15 to 30% (wt/wt) sucrose in a buffer containing 0.05 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4), 0.01 M EDTA, and 0.2% SDS. Centrifugation was performed at 24°C for 6.5 h (for SR-RSV-B-infected cells) or 7.5 h (all others) at 40,000 rpm with a Beckman SW40 rotor.

Sedimentation values were calculated from the 18S and 28S rRNA present in the same gradient when total cell RNA was used or in a parallel gradient when poly(A) RNA was used. ^{32}P-labeled 35S RAV-2 RNA was also included in all gradients (see below). Molecular weights of viral RNAs were calculated from Spirin's formula: molecular weight = $1{,}550 \times S^{2.1}$ (54). Genetic complexities (number of nucleotides per RNA molecule) were calculated by dividing the molecular weight of the RNA by 323, the average molecular weight of a nucleotide in RSV RNA (2). Under our conditions of centrifugation, heat-dissociated RAV-2 virion RNA sedimented at 35S relative to 18S and 28S rRNA's; SR-RSV-B RNA sedimented at 39S. The molecular weights calculated from these sedimentation values are 2.7×10^6 (8,400 nucleotides) and 3.4×10^6 (10,500 nucleotides) for RAV-2 and SR-RSV-B virion RNAs, respectively, in good agreement with published values (2, 3, 9, 10, 43).

Preparation of poly(A)-containing fragments of RAV-2 RNA. A 100-μg amount of RAV-2 35S RNA, containing approximately 10^5 cpm of [^{32}P]RNA to aid in identifying fragments, was dissolved in ETS buffer and randomly fragmented by incubating separate aliquots of the RNA for 10 and 30 min at 100°C. The aliquots were combined and applied to a poly(U)-Sepharose column, and the column was

washed extensively with ETS. Poly(A)-containing fragments were eluted with 50% formamide, concentrated by ethanol precipitation, and fractionated by sucrose gradient centrifugation. The fragments were distributed over a broad size range, from approximately 10 to 35S (Fig. 1, top). Selected fractions from the gradient were combined into four pools (Fig. 1). At this stage the isolated fragments were not sufficiently pure to be useful in hybridization experiments. Further purification was achieved by repeating the poly(U)-Sepharose and sucrose gradient centrifugation steps with each of the RNA pools. The two peak fractions from each gradient (Fig. 1, bottom) were combined and precipitated with ethanol, using 50 μg of yeast tRNA as carrier. The sedimentation values for the four poly(A)-fragment preparations were 27–29S, 23–25S, 19–21S, and 14–16S. The corresponding genetic complexities, calculated from the highest sedimentation value for each pool, were 5,700, 4,500, 2,900, and 1,600 nucleotides per fragment.

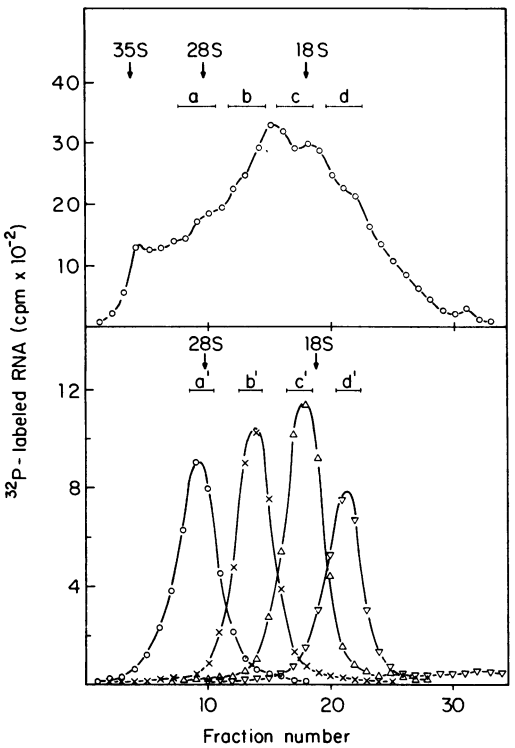

FIG. 1. *Preparation of poly(A)-containing RAV-2 RNA fragments. Randomly fragmented RAV-2 RNA was passed through poly(U)-Sepharose, and poly(A)-containing RNA fragments were recovered (see text). This RNA was then fractionated by rate zonal centrifugation (top), and appropriate fractions were combined into four pools (a–d). Each RNA pool was again passed through poly(U)-Sepharose. Bound material was eluted and centrifuged as before (bottom; results of four separate gradients are plotted on the same illustration). The two peak fractions from each gradient (a'–d') were collected, precipitated with ethanol, and used for hybridization experiments. Ribosomal 28S and 18S marker RNAs were centrifuged in parallel gradients.*

Synthesis of [^{3}H]DNA complementary to viral RNA. Viral cDNA was synthesized from detergent-treated virions as described previously (20), except that higher nucleotide concentrations and a lower Mg^{2+} concentration were used, to increase the size of the cDNA product (46). Virus stocks were used immediately after purification, and optimum detergent concentrations were determined for maximum incorporation with each virus preparation (29). The reaction mixture contained 20 mM Tris-hydrochloride (pH 8.1); 3 mM $MgCl_2$; 15 mM dithiothreitol; 100 μg of actinomycin D per ml; 1 mM each dGTP, dATP, and dCTP; 0.04 mM [^{3}H]TTP (50 Ci/mmol); 0.01 to 0.025% Nonidet P-40 (as determined by preliminary testing for detergent optimum); and purified virus (0.5 to 2 mg of viral protein per ml). The mixture was incubated at 38°C for 8 to 12 h, and the reaction was stopped by addition of 2 volumes of ETS buffer. The specific activity of the ^{3}H-cDNA product was 2×10^7 cpm/μg. The DNA was treated with proteinase K-SDS, extracted with phenol-chloroform as described above for viral RNA, and precipitated twice with ethanol, using 50 μg of yeast tRNA as carrier. The sample was treated with alkali (0.2 N NaOH, 30 min at 50°C) to hydrolyze viral RNA to small fragments and centrifuged on alkaline sucrose gradients (33). High-molecular-weight DNA molecules of appropriate length were recovered from the gradients and used for isolation of specific probes, as described in Results. Before hybridization, cDNA was cleaved by depurination (33) to give an average fragment length of approximately 150 nucleotides. Depurination was achieved by incubating DNA for 25 min at 70°C in 0.1 M sodium acetate (pH 4.2); NaOH was then added to 0.2 N, and the solution was incubated 20 min at 100°C. The solution was neutralized with sodium acetate (pH 4.2), and DNA was precipitated with ethanol.

Nucleic acid hybridization. All hybridizations were performed under conditions of moderate stringency to permit annealing between closely related but imperfectly matched nucleic acid sequences (21). The conditions used were sufficiently stringent, however, to exclude base-pairing between nonhomologous nucleic acids (21). Annealing mixtures contained 30% formamide; 0.45 M NaCl; 0.045 M sodium citrate, pH 7.2; 0.005 M EDTA; 0.2% SDS; and appropriate amounts of cDNA and RNA, as indicated in figure legends. Reaction mixtures were incubated in sealed capillaries at 50°C for varying times as required for each experiment. Hybridization of ^{3}H-cDNA was monitored by S-1 nuclease treatment as described previously (19); hybridization of [^{32}P]RNA was detected by treatment with RNase A (Worthington Biochemicals, Corp., Freehold, N. J.) (50 μg/ml; 60 min at 37°C; in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.2). All data have been corrected for background levels of approximately 1% for ^{3}H-cDNA and 4% for [^{32}P]RNA.

To evaluate saturation levels in hybridizations between ^{3}H-cDNA and [^{32}P]RNA (experiments shown in Fig. 2 and Table 3) it was important to

know the ratio of DNA to RNA in each reaction mixture. Because the specific activity values for the ^{3}H-cDNA (based on the specific activity of the substrate [^{3}H]TTP and the counting efficiency for ^{3}H) and for the [^{32}P]RNA (based on the specific activity of the RNA from the cells used to produce the ^{32}P-labeled virus) were subject to some error, it was necessary to have a method for determining DNA/RNA ratios that was independent of these values. For this purpose, a portion of each [^{32}P]RNA preparation was annealed to an approximately equal amount of ^{3}H-cDNA, and the resulting hybrid was treated with S-1 nuclease to digest unhybridized DNA and RNA. (S-1 nuclease digests 99% of single-stranded cDNA and 95 to 96% of single-stranded RNA.) The ratio of ^{3}H to ^{32}P in the remaining symmetrical hybrid provided an accurate measurement of the relative specific activities of the ^{3}H-cDNA and [^{32}P]RNA. The DNA/RNA ratios in hybridization experiments were then calculated by comparing the input ^{3}H/^{32}P ratio in each hybridization mixture to the ^{3}H/^{32}P ratio of the symmetrical hybrid.

The concentration of virus-specific RNA in normal or infected cells was determined by comparing the kinetics of hybridization between ^{3}H-cDNA and cell RNA to that between ^{3}H-cDNA and purified viral RNA, using $C_rt_{1/2}$ values as a measure of reaction rate (20, 34). C_rt equals the product of RNA concentration (moles of nucleotide per liter) times reaction time (seconds); $C_rt_{1/2}$ equals the C_rt value at which half-maximal hybridization is attained. The number of copies of viral RNA per cell was calculated from the following formula: copies per cell = [($C_rt_{1/2}$ for viral RNA)/($C_rt_{1/2}$ for cell RNA)] × [(grams of RNA per cell)/(grams of RNA per viral RNA subunit)]. $C_rt_{1/2}$ values were determined experimentally; grams of RNA per cell = 10^{-11}; grams of RNA per viral subunit = 4.5×10^{-18} (RAV-2) or 5.7×10^{-18} (SR-RSV-B).

Sucrose gradient fractions were analyzed for the presence of viral RNA by mixing 3 μl of each fraction with an equal volume of hybridization mixture containing ^{3}H-cDNA probe (300 to 400 cpm per hybrid reaction) and other reaction components in appropriate amounts to give the desired final concentrations. Incubation times (shown in figure legends) were adjusted in each case to give hybridization values of up to 30 to 40%, a range in which the relationship between RNA concentration and percent hybridization is approximately linear when RNA is in sufficient excess, as was the case in these experiments. Thus, the hybridization values shown for different gradients do not reflect the absolute concentrations of viral RNA, but they do provide a reasonable estimate of the relative amounts (numbers of copies) of each size class of viral RNA within a single gradient.

RESULTS

Isolation of gene-specific ^{3}H-cDNA probes. The viral RNAs and cDNA's used to select the specific cDNA probes, the procedures used for their isolation, and the predicted characteristics of each probe are outlined in Table 1. Viruses deleted within the *src* gene (td mutants and leukosis viruses) and the *env* gene (BH-RSV and SR-RSV-N8) have been well characterized (9, 10, 31, 65), and the RNAs from these viruses can be used to select *src*-specific (57) and *env*-specific (22, 60) cDNA probes. However, no suitable deletion mutants within the other viral genes are currently available. Isolation of probes specific for the *gag* and *pol* genes ($cDNA_{gag}$ and $cDNA_{pol}$) was effected by selective hybridizations with poly(A)-containing RNA fragments of appropriate length and was based on the approximate map locations of these genes (27, 28, 65, 66). For the present studies we have assumed that *gag* is located within the 2,500 to 2,800 nucleotides adjacent to the 5′ terminus of the viral RNA and that *pol* is located within the next segment of approximately 2,500 nucleotides. Since the precise map locations of these genes have not been firmly established, the designations of $cDNA_{gag}$ and $cDNA_{pol}$ should be considered tentative. $cDNA_c$ was prepared by selecting sequences that hybridized both to short poly(A)-containing RAV-2 RNA fragments (thus excluding 5′-proximal sequences) and to BH-RSV RNA (eliminating *env*-specific sequences), as described in Table 1. This DNA should correspond to the RNA segment located between the poly(A) tract and the *env* gene. [$cDNA_c$ does not hybridize to poly(A) or to nonviral mRNA, which contains poly(A) (data not shown).]

The ^{3}H-cDNA used for these isolations was synthesized from detergent-treated SR-RSV-B (for $cDNA_{src}$) or RAV-2 (all others), as described in Materials and Methods. The cDNA product was fractionated by alkaline sucrose gradient centrifugation into three size classes, with molecular weights of approximately 0.5×10^6 to 1×10^6, 1×10^6 to 1.5×10^6, and $>1.5 \times 10^6$. These three pools represented approximately 45, 20, and 8%, respectively, of the total ^{3}H-cDNA product. The largest DNA, which was highly representative of the total genome (see below), was used to isolate sequences located near the 5′ terminus of the genome (i.e., *gag* and *pol*); the smaller DNAs, which were enriched for 3′ sequences, were used to select the remaining probes. Each size class of DNA was fragmented to an average length of approximately 150 nucleotides before hybridization with RNA, to permit better separation between hybridized and unhybridized DNA sequences in the subsequent hydroxylapatite chromatography step. After the annealing of cDNA to the appropriate viral RNA (see Table 1), the hybridized and unhybridized DNA sequences were separated by hydroxylapatite chromatography (22). The selected cDNA was purified and

TABLE 1. *Isolation of gene-specific cDNA probes*

Probe	Method of isolation: Nucleic acids hybridized[a]		cDNA sequences selected[d]	Final recovery (%)[e]	Expected portion of genome represented in probe[f]
	^{3}H-cDNA (MW × 10^{-6})[b]	Viral RNA[c]			
$cDNA_{gag}$	RAV-2 cDNA (1.5–3)	Poly(A) fragment (5,700 nucleotides)	Unhybridized	10	5,700–8,400 nucleotides from 3′ end of RAV-2 RNA
$cDNA_{pol}$	Step 1: RAV-2 cDNA (1.5–3)	Poly(A) fragment (5,700 nucleotides)	Hybridized		200–5,700-nucleotide interval
	Step 2: Hybridized cDNA (repurified) from step 1	Poly(A) fragment (2,900 nucleotides)	Unhybridized	15	2,900–5,700-nucleotide interval
$cDNA_{env}$	RAV-2 cDNA (1–1.5)	BH-RSV 35S RNA	Unhybridized	25–30	*env* region, deleted in BH-RSV
$cDNA_{src}$	SR-RSV-B cDNA (>1)	RAV-2 35S RNA	Unhybridized	15	*src* region, deleted in RAV-2
$cDNA_{r}$	Step 1: RAV-2 cDNA (0.5–1)	Poly(A) fragment (1,600 nucleotides)	Hybridized[g]		200–1,600-nucleotide interval
	Step 2: Hybridized cDNA (repurified) from step 1	BH-RSV 35S RNA	Hybridized[g]	25–30	Region between *env* (as defined by BH-RSV deletion) and poly(A)

[a] Hybridization was performed as described in the text, using 0.1 to 0.2 μg of cDNA and 10 to 20 μg of RNA per ml. Hybridizations were carried out to C_0t values of 0.3 to 0.5 mol·s/liter with poly(A)-containing RNA fragments or 2 to 5 mol·s/liter with 35S virion RNA. Two cycles of hybridization and hydroxylapatite chromatography were performed with each sample to ensure complete removal of undesired sequences.

[b] ^{3}H-cDNA was fractionated by alkaline sucrose gradient centrifugation, and DNA molecules of the indicated sizes were pooled. DNA was fragmented to an average length of 150 nucleotides before hybridization. MW, Molecular weight.

[c] 35S RNA was extracted from purified RAV-2 or BH-RSV, as indicated. Poly(A) fragments of appropriate length were prepared from RAV-2 35S RNA (Fig. 1).

[d] Hybridized and unhybridized DNA sequences were separated by hydroxylapatite chromatography (20). The DNA-RNA hybrid fraction was treated with S-1 nuclease to digest single-stranded "tails," and DNA was repurified by proteinase K-SDS digestion and phenol-chloroform extraction. RNA was removed by alkaline hydrolysis.

[e] Percentage of input ^{3}H-cDNA recovered in the purified cDNA probe.

[f] Intervals represent nucleotide distance from the 3′ end of RAV-2 RNA. (Total length of RAV-2 RNA = 8,400 nucleotides). The first 200 nucleotides are poly(A) (43, 62), which is apparently not transcribed into DNA, since the cDNA's do not hybridize to poly(A).

[g] Hydroxylapatite chromatography was omitted. DNA-RNA hybrids were treated directly with S-1 nuclease, and DNA was repurified as described in footnote *d*.

recycled through the hybridization and hydroxylapatite steps, to ensure complete removal of undesired sequences. Hybridizations were performed under conditions of moderate stringency (21) to permit annealing between any partially mismatched nucleotide sequences that might be present within the common regions of the viral nucleic acids used for selections of $cDNA_{src}$ and $cDNA_{env}$. Other details are described in Table 1.

Specificity of selected cDNA's and homologies among different viral nucleic acids. The specificities of $cDNA_{src}$ and $cDNA_{env}$ for their respective genes were tested by hybridization to viral RNAs from various transforming and nontransforming viruses and from two viruses deleted within the *env* gene. As shown in Table 2, $cDNA_{src}$ hybridized extensively (87 to 93%) with RNAs from four different sarcoma viruses, but did not hybridize (<3%) with RNAs from three nontransforming viruses, RAV-0, RAV-2, and RAV-7. Likewise, $cDNA_{env}$ hybridized with

TABLE 2. *Extent of homology between gene-specific cDNA probes and viral RNAs*[a]

Probe	Viral RNA						
	RAV-0	RAV-2	RAV-7	SR-RSV-B	PR-RSV-B	BH-RSV	SR-RSV-N8
$cDNA_{gag}$	87	93	90	91	86	90	91
$cDNA_{pol}$	78	89	ND[b]	86	ND	87	ND
$cDNA_{env}$	78	95	83	92	85	1.1	1.8
$cDNA_{src}$	2.5	1.5	2.2	93	87	90	92
$cDNA_c$	45	95	89	91	81	94	89

[a] Maximum extent of hybridization (percent ^{3}H-cDNA hybridized) between cDNA and 30–40S RNAs from the indicated viruses. Hybridizations were performed in quadruplicate, using 400 to 500 cpm of ^{3}H-cDNA (specific activity, 2×10^7 cpm/μg) per reaction and viral 70S RNA at 5 to 15 μg/ml. Hybrid reactions were incubated 20 to 40 h at 50°C, to C_rt values of 2 to 5 mol·s/liter. $C_rt_{1/2}$ values for all reactions were between 0.02 and 0.04 mol·s/liter.

[b] ND, Not determined.

RNAs from *env*-positive viruses of various subgroups, but not with RNA from the two known viruses deleted within this gene, BH-RSV and SR-RSV-N8. Thus, both of these probes appear to be highly specific.

$cDNA_{gag}$, $cDNA_{pol}$, and $cDNA_c$ were also hybridized with different viral RNAs to determine the extents of homology between RAV-2 and other viruses within these three regions. High levels of hybridization (>80%) were obtained with RNAs from all of the viruses except RAV-0 (Table 2), indicating close homologies within the *gag*, *pol*, and *c* regions. Somewhat lower levels of hybridization were observed with RAV-0 RNA, particularly within the *c* region. Only 45% of the $cDNA_c$ annealed to RAV-0 RNA, compared with 81 to 95% annealing with RNAs from the other three viruses.

Genetic complexity of specific probes. The portion of the viral genome represented in each of the probes was estimated by annealing cDNA to ^{32}P-labeled viral RNA, with DNA in excess, and determining the fraction of the RNA that was protected from RNase digestion. The high-molecular-weight ($>1.5 \times 10^6$) RAV-2 cDNA, used for isolation of $cDNA_{gag}$ and $cDNA_{pol}$, protected at least 95% of the RAV-2 RNA (Fig. 2) and thus contains sequences representing essentially the entire viral genome. Furthermore, 85% protection was obtained at a DNA/RNA ratio of 1, demonstrating that this DNA contains a fairly uniform representation of all of the viral sequences. The high-molecular-weight cDNA from SR-RSV-B protected 96% of its homologous RNA at a DNA/RNA ratio of 10 and 80% at a ratio of 1 (data not shown). In contrast, each of the selected probes protected only a portion of the viral RNA. The plateau values for the five probes, especially $cDNA_c$, $cDNA_{src}$, and $cDNA_{env}$, were quite stable over a 10- to 20-fold range of DNA/RNA ratios, showing that these probes are relatively free of contaminating sequences from other portions of the genome. $cDNA_{src}$ protected approximately 19% of the SR-RSV-B RNA but only about 1% of the RAV-2 RNA, confirming the specificity of this probe for sarcoma virus-specific sequences.

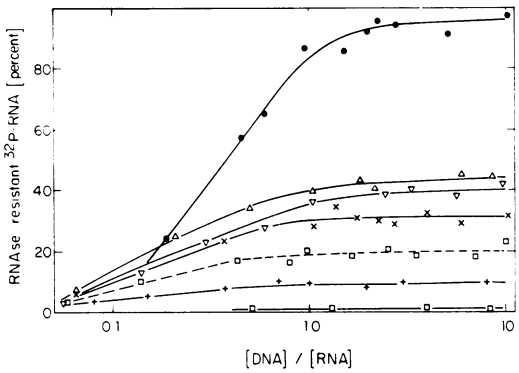

FIG. 2. *Genetic complexity of gene-specific cDNA probes. ^{3}H-cDNA probes were hybridized to ^{32}P-labeled 30–40S virion RNA from RAV-2 (solid lines) or SR-RSV-B (broken line), with increasing DNA/RNA ratios, to determine the percentage of the viral RNA that could be protected from RNase digestion. Probes were $cDNA_{gag}$ (△), $cDNA_{pol}$ (▽), $cDNA_{env}$ (×), $cDNA_{src}$ (□), $cDNA_c$ (+), and unselected high-molecular-weight ($>1.5 \times 10^6$) RAV-2 cDNA (●). cDNA was used at concentrations of 0.2 to 0.4 μg/ml. Concentrations of [^{32}P]RNA (specific activity, 0.5×10^7 to 1×10^7 cpm/μg) varied, as indicated by the DNA/RNA ratios.*

Table 3 provides a summary of hybridization data obtained with different combinations of cDNA probes and viral RNAs and shows the estimated genetic complexity of each probe (i.e., the size of the region protected by the probe, expressed in terms of nucleotides). The complexities calculated from the hybridization data may slightly overestimate the size of the regions represented by each of the probes. Since the hybridizations shown in Table 3 were per-

TABLE 3. *Genetic complexities of cDNA probes and extent of overlapping between probes*

[^{32}P]RNA	^{3}H-cDNA	% Hybridized		% Overlap[c]	Complexity[d]
		Experimental[a]	Predicted[b]		
RAV-2	RAV-2 cDNA[e]	96			8,100
	$cDNA_{gag}$	41			3,400
	$cDNA_{pol}$	38			3,200
	$cDNA_{env}$	30			2,500
	$cDNA_{src}$	1			
	$cDNA_{c}$	9			750
	$cDNA_{gag}$ + $cDNA_{pol}$	68	79	11	5,700
	$cDNA_{gag}$ + $cDNA_{env}$	69	71	2	5,800
	$cDNA_{gag}$ + $cDNA_{c}$	50	50	0	4,200
	$cDNA_{pol}$ + $cDNA_{env}$	62	68	6	5,200
	$cDNA_{pol}$ + $cDNA_{c}$	47	47	0	3,900
	$cDNA_{env}$ + $cDNA_{c}$	38	39	1	3,200
	$cDNA_{gag}$ + $cDNA_{pol}$ + $cDNA_{env}$ + $cDNA_{c}$	97			8,100
SR-RSV-B	SR-RSV cDNA[f]	95			9,500
	$cDNA_{env}$	21			2,100
	$cDNA_{src}$	19			1,900
	$cDNA_{src}$ + $cDNA_{env}$	39	40	1	3,900
	$cDNA_{gag}$ + $cDNA_{pol}$ + $cDNA_{env}$ + $cDNA_{src}$ + $cDNA_{c}$	94			9,400

[a] Percent [^{32}P]RNA protected from RNase digestion, as shown in Fig. 2, using a DNA/RNA ratio of 5 to 10. (DNA concentration was adjusted to reflect the approximate complexity of each probe, to have similar ratios of the corresponding DNA and RNA sequences in each hybrid reaction.) The concentration of each cDNA probe, when used in combination with other probes, was the same as that used for each probe separately. 500 to 1,000 cpm of [^{32}P]RNA (5×10^6 to 10×10^6 cpm/μg) was used for each reaction. Values represent the average of four to five determinations. Standard deviations were 1.7% or less of the total [^{32}P]RNA.

[b] Sum of the hybridization values obtained with each probe separately.

[c] Difference between the predicted value (sum of the individual values) and the actual experimental value obtained with the combination of probes. Overlap values of 2% or less are not statistically significant.

[d] Genetic complexity (in nucleotides) calculated from the percentage of the ^{32}P-labeled viral RNA protected from RNase digestion (i.e., percent hybridized, experimental value). For purposes of calculation, the genetic complexity of RAV-2 RNA was assumed to be 8,400 nucleotides (calculated from our sedimentation value of 35S), and for SR-RSV-B RNA the complexity was assumed to be 10,000 nucleotides (2, 3, 10, 43; our calculated value is slightly higher [10,500 nucleotides]).

[e] Unselected high-molecular-weight ($>1.5 \times 10^6$) cDNA from RAV-2 (used for preparation of $cDNA_{gag}$ and $cDNA_{pol}$).

[f] Unselected high-molecular-weight ($>10^6$) cDNA from SR-RSV-B (used for preparation of $cDNA_{src}$).

formed with DNA in 5 to 10-fold excess over RNA, contaminating sequences present in very low amounts would contribute to the levels of protection observed. However, the complexities of $cDNA_{src}$ (1,900 nucleotides), $cDNA_{env}$ (2,100 to 2,500), and $cDNA_{c}$ (750) are in reasonable agreement with the sizes of these regions estimated by Wang and co-workers (9, 65, 66) and Joho et al. (27, 28). The complexities of $cDNA_{gag}$ (3,400 nucleotides) and $cDNA_{pol}$ (3,200 nucleotides), on the other hand, are somewhat higher than the sizes of the corresponding regions [2,700 and 2,800 nucleotides, respectively, as calculated from the size of the poly(A) fragments used for their isolation], suggesting that these two probes contain some contaminating cDNA sequences from other regions of the genome (see below).

Each cDNA probe was hybridized in combination with various other probes to determine whether they contained any sequences in common. If two probes contain overlapping sequences, the level of protection obtained with the combination of the two probes would be less than the sum of the values obtained with each probe separately; the extent of overlap would be equal to the difference between the sum of the individual values and the value obtained with the two probes together. $cDNA_{src}$, $cDNA_{env}$, and $cDNA_{c}$ appear to be specific for different portions of the genome, since the various combinations of these probes give essentially addi-

tive values (Table 3). However, $cDNA_{gag}$ and $cDNA_{pol}$ appear to extend somewhat into adjacent regions. An approximately 11% overlap was observed between these two probes, and an approximately 6% overlap was observed between $cDNA_{pol}$ and $cDNA_{env}$. The heterogeneity of $cDNA_{gag}$ and $cDNA_{pol}$ may be due to the slight heterogeneity in the lengths of the poly(A)-containing RNA fragments used for the selection of these probes or to the presence in the poly(A)-fragment preparations of small amounts of contaminating RNA sequences from other portions of the viral genome. Nevertheless, $cDNA_{gag}$ and $cDNA_{pol}$ contain primarily unique sequences, and should thus be satisfactory reagents for analysis of their respective portions of the viral genome. A combination of all five probes provided essentially complete protection (94%) of SR-RSV-B RNA; likewise, the four leukosis virus-specific probes protected 96% of the RAV-2 RNA. Thus, these probes collectively represent essentially the entire genomes of these two viruses.

Map locations of specific probes. The approximate locations of the RNA sequences corresponding to each of the leukosis virus-specific probes were determined by annealing the probes to poly(A)-containing RAV-2 RNA fragments of different lengths. Poly(A) fragments with lengths of approximately 1,600, 2,900, 4,500, and 5,700 nucleotides, as well as full-length RAV-2 RNA (8,400 nucleotides), were used, thus making it possible to define five different intervals within the viral genome. The annealing patterns obtained with each probe are shown in Table 4, and the percentage of each probe that hybridized specifically within each of the five regions is shown in Table 5. $cDNA_{gag}$ hybridized primarily (78%) within the 2,700-nucleotide interval closest to the 5′ terminus, with some hybridization in the adjacent interval. $cDNA_{pol}$ hybridized 89% to sequences located within the intervals of 2,900 to 4,500 and 4,500 to 5,700 nucleotides from the 3′ end; $cDNA_{env}$ hybridized mostly within the interval of 1,600 to 2,900 nucleotides, with some annealing to the 1,600-nucleotide fragment; and $cDNA_c$ hybridized almost exclusively within the 0 to 1,600-nucleotide interval. These data are in good agreement with the expected map locations of each probe (27, 28, 65, 66). In all cases, very little hybridization was observed outside the expected regions, again demonstrating the specificity of the probes.

The data shown in Tables 2 through 5 make it possible to estimate the size and map location of the RNA sequences corresponding to each of the selected probes. The approximate position of each probe relative to the genetic map of RSV is shown in Fig. 3.

TABLE 4. *Hybridization of specific cDNA probes to poly(A)-containing RAV-2 RNA fragments of different lengths*

Probe	^{3}H-cDNA hybridized (%) to RNA fragment of indicated length[a]				
	1,600	2,900	4,500	5,700	8,400[b]
$cDNA_{gag}$	1	4	8	22	100
$cDNA_{pol}$	1	6	56	95	100
$cDNA_{env}$	30	95	96	98	100
$cDNA_c$	93	95	98	99	100

[a] Hybridizations were performed to a C_rt of 0.3 to 0.5 mol·s/liter. (RNA concentrations for RNA fragments were adjusted to reflect the proportion of the RNA genome represented in the fragment.) Isolation of poly(A)-containing RNA fragments and calculations of fragment length (nucleotides per fragment) are described in the text and in the legend to Fig. 1. All values have been normalized to the maximum hybridization level obtained with 35S RAV-2 RNA.

[b] Unfragmented 35S RAV-2 RNA.

TABLE 5. *Specific annealing of cDNA probes within defined segments of RAV-2 RNA*

Probe	^{3}H-cDNA hybridized (%) within indicated intervals[a]				
	0–1,600	1,600–2,900	2,900–4,500	4,500–5,700	5,700–8,400
$cDNA_{gag}$	1	3	4	14	78
$cDNA_{pol}$	1	5	50	39	5
$cDNA_{env}$	30	65	1	2	2
$cDNA_c$	93	2	3	1	1

[a] Intervals are defined by the lengths of the poly(A)-containing RNA fragments listed in Table 4 and are expressed in terms of the nucleotide distance from the 3′ end of the genome of RAV-2. The percent ^{3}H-cDNA hybridized within each interval was calculated by subtracting the percent hybridized to a specific fragment (Table 4) from the percent hybridized to the next-larger fragment.

Amounts of gene-specific viral RNAs in virus-infected cells. RNA from RAV-0-, RAV-2-, or SR-RSV-B-infected cells was hybridized to each of the selected cDNA probes to determine the relative abundance of RNA sequences transcribed from different portions of the proviral genome. RNA content was calculated from the hybridization kinetics, using purified virion RNA as a standard (see Materials and Methods). Standard curves were constructed for each cDNA-viral RNA combination to minimize errors due to differences in hybridization kinetics that might be specific to the various probes or DNA-RNA pairs (see Discussion).

All of the viral genes were represented at high levels (1,600 to 20,000 copies per cell) in the RNA of the infected cells (Table 6), with the exception of the leukosis virus-infected cells, which contained only small amounts of *src*-specific RNA, as expected. The *src*-specific RNA in these cells was probably transcribed from the endogenous *src*-related gene described by Stehelin et al. (58), since similar amounts of *src* RNA were present in uninfected cells (Table 6; see also accompanying paper [67]). Expression of the endogenous *src* gene was apparently not affected by the presence of RAV-2 in the cell.

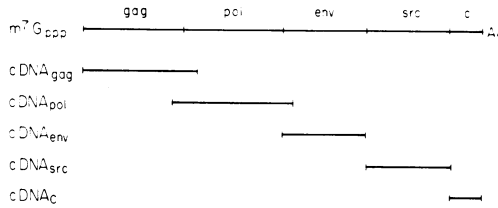

FIG. 3. *Relationship between the genetic map of RSV and gene-specific cDNA probes. Approximate map location of each probe was estimated from the data in Tables 2 through 5, in conjunction with the genetic mapping data of Wang et al. (65, 66) and Joho et al. (27, 28). The size of the region corresponding to each probe was based on the data in Fig. 2 and Table 2.*

TABLE 6. *Content of gene-specific RNAs in virus-infected cells*

Region analyzed[b]	RNA content (copies/cell)[a]			
	Uninfected cells[c]	RAV-0-producing cells[d]	RAV-2-infected cells[c]	SR-RSV-B-infected cells[c]
gag	0.2	1,600	9,800	10,000
pol	ND[e]	1,700	9,400	10,200
env	0.5	3,300	16,000	20,000
src	5	ND	7	18,000
c	ND	ND	17,000	19,500

[a] Viral RNA content was calculated from the kinetics of hybridization between ^{3}H-cDNA probe and cell RNA, as described in the text. Standard C_rt curves, obtained with purified virion 30-40S RNA, were prepared for each cDNA-viral RNA pair, to minimize possible errors due to probe-specific or viral RNA-specific kinetic differences. Values thus represent "genome equivalents" and do not reflect possible duplications of sequences in the viral genome (see text).

[b] Analyses were performed using $cDNA_{gag}$, $cDNA_{pol}$, $cDNA_{env}$, $cDNA_{src}$, and $cDNA_c$, respectively.

[c] Uninfected or infected gs$^-$chf$^-$ cells.

[d] Line 7 × 15 cells, which spontaneously release RAV-0 (6).

[e] ND, Not determined.

In all three types of infected cell the *gag* and *pol* sequences were relatively less abundant than the other viral sequences. The differences were not large (approximately twofold), but they were consistently observed in different experiments. The unequal representation of viral sequences presumably reflects differences among the smaller viral RNA transcripts, since the 30-40S RNAs should contain equal amounts of all viral sequences. Since the full-length transcripts would partially obscure any contribution of the smaller RNAs in these analyses, the differences in sequence representation among the smaller RNAs might be much greater than twofold.

Size and genetic content of viral RNAs in leukosis virus-infected cells. RNA from RAV-2-infected cells was fractionated on sucrose gradients, and aliquots from each fraction were hybridized with each of the leukosis virus-specific cDNA probes. Two distinct peaks of viral RNA were detected, with sedimentation values of approximately 35S and 21S (Fig. 4, top). The 35S RNA, which cosedimented with the ^{32}P-labeled 35S virion RNA marker, hybridized with all four probes and thus contains complete leukosis virus genetic information. (The marker 35S RNA was present at a concentration approximately 1/1,000 that of the cellular virus-specific RNA, and would not be detected by hybridization under the conditions used.) In contrast, the 21S RNA hybridized only with $cDNA_{env}$ and $cDNA_c$. No distinguishable peaks of *pol* or *gag* RNA were detected in the lower-molecular-weight (10 to 30S) region of the gradient.

To determine whether these viral RNAs are polyadenylated, the total cell nucleic acid was fractionated by affinity chromatography on poly(U)-Sepharose, which binds poly(A)-containing polynucleotides. Only 3 to 5% of the total cell RNA was bound to the poly(U)-Sepharose, but 85% of the virus-specific RNA was bound, as determined by kinetic hybridization analysis of the bound and unbound RNA fractions. A similar percentage (80 to 85%) of the ^{32}P-labeled viral 35S RNA marker was bound. The two RNA pools (bound and unbound) were fractionated on sucrose gradients and analyzed for the presence of viral RNA. Both the 35S and the 21S virus-specific RNAs were present in the poly(A) fraction (Fig. 4, middle); the pattern observed was virtually identical to that obtained with total cell RNA (Fig. 4, top). In contrast, viral sequences were barely detectable (at the same level of sensitivity) in the gradient containing non-polyadenylated RNA (Fig. 4, bottom), and no distinct peaks could be resolved by using either $cDNA_{gag}$, $cDNA_{pol}$, or $cDNA_{env}$.

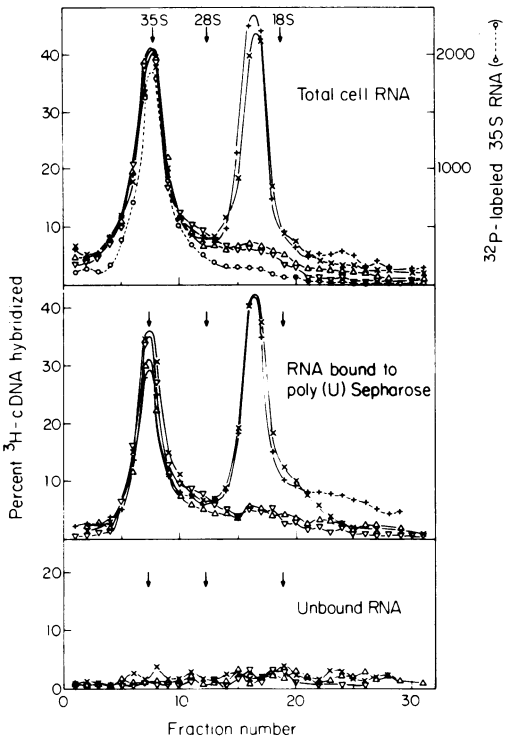

FIG. 4. *Size of gene-specific viral RNAs in RAV-2-infected cells. RNA from RAV-2-infected cells was fractionated by rate zonal centrifugation, and aliquots from each gradient fraction where hybridized to ³H-labeled cDNA$_{gag}$ (△), cDNA$_{pol}$ (▽), cDNA$_{env}$ (×), or cDNA$_c$ (+). Top: 70 μg of total cell RNA was applied to the gradient; hybridizations were carried out for 20 to 40 h. Middle: poly(A)-containing RNA from 300 μg of total cell RNA; hybridizations were carried out for 5 to 10 h. Bottom: non-poly(A)-containing cell RNA (60 μg); hybridizations were carried out for 25 to 50 h. ³²P-labeled RAV-2 35S RNA (○), added to cells immediately before RNA extraction, serves both as a size marker and as a monitor for RNA breakdown. Conditions for sucrose gradient centrifugation and hybridization are described in the text.*

Thus, only two major species of virus-specific RNA are present in RAV-2-infected cells, and both are polyadenylated. The 35S RNA is indistinguishable in sedimentation value, genetic content, and poly(A) content from the 35S RNA in the virion. The 21S RNA, however, contains only *env*, *c*, and poly(A) and thus appears to be a transcript of the 3′ portion of the viral genome. The molecular weight of the smaller RNA, calculated from its sedimentation value, would be approximately 10^6, corresponding to a genetic complexity of roughly 3,000 nucleotides. This is consistent with the apparent genetic content of this molecule, *env* (approximately 2,300 nucleotides), *c* (450 to 750 nucleotides), and poly(A) (perhaps 50 to 200 nucleotides).

Line 7 × 15 cells, which spontaneously release the endogenous virus, RAV-0, were also analyzed. As shown in Table 6, these cells contain significant amounts of virus-specific RNA (although considerably less than RAV-2- or SR-RSV-B-infected cells). The pattern of virus-specific RNA in these cells (Fig. 5) was virtually identical to that in RAV-2-infected cells. Two RNA species were detected, a 35S RNA hybridizable to all four leukosis virus-specific probes, and a 21S RNA hybridizable only to cDNA$_{env}$ and cDNA$_c$. It should be noted, however, that the RAV-0 RNAs were not completely homologous with the cDNA probes prepared from RAV-2 (see Table 2). The data in Fig. 5 have been normalized to the maximum level of hybridization for each probe with RAV-0 virion RNA.

Size and genetic content of viral RNAs in sarcoma virus-infected cells. Sarcoma viruses contain one additional gene, *src*, which is not present in the leukosis viruses. The *src* gene, coding for sarcomagenic transformation, is located near the 3′ end of the genome, between *env* and *c* (27, 65). Because of this additional genetic information, the RNA of nondefective sarcoma viruses is 20 to 25% larger than that of leukosis viruses. Under our conditions, SR-RSV-B RNA sediments at approximately 39S (corresponding to a molecular weight of 3.4 × 10^6), whereas RAV-2 RNA sediments at 35S (2.7 × 10^6).

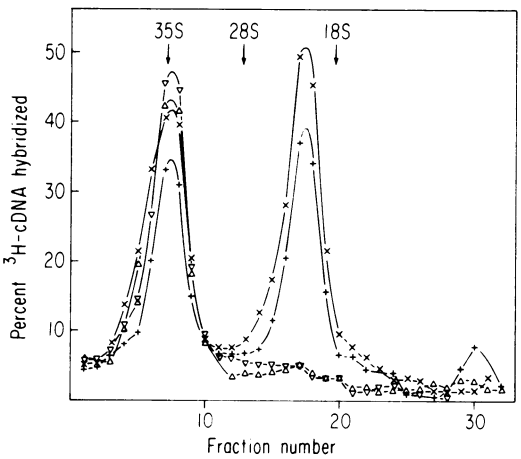

FIG. 5. *Size of gene-specific viral RNAs in RAV-0-producing line 7 × 15 cells. Poly(A)-containing RNA from 1 mg of line 7 × 15 cell RNA was fractionated by rate zonal centrifugation, and aliquots of each gradient fraction were hybridized to cDNA$_{gag}$ (△), cDNA$_{pol}$ (▽), cDNA$_{env}$ (×), or cDNA$_c$ (+). Hybridizations were carried out for 35 to 50 h.*

Three size classes of virus-specific RNA were detected in SR-RSV-B-infected cells (Fig. 6). The largest of these, sedimenting at 39S, was detectable with all five gene-specific probes and thus appears to be a complete transcript of the viral genome. The two smaller RNAs, a major peak at 28S and a minor peak at 21S, were detectable only with $cDNA_{env}$, $cDNA_{src}$, and $cDNA_c$. All three size classes of viral RNA were polyadenylated. [The data in Fig. 6 were obtained by using poly(A)-containing RNA.] As with RAV-2-infected cells, *gag* and *pol* sequences were not detectable in significant amounts in the lower-molecular-weight region, either in the poly(A)-containing RNA (Fig. 6) or in the total cell RNA (data not shown). The profiles obtained with total cell RNA were virtually identical to those shown in Fig. 6.

The genetic complexity of the 28S RNA, calculated from its sedimentation value, would be approximately 5,300 nucleotides, consistent with its apparent genetic content of *env* (2,300 nucleotides), *src* (1,600 to 1,900 nucleotides), *c* (450 to 750 nucleotides), and poly(A) (50 to 200 nucleotides), a total of 4,400 to 5,150 nucleotides. (We have some evidence, based on hybridization kinetics, that the *c* region may be duplicated in the 28S RNA, which would add another 450 to 750 nucleotides to this value [see Discussion].) This RNA species thus corresponds to the 3′ half of the SR-RSV-B genome. The 21S RNA was also detected with the same three cDNA probes, specific for *env*, *src*, and *c*. Since a molecule of this size could not accommodate these three genes, it seems likely that the 21S peak actually represents two different RNA molecules, one of which is specific for the *env* gene and the other for *src*. One possibility is that the *env*-specific RNA is derived from td virus present in the sarcoma virus stock. td viruses are known to continually segregate from the sarcoma virus population through deletion of the *src* gene and are thus present in nearly all sarcoma virus stocks (30, 62). Since the td virus is essentially the same as leukosis viruses, it would be expected to contribute the two RNA species present in RAV-2- and RAV-0-infected cells (i.e., 35S RNA and a 21S *env*-specific RNA). Small amounts of td virus (roughly 10% of the virus population) could account for the small peak of 21S *env*-specific RNA detected in the SR-RSV-B-infected cells and also for the slight shoulder in the 35S region of the profiles obtained with $cDNA_{gag}$ and $cDNA_{pol}$ (Fig. 6).

To test this possibility, we recloned the SR-RSV-B to remove td virus and isolated RNA from cells infected after only one virus passage. The RNA pattern from such recloned virus is shown in Fig. 7. As can be seen, the 21S *src*- and *c*-specific RNA peak was clearly present, whereas the *env*-specific 21S RNA was barely detectable. Thus, we conclude that the *env*-specific 21S RNA is derived from td virus and not from SR-RSV-B.

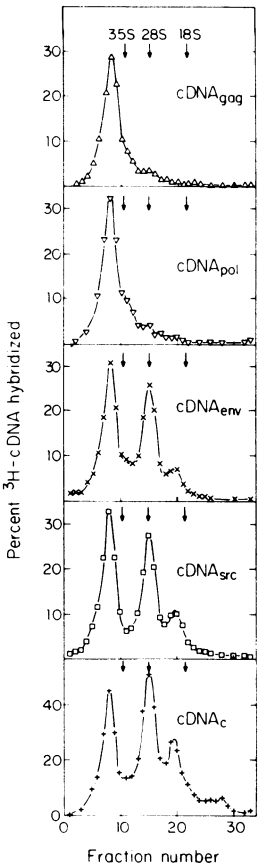

FIG. 6. *Size of gene-specific viral RNAs in SR-RSV-B-infected cells. Poly(A)-containing RNA from 500 μg of SR-RSV-B-infected cell RNA was fractionated by rate zonal centrifugation, and aliquots from each gradient fraction were hybridized with each of the five gene-specific probes, as indicated on the illustrations. Results for each probe are plotted separately, so that the individual profiles can be readily distinguished. Hybridizations were carried out for 2.5 to 5 h.*

Virus-specific RNA in cells infected with a defective sarcoma virus. BH-RSV contains a deletion within the *env* gene but is competent in all other viral functions, including cell transformation. The RNA of this virus is, consequently, approximately 20% smaller than that of SR-RSV-B (9). Cells infected with BH-RSV contained only two identifiable virus-specific RNAs, genome-length (35S) RNA, and a 21S RNA species detectable with $cDNA_{src}$ and $cDNA_c$ (Fig. 8). The latter RNA species would

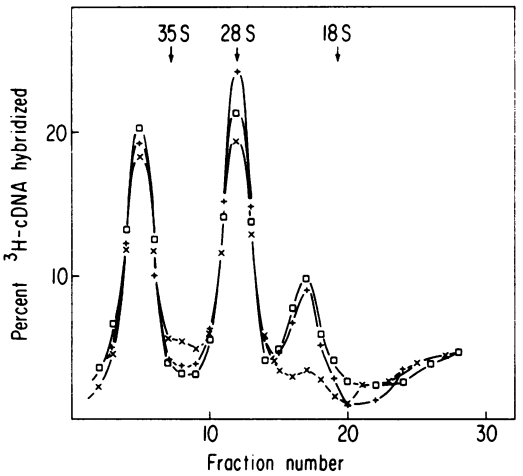

FIG. 7. *Presence of gene-specific RNAs in cells infected with recloned SR-RSV-B. SR-RSV-B was recloned by colony isolation in soft agar to remove td virus. Total RNA (50 μg) from cells infected with recloned virus was fractionated by rate zonal centrifugation, and aliquots were hybridized with cDNA$_{env}$ (×), cDNA$_{src}$ (□), or cDNA$_c$ (+). Hybridizations were carried out for 25 to 50 h.*

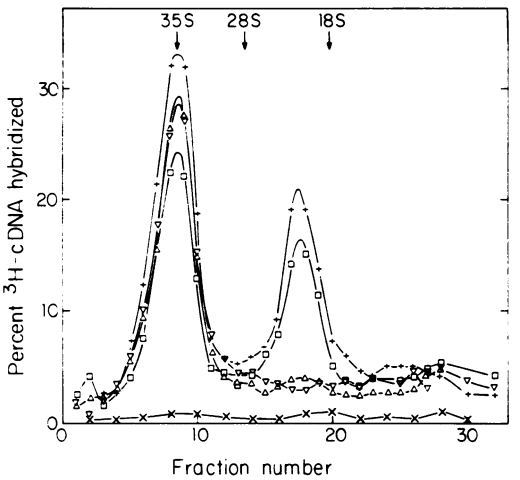

FIG. 8. *Size of viral RNAs in BH-RSV-infected cells. Total cell RNA (100 μg) from BH-RSV-infected cells was fractionated as before, and aliquots were hybridized to cDNA$_{gag}$ (△), cDNA$_{pol}$ (▽), cDNA$_{env}$ (×), cDNA$_{src}$ (□), or cDNA$_c$ (+). Hybridizations were carried out for 50 to 80 h.*

appear to be similar to the *src*-specific 21S RNA in SR-RSV-B-infected cells. As expected, cDNA$_{env}$ did not hybridize significantly to the RNA in BH-RSV-infected cells. Likewise, the 28S RNA species found in SR-RSV-B-infected cells, which contains *env*-specific information, was not present in these cells.

DISCUSSION

The study of viral RNA transcription in RNA tumor virus-infected cells is complicated by the presence of vast excesses of host cell RNA. Unlike many animal viruses, the RNA tumor viruses do not shut off the expression of cellular genes. Furthermore, it has not been possible to selectively inhibit host cell transcription, since viral RNA, like cell RNA, is synthesized from double-stranded DNA, apparently utilizing cellular RNA polymerase II (8, 26, 47), and is thus sensitive to the same metabolic inhibitors. Nucleic acid hybridization, using radiolabeled viral cDNA as a probe, has proven to be extremely useful for circumventing these difficulties. The cDNA is a highly specific and sensitive reagent that can be used to detect virus-specific RNA representing less than 0.0001% of the total RNA in the cell. The recent isolations of cDNA probes specific for different portions of the viral genome, including the *src* (57) and *env* (22, 60) genes of avian oncoviruses and sarcoma virus-specific sequences in the murine system (12, 50), have extended the usefulness of this technique, making it possible to analyze the expression of individual viral genes.

The five different cDNA probes prepared in this study are highly specific for different portions of the viral genome. cDNA$_{gag}$ and cDNA$_{pol}$ overlap somewhat with adjacent probes, but even these two probes are at least 80% specific for their selected regions (Table 5). The genetic complexities of the probes (Table 3) are in reasonable agreement with the estimated sizes of the corresponding genes in RSV (27, 28, 65, 66). The specificity of these probes is confirmed by the clear distinctions that could be made between different viral RNA species in the cell, based on the hybridization patterns obtained with each of the probes. It should be stressed, however, that these probes do not necessarily conform exactly to the corresponding viral genes. The genetic map of RSV was derived by oligonucleotide analyses of viral RNAs containing deletions within the *src* and *env* genes and point mutations within the *pol* and *gag* genes (27, 28, 65, 66). Since these mutations do not precisely define the limits of each gene (particularly *gag* and *pol*), the gene locations, and hence our isolation of gene-specific probes based on these mapping data, must be considered only approximate.

An analysis of sequence homologies between the specific cDNA probes prepared from SR-RSV-B (cDNA$_{src}$) or RAV-2 (all other cDNA's), and RNAs from various avian oncoviruses demonstrated substantial similarities within most of the genetic regions of these viruses (Table 2).

The lowest levels of homology were observed with RAV-0 RNA, consistent with previously published results (21, 38). The most striking difference was found within the *c* region of this virus, which hybridized to only 45% of $cDNA_c$, as compared with over 80% hybridization with RNAs from the other viruses tested. This low level of homology suggests either a deletion of a portion of this region or a substantial difference in base sequence. RAV-0 differs from the other viruses in several properties, such as its poor growth on quail cells (17) and certain chicken lines (35, 45) and its failure to induce leukemia in infected chickens (42). Thus, the difference within the *c* region of RAV-0 could be related to one of these properties. It is not known whether the *c* region codes for a viral protein. However, if our estimate of its size (750 nucleotides) is correct, the *c* region would be sufficiently large to code for a protein with a molecular weight of approximately 25,000. Our estimate, however, is somewhat higher than that of Wang et al. (450 nucleotides) (66).

During the characterization of these probes, we observed that $cDNA_c$ hybridized more rapidly (roughly twofold) with SR-RSV-B RNA than with other viral RNAs (data not shown). One explanation for this would be that the *c* region is duplicated in SR-RSV-B. These accelerated kinetics were also observed with the 28S RNA in SR-RSV-B-infected cells, suggesting that this apparent gene duplication is located on the 3′ half of the viral genome. Further analysis is necessary, however, to verify this conclusion. We also observed some variations in the annealing kinetics of different probes that were probe specific, rather than RNA specific. For example, hybridization with $cDNA_{env}$ was more rapid (roughly twofold) than that with other probes, regardless of the source of the viral RNA. We have not determined whether these variations were related to small differences in the sizes of the probes or to variations in the guanine-plus-cytosine content of the different viral genes. Since the amounts of gene-specific RNAs in virus-infected cells were calculated from standard curves, using the appropriate cDNA-viral RNA combinations, the data in Table 6 do not reflect these kinetic differences or the possible duplication of the *c* region in SR-RSV-B. Likewise, the hybridization times used for analyzing RNA probes (Fig. 4 through 8) were adjusted to compensate for probe-specific kinetic differences.

Analyses of viral RNA content in infected cells (Table 6) indicate that sequences located near the 3′ terminus of the viral genome are roughly twofold more abundant than 5′-proximal sequences. This is true for all three types of infected cell tested. The unequal sequence representation presumably reflects the fact that the subgenome-length RNAs, which constitute 40 to 60% of the virus-specific RNA molecules in the cells, contain only 3′-proximal sequences. SR-RSV-B-infected cells contain approximately 20,000 copies of *src*-specific RNA and 10,000 copies of *gag*- and *pol*-specific RNA per cell (Table 6). From these values and the data in Fig. 6 and 7, we can estimate that each cell contains roughly 8,000 to 10,000 molecules of 39S RNA, 8,000 to 10,000 molecules of 28S RNA, and 3,000 to 4,000 molecules of 21S RNA. *gag* and *pol* sequences are present only in the 39S RNA species, whereas *src* is present in all three RNA species.

The different virus-specific RNAs detected in cells infected with RAV-0, RAV-2, SR-RSV-B, or BH-RSV are shown schematically in Fig. 9. The SR-RSV-B-infected cells contained, in addition to the RNAs shown, a small amount of *env*-specific 21S RNA. However, this RNA was probably derived from td virus present in the virus stock. Recloning of the sarcoma virus resulted in a significant reduction in the amount of 21S *env*-specific RNA but did not alter the level of 21S *src*-specific RNA.

The subgenome-length RNAs in each type of infected cell appear to represent the 3′ portion of the respective viral genome (Fig. 9). These smaller RNAs could be generated either by independent primary transcription of limited regions of the proviral DNA or by specific enzymatic cleavage of the higher-molecular-weight viral RNAs. According to the first model, RNA synthesis in SR-RSV-B-infected cells would initiate at three different promoters, located at (or external to) a site on the provirus corresponding to the 5′ terminus of the viral RNA and at internal sites located between *pol* and *env* and between *env* and *src*. Only one termination site would be required, since all three RNA species in these cells contain the *c* region and thus apparently extend to the 3′ terminus of the genome. The second model would require only one initiation site. The subgenome-length RNAs would be generated by cleavage of genome-

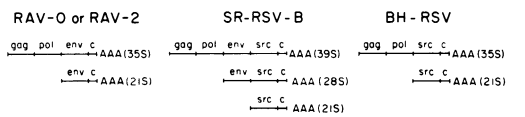

FIG. 9. *Virus-specific RNAs detectable in cells infected with avian leukosis or sarcoma viruses. Genetic content and sedimentation values for each RNA species were derived from the data in Fig. 4 through 8.*

length RNA at the two internal sites mentioned above. Sequential processing of the 39S RNA would generate first the 28S RNA by removal of the 5′ sequence *gag-pol* and then the 21S RNA by removal of *env*. The 5′ fragment generated by each cleavage event would presumably be rapidly degraded, since these RNA species were not detected in either total cell RNA, poly(A) RNA, or non-poly(A) RNA under the steady-state conditions analyzed in these experiments. A model of this type, involving processing on the polyribosomes, has been suggested by Mueller-Lantzsch and Fan (36) for murine leukemia virus. These authors have predicted the presence of an additional intermediate, containing *pol* at the 5′ terminus, which we did not detect in our experiments. However, we cannot rule out the possibility that this RNA (which would sediment at roughly 33 to 35S in the case of SR-RSV-B) is present at very low concentration and is thus obscured by the major peak of 39S viral RNA. Haseltine and Baltimore (19) have reported the presence in murine leukemia virus-infected cells of a viral RNA species that is larger than genome length and have suggested that this might be a precursor to the 30–40S viral RNA. This high-molecular-weight RNA was present in very low amounts, however, and would probably not be detected under the conditions used in our experiments.

Approximately 80 to 85% of the virus-specific RNA in the cell contains poly(A), as shown by its binding to poly(U)-Sepharose. Whether the nonbinding RNA represents non-adenylated mRNA, intermediates in the processing or degradation of viral RNA, or RNA fragments generated during the handling of the RNA, or whether it simply reflects the efficiency of binding to poly(U)-Sepharose, is not known. However, in the one case where non-adenylated RNA was analyzed (from RAV-2-infected cells), we could not distinguish any major peaks of virus-specific RNA.

Both the genome-length and subgenome-length viral RNAs detected in these experiments contain poly(A). Since these two classes of RNA have been found associated with polyribosomes in cells infected with avian or murine leukemia viruses (11, 14, 49, 51), it seems likely that these RNAs function as mRNA's. From our data we would conclude that the genome-length RNA is the messenger for the *gag* and *pol* genes, since these sequences are not found in the lower-molecular-weight RNAs. This is consistent with the observation by Mueller-Lantzsch and Fan (36) that polyribosomes immunoprecipitated with anti-p30 serum contain only 35S RNA and with the observation in cell-free translating systems that 35S viral RNA (either from the virus particle or from infected cells) can direct the synthesis of only the *gag* (and possibly *pol*) gene products (32, 37, 40, 63). The subgenome-length viral RNAs would presumably be the primary messengers for the *env* and *src* genes. The mRNA's for the envelope glycoprotein would be the 28S RNA in SR-RSV-B-infected cells, which contains *env* at its 5′ end, and the 21S RNA in leukosis virus-infected cells. The *src* mRNA would be the 21S RNA in SR-RSV-B- or BH-RSV-infected cells. This interpretation is consistent with the demonstration by Stacey et al. (55) that 21S RNA from RAV-2-infected cells can function as messenger for the envelope glycoprotein in vivo and with the experiments of Gielkens et al. (13), who have shown that 20–22S RNA from murine leukemia virus-infected cells can direct the cell-free synthesis of a polypeptide that appears to be virus specific.

It is interesting that only 3′-proximal sequences are represented in the subgenome-length viral RNAs. (An exception to this was found in one type of uninfected cell that contains a 19S *gag*-specific RNA transcribed from the endogenous provirus [67]. This may represent an aberrant situation, however, since these cells do not produce virus particles and since the provirus in these cells appears to be partially defective.) Jacobson and Baltimore (25) have suggested that ribosomes bind only at the 5′ end of the mRNA in eukaryotic cells. Thus, genes located distal to the 5′ end of a large polycistronic messenger, such as viral 35S RNA, might be translated very inefficiently. This, in fact, is observed in vitro with viral 35S RNA, as mentioned above. The virus particle contains approximately 5,000 molecules of each of the *gag* proteins (59) but only 50 to 100 polymerase molecules (39), and a similar ratio is found in the infected cell (5, 39). Thus, occasional read-through from *gag* into *pol* would be sufficient to supply these small amounts of polymerase. The subgenome-length RNAs would presumably be more efficient messengers for the 5′-distal genes, *env* and *src*. In addition, the presence of different mRNA transcripts would provide a possible means for independently regulating the synthesis of each viral gene product. The presence of both subgenome-length and genomic RNAs functioning as mRNA's has also been described in cells infected with Sindbis virus (53) and with some plant viruses (24, 52).

Different size classes of intracellular viral RNA, with properties similar to those described

here, have also been found independently by Weiss et al. (S. Weiss, B. Baker, H. E. Varmus, and J. M. Bishop, manuscript in preparation).

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LITERATURE CITED

1. **Baltimore, D.** 1975. Tumor viruses: 1974. Cold Spring Harbor Symp. Quant. Biol. 39:1187-1200.
2. **Beemon, K., P. H. Duesberg, and P. K. Vogt.** 1974. Evidence for crossing-over between avian tumor viruses based on analysis of viral RNAs. Proc. Natl. Acad. Sci. U.S.A. 71:4254-4258.
3. **Billeter, M. A., J. T. Parson, and J. M. Coffin.** 1974. The nucleotide sequence complexity of avian tumor virus RNA. Proc. Natl. Acad. Sci. U.S.A. 71:3560-3564.
4. **Bishop, J. M., D.-T. Deng, B. J. W. Mahy, N. Quintrell, E. Stavnezer, and H. E. Varmus.** 1976. Synthesis of viral RNA in cells infected by avian sarcoma viruses, p. 1-20. *In* D. Baltimore, A. S. Huang, and C. F. Fox (ed.), Animal virology. Academic Press Inc., New York.
5. **Chen, J. H., W. S. Hayward, and H. Hanafusa.** 1974. Avian tumor virus proteins and RNA in uninfected chicken embryo cells. J. Virol. 14:1419-1429.
6. **Crittenden, L. B., J. V. Motta, and E. J. Smith.** 1977. Genetic control of RAV-0 production in chickens. Virology 76:90-97.
7. **Deng, C. T., D. Stehelin, J. M. Bishop, and H. E. Varmus.** 1977. Characteristics of virus-specific RNA in avian sarcoma virus-transformed BHK-21 cells and revertants. Virology 76:313-330.
8. **Dinowitz, M.** 1975. Inhibition of Rous sarcoma virus by α-amanitin: possible role of cell DNA-dependent RNA polymerase form II. Virology 66:1-9.
9. **Duesberg, P. H., S. Kawai, L.-H. Wang, P. K. Vogt, H. M. Murphy, and H. Hanafusa.** 1975. RNA of replication-defective strains of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 72:1569-1573.
10. **Duesberg, P. H., and P. K. Vogt.** 1973. RNA species obtained from clonal lines of avian sarcoma and avian leukosis virus. Virology 54:207-219.
11. **Fan, H., and D. Baltimore.** 1973. RNA metabolism of murine leukemia virus: detection of virus-specific RNA sequences in infected and uninfected cells and identification of virus-specific messenger RNA. J. Mol. Biol. 80:93-117.
12. **Frankel, A. E., R. L. Neubauer, and P. J. Fischinger.** 1976. Fractionation of DNA nucleotide transcripts from Moloney sarcoma virus and isolation of sarcoma virus-specific complementary DNA. J. Virol. 18:481-490.
13. **Gielkens, A. L., D. Van Zaane, H. P. J. Bloemers, and H. Bloemendal.** 1976. Synthesis of Rauscher murine leukemia virus-specific polypeptides in vitro. Proc. Natl. Acad. Sci. U.S.A. 73:356-360.
14. **Gielkens, A. L. J., M. H. L. Salden, and H. Bloemendal.** 1974. Virus-specific messenger RNA on free and membrane-bound polyribosomes from cells infected with Rauscher leukemia virus, Proc. Natl. Acad. Sci. U.S.A. 71:1093-1097.
15. **Hanafusa, H.** 1969. Rapid transformation of cells by Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 63:318-325.
16. **Hanafusa, H., T. Aoki, S. Kawai, T. Miyamoto, and R. E. Wilsnack.** 1973. Presence of antigen common to avian tumor virus envelope antigen in normal chick embryo cells. Virology 56:22-32.
17. **Hanafusa, H., W. S. Hayward, J. H. Chen, and T. Hanafusa.** 1975. Control of expression of tumor virus genes in uninfected chicken cells. Cold Spring Harbor Symp. Quant. Biol. 39:1139-1144.
18. **Hanafusa, T., H. Hanafusa, T. Miyamoto, and E. Fleissner.** 1972. Existence and expression of tumor virus genes in chick embryo cells. Virology 47:475-482.
19. **Haseltine, W. A., and D. Baltimore.** 1976. Size of murine RNA tumor virus-specific nuclear RNA molecules. J. Virol. 19:331-337.
20. **Hayward, W. S., and H. Hanafusa.** 1973. Detection of avian tumor virus RNA in uninfected chicken embryo cells. J. Virol. 11:157-167.
21. **Hayward, W. S., and H. Hanafusa.** 1975. Recombination between endogenous and exogenous RNA tumor virus genes as analyzed by nucleic acid hybridization. J. Virol. 15:1367-1377.
22. **Hayward, W. S., and H. Hanafusa.** 1976. Independent regulation of endogenous and exogenous avian RNA tumor virus genes. Proc. Natl. Acad. Sci. U.S.A. 73:2259-2263.
23. **Hayward, W. S., S. Y. Wang, E. Urm, and H. Hanafusa.** 1976. Transcription of the avian RNA tumor virus glycoprotein gene in infected and uninfected cells, p. 21-35. *In* D. Baltimore, A. S. Huang, and C. F. Fox (ed.), Animal virology. Academic Press Inc., New York.
24. **Hunter, T. R., T. Hunt, J. Knowland, and D. Zimmern.** 1976. Messenger RNA for the coat protein of tobacco mosaic virus. Nature (London) 260:759-764.
25. **Jacobson, M. F., and D. Baltimore.** 1968. Polypeptide cleavage in the formation of poliovirus proteins. Proc. Natl. Acad. Sci. U.S.A. 61:77-84.
26. **Jacquet, M., Y. Groner, J. Monroy, and J. Hurwitz.** 1974. The in vitro synthesis of avian myeloblastosis viral RNA sequences. Proc. Natl. Acad. Sci. U.S.A. 71:3045-3049.
27. **Joho, R. H., M. A. Billeter, and C. Weissmann.** 1975. Mapping of biological functions on RNA of avian tumor virus: location of regions required for transformation and determination of host range. Proc. Natl. Acad. Sci. U.S.A. 72:4772-4776.
28. **Joho, R. H., E. Stoll, R. R. Friis, M. A. Billeter, and C. Weissmann.** 1976. A partial genetic map of Rous sarcoma virus RNA: location of polymerase, envelope and transformation markers, p. 127-145. *In* D. Baltimore, A. S. Huang, and C. F. Fox (ed.), Animal virology. Academic Press Inc., New York.
29. **Junghans, R. P., P. Duesberg, and C. A. Knight.** 1975. In vitro synthesis of full-length DNA transcripts of Rous sarcoma virus RNA by viral DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 72:4895-4899.
30. **Kawai, S., and H. Hanafusa.** 1972. Genetic recombination with avian tumor virus. Virology 49:37-44.
31. **Kawai, S., and H. Hanafusa.** 1973. Isolation of a defective mutant of avian sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 70:3494-3497.
32. **Kerr, I. M., U. Olshevsky, H. F. Lodish, and D. Baltimore.** 1976. Translation of murine leukemia virus RNA in cell-free systems from animal cells. J. Virol. 18:627-635.
33. **Khoury, A. T., and H. Hanafusa.** 1976. Synthesis and integration of viral DNA in chicken cells at different times after infection with various multiplicities of avian oncornavirus. J. Virol. 18:383-400.
34. **Leong, J.-A., A.-C. Garapin, N. Jackson, L. Fanshier, W. Levinson, and J. M. Bishop.** 1972. Virus-specific ribonucleic acid in cells producing Rous sarcoma virus: detection and characterization. J. Virol. 9:891-902.

35. Linial, M., and P. E. Neiman. 1976. Infection of chick cells by subgroup E viruses. Virology 73:508–520.
36. Mueller-Lantzsch, N., and H. Fan. 1976. Monospecific immunoprecipitation of murine leukemia virus polyribosomes: identification of p30 protein specific messenger RNA. Cell 9:579–588.
37. Naso, R. B., L. J. Arcement, T. G. Wood, and R. B. Arlinghaus. 1975. The cell-free translation of Rauscher leukemia virus RNA into high molecular weight polypeptides. Biochim. Biophys. Acta 383:195–206.
38. Neiman, P. E., S. E. Wright, and H. G. Purchase. 1975. Studies of the interrelationship of chicken leukosis virus and host cell genomes by RNA-DNA hybridization. Cold Spring Harbor Symp. Quant. Biol. 39:875–883.
39. Panet, A., D. Baltimore, and T. Hanafusa. 1975. Quantitation of avian RNA tumor virus reverse transcriptase by radioimmunoassay. J. Virol. 16:146–152.
40. Pawson, T., G. S. Martin, and A. E. Smith. 1976. Cell-free translation of virion RNA from nondefective and transformation-defective Rous sarcoma viruses. J. Virol. 19:950–967.
41. Perry, R. P., J. LaTorre, D. E. Kelley, and J. R. Greenberg. 1972. On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes. Biochim. Biophys. Acta 262:220–226.
42. Purchase, H. G., W. Okazaki, P. K. Vogt, H. Hanafusa, B. R. Burmester, and L. B. Crittenden. 1977. Oncogenicity of avian leukosis viruses of different subgroups and of mutants of sarcoma viruses. Infect. Immun. 15:423–428.
43. Quade, K. E., R. E. Smith, and J. L. Nichols. 1974. Evidence for common nucleotide sequences in the RNA subunits comprising Rous sarcoma virus 70S RNA. Virology 61:287–291.
44. Rho, H. M., and M. Green. 1974. The homopolyadenylate and adjacent nucleotides at the 3′ terminus of 30–40S RNA subunits in the genome of murine sarcoma-leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 71:2386–2390.
45. Robinson, H. L. 1976. Intracellular restriction on the growth of induced subgroup E avian type C viruses in chicken cells. J. Virol. 18:856–866.
46. Rothenberg, E., and D. Baltimore. 1976. Synthesis of long, representative DNA copies of the murine RNA tumor virus genome. J. Virol. 17:168–174.
47. Rymo, L., J. T. Parsons, J. M. Coffin, and C. Weissmann. 1974. In vitro synthesis of Rous sarcoma virus-specific RNA is catalyzed by a DNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 71:2782–2786.
48. Scheele, C. M., and H. Hanafusa. 1971. Proteins of helper-dependent RSV. Virology 45:401–410.
49. Schincariol, A. L., and W. K. Joklik. 1973. Early synthesis of virus-specific RNA and DNA in cells rapidly transformed with Rous sarcoma virus. Virology 56:532–548.
50. Scolnick, E. M., R. S. Howk, A. Anisowicz, P. T. Peebles, C. D. Scher, and W. D. Parks. 1975. Separation of sarcoma virus-specific and leukemia virus-specific genetic sequences of Moloney sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 72:4650–4654.
51. Shanmugam, G., S. Bhaduri, and M. Green. 1974. The virus-specific RNA species in free and membrane-bound polyribosomes of transformed cells replicating murine sarcoma-leukemia viruses. Biochem. Biophys. Res. Commun. 56:697–704.
52. Shih, D. S., L. C. Lane, and P. Kaesberg. 1972. Origin of the small component of Brome mosaic virus. J. Mol. Biol. 64:353–362.
53. Simmons, D. T., and J. H. Strauss. 1974. Translation of Sindbis virus 26S and 49S RNA in lysates of rabbit reticulocytes. J. Mol. Biol. 86:397–409.
54. Spirin, A. S. 1963. Some problems concerning macromolecular structure of RNA. Prog. Nucleic Acid Res. Mol. Biol. 1:301–345.
55. Stacey, D. W., V. G. Allfrey, and H. Hanafusa. 1977. Microinjection analysis of envelope messenger activities of avian leukosis viral RNAs. Proc. Natl. Acad. Sci. U.S.A. 74:1614–1618.
56. Stavnezer, E., G. Ringold, H. E. Varmus, and J. M. Bishop. 1976. RNA complementary to the genome of RNA tumor viruses in virions and virus-producing cells. J. Virol. 20:342–347.
57. Stehelin, D., R. V. Guntaka, H. E. Varmus, and J. M. Bishop. 1976. Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses. J. Mol. Biol. 101:349–365.
58. Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt. 1976. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature (London) 260:170–173.
59. Stromberg, K., N. E. Hurley, N. L. Davis, R. R. Rueckert, and E. Fleissner. 1974. Structural studies of avian myeloblastosis virus: comparison of polypeptides in virion and core component by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Virol. 13:513–528.
60. Tal, J., D. J. Fujita, S. Kawai, H. E. Varmus, and J. M. Bishop. 1977. Purification of DNA complementary to the *env* gene of avian sarcoma virus and analysis of relationships among the *env* genes of avian leukosis-sarcoma viruses. J. Virol. 21:497–505.
61. Tsuchida, N., M. S. Robin, and M. Green. 1972. Viral RNA subunits in cells transformed by RNA tumor viruses. Science 176:1418–1419.
62. Vogt, P. K. 1971. Spontaneous segregation of nontransforming viruses from cloned sarcoma viruses. Virology 46:939–946.
63. Von der Helm, K., and P. H. Duesberg. 1975. Translation of Rous sarcoma virus RNA in a cell-free system from Ascites Krebs II cells. Proc. Natl. Acad. Sci. U.S.A. 72:614–618.
64. Wang, L.-H., and P. Duesberg. 1974. Properties and location of poly(A) in Rous sarcoma virus RNA. J. Virol. 14:1515–1529.
65. Wang, L.-H., P. H. Duesberg, S. Kawai, and H. Hanafusa. 1976. Location of envelope-specific and sarcoma-specific oligonucleotides on the RNA of Schmidt-Ruppin Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 73:447–451.
66. Wang, L.-H., D. Galehouse, P. Mellon, P. Duesberg, W. S. Mason, and P. K. Vogt. 1976. Mapping oligonucleotides of Rous sarcoma virus RNA that segregate with polymerase and group-specific antigen markers in recombinants. Proc. Natl. Acad. Sci. U.S.A. 73:3952–3956.
67. Wang, S. Y., W. S. Hayward, and H. Hanafusa. 1977. Genetic variation in the RNA transcripts of endogenous virus genes in uninfected chicken cells. J. Virol. 24:64–73.