Restricted Addition of Proviral DNA in Target Tissues of Chickens infected with Avian Myeloblastosis Virus

M. A. Baluda, M. Shoyab, M. Ali, P. D. Markham and W. N. Drohan

University of California
Department of Microbiology & Immunology
Los Angeles, California USA 90024

Avian myeloblastosis virus (AMV) is an avian RNA tumor virus which can replicate in every cell from susceptible chickens but expresses its oncogenic effect only in specific target cells. In chickens, the BAI Strain A of AMV used in these studies gives rise to highly differentiated neoplasias, i. e., myeloblastic leukemia, chronic lymphoid leukemia, nephroblastoma and osteopetrosis. *In vitro*, AMV can transform cells from hematopoeitic tissues and from the Bursa of Fabricius (c. f. Baluda, 1962).

AMV, as all RNA tumor viruses do, replicates its RNA via a DNA template (v-DNA) (Temin, 1963, 1964; Bader, 1964, 1965; Baluda & Nayak, 1970; Baltimore, 1970; Temin & Mizutani, 1970). This was established by isolation of infectious DNA from virus transformed cells and by detection of viral specific DNA sequences in infected cells and of reverse transcriptase in virions (Baluda & Nayak, 1970; Baltimore, 1970; Temin & Mizutani, 1970; Hill & Hillova, 1971; Svoboda et al., 1972 Neiman, 1972; Shoyab et al., 1974). Furthermore, some strains of sarcoma viruses, avian and murine which do not contain active RNA directed DNA polymerases are noninfectious (Hanafusa & Hanafusa, 1971; May et al., 1972; Peebles et al., 1972). Due to the vertical transmission of endogenous proviral DNA, even apparently normal chicken cells contain DNA sequences which are complementary to the RNA of AMV (Baluda & Nayak, 1970; Baluda, 1972; Neiman, 1972; Shoyab et al., 1974 a, b, c; Shincariol et al., 1974; Varmus et al., 1972). After infection of chicken cells with an RNA tumor virus, the amount of viral specific sequences is increased due to the acquisition of qualitatively different virus specific sequences (Baluda, 1972 Shoyab et al., 1974 a, b). Addition of viral DNA after infection with avian oncornaviruses also takes place in mouse, rat or duck cells which do not contain DNA sequences homologous to the RNA of avian oncornaviruses (Baluda, 1972; Harel et al., 1972; Varmus et al., 1973; Shoyab et al., 1974; Shoyab et al., 1975).

Endogenous Viral DNA in Normal Chicken Cells. AMV RNA hybridizes with normal chicken DNA 50–65 % as much as with DNA from leukemic cells transformed by AMV (Baluda, 1972; Shoyab et al., 1974 a, b, c). We had postulated that hybridization between AMV RNA and normal chicken DNA might be due to homology between AMV RNA and the RAV-0 like endogenous virus genome.

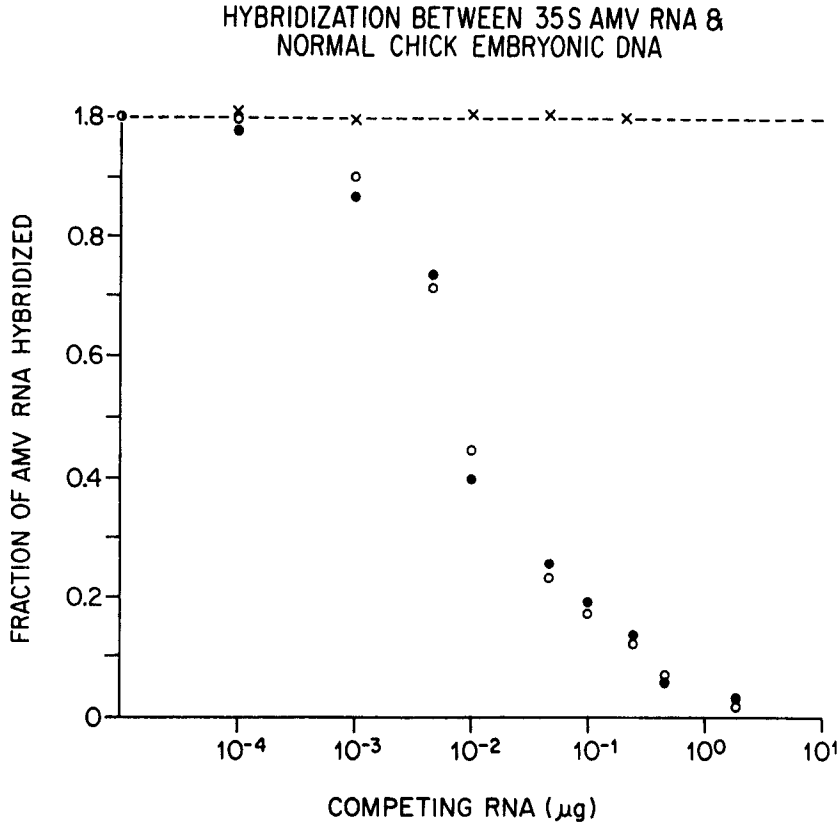

Fig. 1: Inhibition of hybridization between ^{3}H-35S AMV RNA and normal chicken DNA by 35S RAV-0 RNA: The hybridization mixture contained 1.6 mg of sonicated DNA, 6×10^{-4} μg of ^{3}H-labeled 35S viral RNA (specific activity: 1.9×10^6 cpm per μg), 0–2 μg of unlabeled 35S viral RNA and 0.1 % SDS in 0.4 ml of 0.4M phosphate buffer pH 6.8. The hybridization mixture was placed in tightly silicone-stoppered tubes, boiled for 3 minutes in a water ethylene-glycol bath, quickly transferred to a water bath at 65 C and incubated for 64 h to reach a Cot of approximately 15,000 (concentration of nucleotides in moles per liter x time in seconds). The mixture was then diluted with cold water and processed to determine the fraction of ^{3}H-labeled RNA which became RNase resistant. Viral RNA which hybridized with embryonic mouse DNA under similar conditions (6 %) was subtracted from the experimental values. The values obtained in the presence of yeast RNA but in the absence of unlabeled viral RNA were normalized to 100 percent and competition presented as percent of maximum hybridization.

○—○ Competition by 35S AMV RNA
●—● Competition by 35S RAV-0 RNA

That is the case as shown in Figure 1; 35S RNA from RAV-0 inhibited by 98 % hybrid formation between normal chicken DNA and 35S AMV RNA.

Similar conclusions are obtained with another competition hybridization technique (Table I). If normal chicken DNA is first hybridized with an excess of unlabeled 70S RAV-0 RNA, there is no subsequent hybridization with ^{3}H-35S AMV RNA. If yeast RNA replaced RAV-0 RNA in the first hybridization, 288 cpm of AMV RNA hybridized with normal chicken DNA. Also, 70S RAV-0 RNA inhibits by 84 % hybridization between ^{3}H-labeled 35S AMV RNA and DNA from leukemic chicken myeloblasts. This findings is in agreement with other work which shows that there is approximately 70 % homology between the genomes of RAV-0 and AMV. This was determined by RNA excess competition of DNA driven RNA-DNA hybridization. Hybridization between ^{3}H-labeled 35S AMV RNA and

Table I: Filter hybridization of ^{3}H-35S AMV RNA with normal or leukemic chicken DNA with or without prehybridization with unlabeled 70S RAV-0 RNA

	CPM Hybridized/100 μg DNA Prehybridized With:		
DNA	Unlabeled 70S RAV-0 RNA (A)	Yeast RNA (B)	$\frac{A}{B}$
Normal Chicken Embryos	0	288 ± 5	0
Leukemic Chickens	97 ± 7	616 ± 8	0.16

Two hybridization vials were used. In the first vial, the hybridization mixture contained 10 μg/ml of 70S unlabeled RAV-0 RNA and .05 % SDS in 4 X SSC. In the second vial, yeast RNA replaced RAV-0 RNA. At the end of the first cycle of hybridization at 70 °C for 20 h, the filters were directly transferred to different vials containing 1.05 X 10^6 cpm of 35S ^{3}H-AMV RNA, 7.5 mg of yeast RNA in 1.5 ml of 4 X SSC plus 0.05 % SDS. After the second hybridization performed for 12 h at 70 °C, the filters were washed, treated with ribonucleases A and T_1 and processed as described earlier (Baluda & Nayak, 1970). Cpm bound to each mouse DNA filter (12 cpm when RAV-0 RNA was used as competitor or 21 cpm when cold yeast RNA was used in the first cycle of hybridization) were deducted from cpm hybridized to experimental filters.

DNA from leukemic chicken myeloblasts was inhibited 92 % by 2 μg per 0.4 ml of unlabeled 35S AMV, and 68 % by 2 μg of unlabeled 35S RAV-0 RNA. Conversely, hybridization between ^{3}H-labeled 35 S RAV-O RNA and leukemic chicken DNA was inhibited 96 % by unlabeled 35S RAV-0 RNA and 67 % by unlabeled 35S AMV RNA.

Appearance of Viral DNA After Infection of Chick Embryo Fibroblasts with Prague Strain of Rous Sarcoma Virus (PR-RSV) or Avian Myeloblastosis Virus (AMV). Proviral DNA is synthesized early after infection. This has been shown by hybridization of ^{3}H-labeled 70S AMV RNA with DNA from chick embryo fibroblasts (CEF) infected with either AMV or PR-RSV (Ali & Baluda, 1974). It was possible to separate v-DNA newly synthesized after infection from the endogenous viral DNA integrated into high molecular weight cellular DNA by the Hirt fractionation procedure. The fractionation of v-DNA into Hirt's supernate (low molecular weight DNA) and pellet (high molecular weight DNA) from cells infected with AMV at a high input multiplicity of infection showed an enrichment of viral DNA in the Hirt supernate as early as 1 h after infection. The synthesis of small molecular weight viral DNA continued for approximately 72 hours (Figure 2). Alkaline sucrose velocity sedimentation analysis of v-DNA in the Hirt supernate suggests that v-DNA is synthesized as molecules equivalent to copies of the 3 X 10^6 daltons viral RNA subunit. Lagging a few hours behind the increase in free v-DNA, there was an increase in the pellet (integrated) v-DNA until it reached a maximum approximately 72 hours after infection (Figure 2).

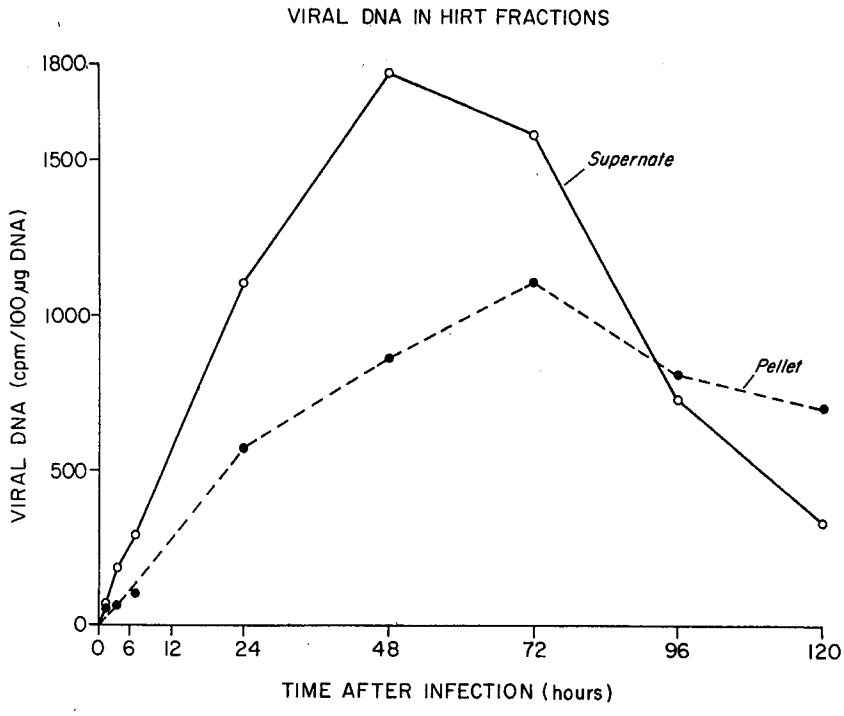

Fig. 2: Proviral DNA synthesis in cells infected with AMV or PR-RSV: CEF cultures were infected with AMV or PR-RSV at input multiplicities of 4 or more and were subjected to Hirt fractionation at various time intervals after infection. Hirt fractionation separates DNA of large molecular weight from DNA of small molecular weight. The cells were lysed in 0.6 % SDS, 0.01M Tris-HCl (pH 7.4) and 0.01M EDTA. The lysate was made 1M with NaCl, allowed to precipitate at 0 °C for 16 hours and centrifuged at 35,000 xg for 1 hour. DNA from supernate or pellet was then extracted, purified, denatured, immobilized on nitrocellulose filters and hybridized at 70 °C for 10 hours with ^{3}H-labeled 70S AMV-RNA (10^6 cpm per ml) in 4 x SSC containing 3 mg of mouse RNA per ml and 0.05 % SDS. After hybridization, the amount of DNA attached to each filter was determined by the Burton diphenylamine reaction.

Three to five filters were made with DNA from each fraction. ^{3}H-AMV-RNA bound by mouse DNA filter was deducted from each experimental filter and the amount of ^{3}H-AMV RNA hybridized per 100 µg of DNA was determined. The ^{3}H AMV RNA hybridized to endogenous viral DNA in noninfected cells was deducted from the cpm hybridized at various time intervals for each fraction. Cpm in the Hirt supernate were also divided by the enrichment factor, i.e., ratio of DNA recovered in the pellet to that recovered in supernate calculated from the respective absorbancy at 260 mµ.

The newly synthesized viral DNA from the Hirt supernatant fraction was subjected to neutral cesium chloride-ethidium bromide density equilibrium sedimentation and 10–20 % of v-DNA sedimented at a density of 1.58–1.60 g/cc, demonstrating the existence of circular molecules. The presence of supercoiled circular molecules was also detected by velocity sedimentation in alkaline sucrose gradients. The free linear and circular viral DNA which is detected early after infection in oncornavirus infected avian cells appears to be synthesized for a short time only after infection, approximately 72 hours.

The sedimentation profiles of minimally sheared cellular DNA in alkaline sucrose velocity gradients suggest that v-DNA is synthesized as small molecules which subsequently are covalently linked to high molecular weight cellular DNA. Table II shows that 60 hours after infection of CEF with PR-RSV, 50 % of the newly synthesized v-DNA still appears as free molecules. By contrast, in leukemic

Table II: Alkaline sucrose velocity sedimentation of virus specific DNA isolated from CEF 60 hours after infection with PR-RSV

Fraction (range of sedimentation values)	DNA[a] per fraction (µg)	Cpm viral RNA hybrizided per 100 µg DNA[b]	Newly synthesized v-DNA %
(0– 18)S	24.8	3,273 ± 35	9.0
(18– 28)S	16.8	7,324 ± 621	27.6
(28– 40)S	14.4	5,331 ± 608	14.6
(40– 80)S	33.1	2,780 ± 380	6.8
(80–122)S	167.8	2,925 ± 232	42.1
Total DNA (uninfected)		2,133 ± 353	0
Total DNA (infected)		4,221 ± 373	

^{3}H-labelled DNA from various fractions of the gradients was purified, denatured, immobilized on filters and hybridized with ^{32}P-labeled 70S AMV RNA in large excess.
[a] Determined from ^{3}H radioactivity before hybridization.
[b] Mean of 2–5 filters ± standard deviation. An average background of 131 cpm per filter of ^{32}P RNA hybridized to mouse DNA filters has been subtracted.

cells several weeks after infection, or in normal cells, 100 % of the v-DNA seems to be integrated (Markham & Baluda, 1973).

Integration of Oncornavirus DNA in Normal Chicken Cells and in Leukemic Cells Transformed by AMV. The integration of proviral DNA into host DNA had been postulated to explain the persistence of the viral genome in cells transformed by oncornaviruses (Bentvelzen et al., 1970; Temin, 1962, 1971). Also, genetic analysis suggested a close association between the cell genome and genetic information responsible for expression of virus specific products (c. f. Markham & Baluda, 1973).

The integration of v-DNA into normal chicken cell DNA and in leukemic myeloblasts transformed by AMV several weeks earlier was demonstrated by the formation of alkali stable bonds between v-DNA and nuclear cellular DNA of large molecular weight (Sambrook et al., 1968; Markham & Baluda, 1973). The sedimentation profile in alkaline sucrose gradients of minimally sheared DNA from leukemic myeloblasts transformed several weeks earlier showed that viral DNA sedimented with high molecular weight cellular DNA (Figure 3). There is no detectable free viral DNA. The presence of viral DNA in pools I and II is due to partial degradation of cellular DNA into fragments containing v-DNA, since the concentration of v-DNA per 100 µg of cellular DNA was similar in pools I, II, III and IV. If some v-DNA had existed in a free state of lower molecular weight, it would have become more concentrated in pools I and II since there was a 3-to-4-fold enrichment of smaller sized DNA in these pools as compared to pools III and IV.

The existence of v-DNA as supercoiled circular molecules of relatively small size with a high sedimentation coefficient, i. e., greater than 93S, can also be ruled

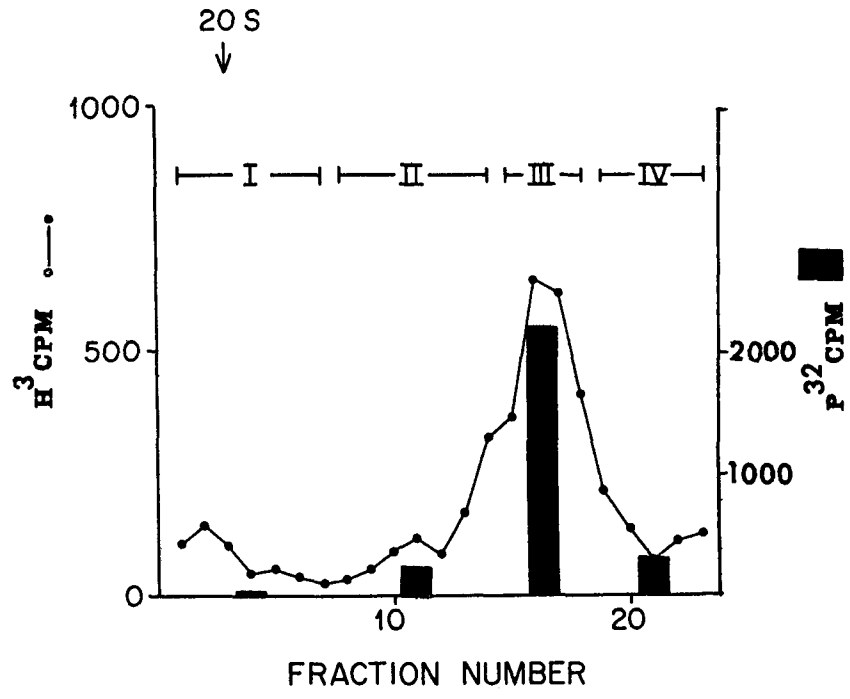

Fig. 3: Hybridization of ^{32}P-labeled 70S AMV RNA with ^{3}H-labeled DNA from cells fractionated by alkaline sucrose velocity sedimentation: Cultured leukemic cells were labeled for 8 h with ^{3}H-thymidine (2 μCi/ml), washed, and incubated in normal medium for an additional 16 h. Cells (2–3 x 10^6) were layered on the top of each alkaline sucrose gradient. The cells were lysed at 4 C for at least 12 h, then the gradients were centrifuged for 7 h at 22,000 rpm at 4 C in a Beckman SW-27 rotor. Fractions were collected from the top of each gradient by pumping 70 % sucrose into the bottom of the centrifuge tube. Samples from each fraction were precipitated with 5 % trichloroacetic acid, filtered, washed, dried, and counted in toluene scintillation fluid. Direction of sedimentation is from left to right. For each cell type, 72 gradients were run, and the DNA was pooled from 60 to 72 gradients according to specified sedimentation values and neutralized with 2 N HCl in 0.4M Tris, pH 7.4. Soluble yeast RNA was added as carrier (5 μg/ml), and the DNA was concentrated by ethanol precipitation, phenol extracted, treated with alkali (0.3 N KOH, 18 h at 37 C), dialyzed, denatured, and trapped on nitrocellulose membrane filters. DNA filters were hybridized with 1.2 x 10^6 counts per min per ml of ^{32}P 70S RNA (specific activity, 5.4 x 10^5 counts per min per μg). The histograms represent cpm of ^{32}P-70S AMV RNA hybridized to each pool of DNA with specified sedimentation values.

out. After neutral cesium chloride-ethidium bromide density equilibrium sedimentation, all the viral DNA was present as linear, double-stranded DNA not separable from linear chicken DNA. In addition, after extraction by Hirt's procedure, the viral DNA precipitated with high molecular weight cellular DNA. Similar results were obtained for endogenous v-DNA in normal chicken cells showing that all the endogenous v-DNA is integrated in nuclear cellular DNA of large molecular weight (Markham & Baluda, 1973).

Integration may be a great advantage for RNA tumor viruses since the DNA provirus becomes part of the cellular genome and stability of infection is insured (Temin, 1971). Also, since the viral information has an RNA phase, activation of viral replication can occur without excision of the integrated viral DNA. Oncornaviruses, therefore, avoid the need for many complicated regulatory mechanisms to control DNA replication, transcription and excision such as those required by temperate phages (Borek & Ryan, 1973; Echols, 1971).

Table III: Filter hybridization of 35S [^{3}H] AMV RNA with DNA from various tissues of normal and leukemic chickens[a]

	CPM Hybridized per 100 μg DNA[b] from								
	Brain	Thigh Muscle	Heart	Liver	Lung	Spleen	Kidney	RBC	Leukemic Myelo-blasts
Normal Chickens	1415 ± 78	1249 ± 99	1049 ± 25	1221 ± 105	1209 ± 67	1123 ± 68	1232 ± 57	1201 ± 93	
Leukemic Chickens	1286 ± 60	1335 ± 108	2038 ± 110	1831 ± 112	1903 ± 70	1662 ± 84	3374 ± 394	2820 ± 240	2810 ± 134

a. Various tissues from 12 normal and from 12 leukemic 3–5 week old chickens were removed and pooled. DNA was isolated, purified, trapped on nitrocellulose filters and hybridized for 10 h at 70 °C. Five filters were hybridized in each vial which contained 5×10^5 cpm of ^{3}H-35S AMV RNA (specific activity: 1.9×10^6 cpm per μg) and 1.5 mg of mouse RNA in 0.5 ml of 4 x SSC plus 0.05 % SDS. After hybridization and processing, each filter contained between 30–40 μg of DNA. ^{3}H-RNA bound to mouse DNA (15–20 cpm per filter) was subtracted as background from each experimental filter.
b. Counts per minute ± standard deviation.

Table IV: Hybridization of 70S AMV ^{3}H-RNA to DNA from different tissues of chickens with kidney tumors[a]

Chicken Number	Cpm hybridized per 100 μg DNA from[b] Breast Muscle	Heart	Liver	Lung	Spleen	RBC	Normal Kidney	Kidney Tumor No. 1	Kidney Tumor No. 2
16307	533 ± 36	530 ± 51	550 ± 50	728 ± 27	Atrophied[c]	976 ± 27	595 ± 53	1083 ± 36	
16322	710 ± 36	657 ± 36	621 ± 36	666 ± 44	604 ± 36	923 ± 36	1376 ± 27	1085 ± 36	1385 ± 36
16343	675 ± 44	n. d.	515 ± 53	n. d.	746 ± 44	911 ± 41		1811 ± 71	1331 ± 63

[a] One-day old chicks were injected intraperitoneally with AMV, and three that developed kidney tumors (embryonal nephromas) detected by abdominal palpation were sacrificed on day 102. Chicken 16307 had a nephroma confined to the upper lobe of the right kidney (kidney tumor no. 1); the other lobes of the right kidney and the entire left kidney (normal kidney) were not affected. Chicknes 16322 and 16343 had bilateral kidney tumors. Chicken 16322 had a large cystic tumor involving the entire left kidney (kidney tumor no. 1) and a smaller cystic tumor of the top lobe of the right kidney (kidney tumor no. 2); the other two lobes of the right kidney appeared normal (normal kidney). Chicken 16343 had a teratoma involving the entire left kidney (kidney tumor no. 1) and a cystic tumor involving the entire right kidney (kidney tumor no. 2); there was no detectable normal kidney tissue.

DNA was isolated from each organ, purified, treated with .3N KOH, denatured, trapped on nitrocellulose filters, and hybridized at 70 °C for 10 hr with 70S AMV ^{3}H-RNA. Each vial contained five experimental filters from one organ in 1 ml of 4 x saline citrate with 0.05 % sodium dodecyl sulfate, 3.0 mg of mouse embryo RNA, and 1 x 10^6 counts per min of AMV ^{3}H-RNA (specific activity of 8.1 x 10^5 counts per min per μg). The counts per minute shown represent ^{3}H radioactivity hybridized per 100 μg of DNA.

[b] Mean of five filters ± standard deviation.

[c] At postmortem it was noted that the spleen of this chicken (16307) was devoid of pulp.

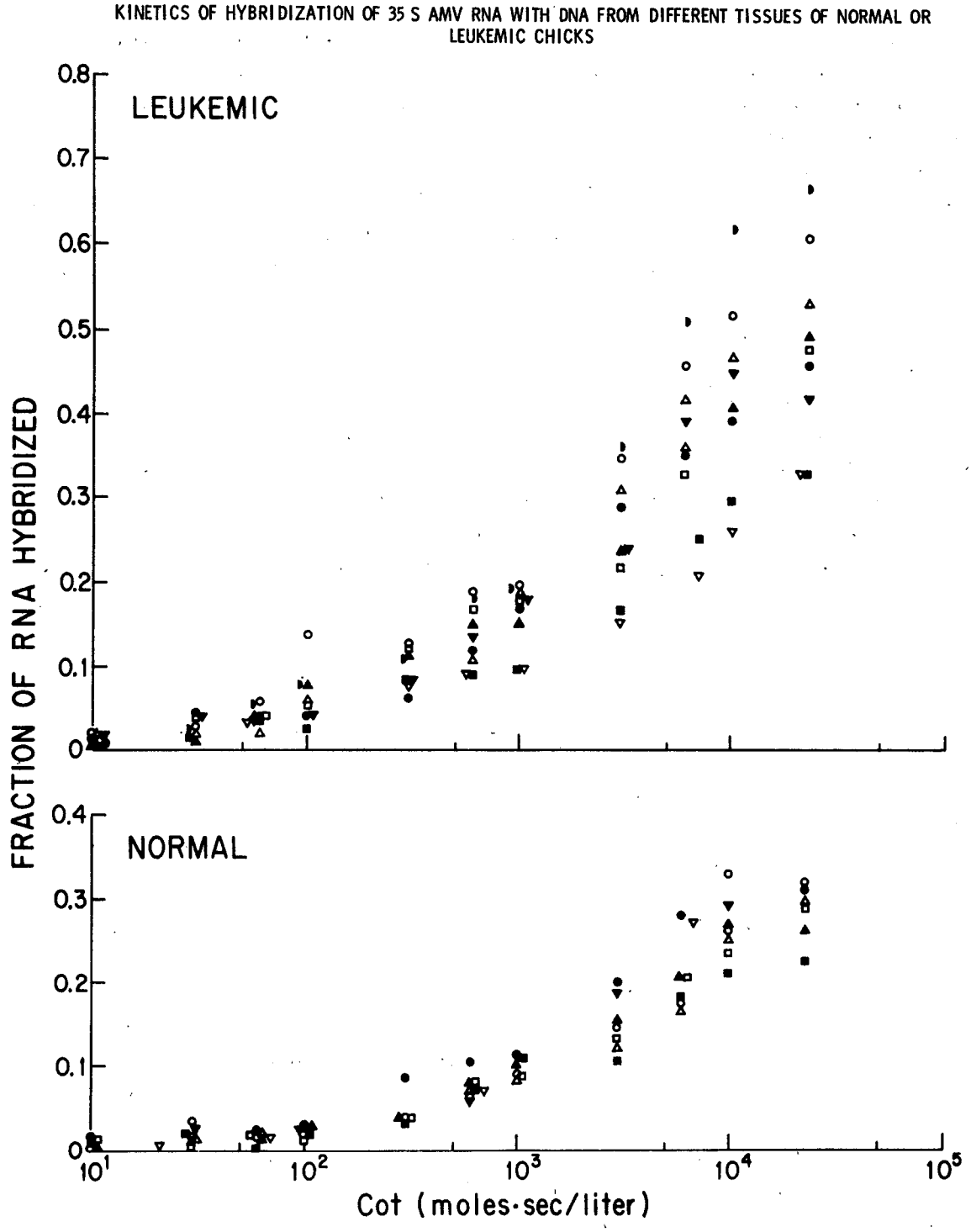

Fig. 4: Hybridization kinetics of ^{3}H-labeled 35S AMV RNA with excess DNA from various tissues of normal and leukemic chickens: Tissues from six normal or from six leukemic chickens were pooled and DNA isolated. The hybridization mixture contained per ml 4 mg of cellular DNA sheared to a fragment size of 6–7S, 2,500 cpm of sonically treated ^{3}H-labeled 35S AMV RNA (size 8 to 10S, specific activity 1.9 x 10^6 cpm per µg) in 0.4M phosphate buffer, pH 6.8 plus 0.1 % SDS. The hybridization was carried out at 65 C in tightly silicone-stoppered tubes. After boiling for 3 min in a waterethylene glycol bath, the mixture was quickly transferred to a water bath at 65 C. Samples of 0.25 ml were taken at different time intervals and diluted with cold water in an ice-water bath. One-half of each sample was then treated with pancreatic A and T_1 ribonucleases to determine the fraction of viral RNA rendered ribonuclease resistant. A background of 2 % obtained a Cot 0 was deducted from all experimental values.

○ RBC DNA
● Spleen DNA
△ Kidney DNA
▲ Lung DNA
□ Heart DNA
■ Muscle DNA
▽ Brain DNA
▼ Liver DNA
◗ Leukemic Myeloblast DNA

Acquisition of Viral DNA in Target Tissues of AMV Infected Chickens. DNA-RNA hybridization studies were carried out to investigate: i) the distribution of vertically transmitted endogenous viral DNA in various tissues of normal chickens, and ii) the distribution of AMV provirus in various tissues of chickens which developed neoplasias after infection with AMV. Two types of nucleic acid hybridization were used: i) denatured cellular DNA immobilized on filters was hybridized to an excess of viral RNA to quantitate the cellular concentration of viral DNA sequences in different tissues, and ii) 35S viral RNA was hybridized to an excess of cellular DNA to determine the proportion of the AMV genome that is present in different tissues.

Filter Hybridization in RNA Excess. Our findings demonstrate that in normal chickens the endogenous viral DNA is present at the same cellular concentration in every tissue that was tested (Table III). This demonstrates the constancy of vertically transmitted endogenous viral DNA in every organ and probably in every cell of normal chickens. By contrast, after injection of AMV into one-day old chicks, AMV specific DNA appears to be acquired only by tumor cells and by target cells in leukemic chickens (Table III). The tissues from leukemic chickens can be divided into three groups: 1) muscle and brain in which the cellular concentration of viral DNA remains the same as before infection with AMV, 2) heart, lung, liver and spleen in which the concentration of v-DNA is increased approximately 50 %, 3) leukemic myeloblasts, RBC and kidneys in which there is a 2–2.5-fold increase in v-DNA. The latter group of tissue contains about the same amount of viral DNA. These tissues are known to contain target cells which can be converted to neoplastic cells by AMV (Baluda & Jamieson, 1961; Baluda et al., 1963; Walter et al., 1962).

AMV-induced kidney tumors provide well defined, localized carcinoma tissue and apparently normal tissues can be obtained from the same chicken due to the absence of metastases. Consequently, 1-day-old chicks were injected intraperitoneally with AMV, and 3 months later three female survivors that had enlarged abdomens with a palpable tumor mass were sacrificed. Examination of their peripheral blood revealed that all three chickens had slightly immature (blue-gray) erythrocytes. In addition to the RBC, six apparently normal tissues (breast muscle, heart, liver, lung, spleen, and kidney) were removed, and their DNA was tested for viral DNA content (Table IV). Tissues known to contain target cells for tumor induction by AMV showed an increase in the average cellular concentration of viral DNA, whereas non-target tissues did not. The lungs, which may be considered a partial target tissue because they contain a large number of leukocytes and erythrocytes, showed a small, but significant (at the 0.01 level using the t test) increase in viral DNA content.

Liquid Hybridization in DNA Excess. To determine what proportion of the AMV genome was present in the various tissues of normal and leukemic chickens, 35S AMV RNA was hybridized in liquid to an excess of cellular DNA (Shoyab et al., 1974). Cellular DNA from every organ of normal chickens tested contained viral DNA sequences which represented approximately the same fraction of the AMV RNA genome (Figure 4). The amount of input viral RNA made RNase resistant by DNA from various tissues of normal chicken varied between 23 to 32 %. This represents hybridization of AMV-RNA with RAV-0 like endogenous viral

DNA. The kinetics of hybridization are almost the same for every normal tissue and are identical to the kinetics of hybridization between AMV RNA and DNA from individual whole normal chicken embryos (Shoyab et al., 1974). These results indicate that the endogenous oncornavirus DNA sequences, estimated at two to three copies per diploid cell genome, are quantitatively and qualitatively similar in every organ of normal chickens.

Unlike the findings obtained with tissues from normal chickens, the kinetics of hybridization of AMV RNA with DNA from tissues of leukemic chickens varied with different tissues (Figure 4). Maximum hybridization was obtained with DNA from leukemic myeloblasts or RBC (64 to 67 %). Hybridization obtained with kidney DNA was about 10 % lower than with DNA from leukemic myeloblasts or RBC. DNA from muscle or brain hybridized 33 % of input viral RNA, the same fraction hybridized by DNA from uninfected chickens. DNA from heart, liver, lung, or spleen hybridizes a fraction (42 to 50 %) of viral RNA intermediate between the first two groups of tissues. These data indicate that there are 4 to 6 DNA copies of the viral genome per leukemic myeloblast.

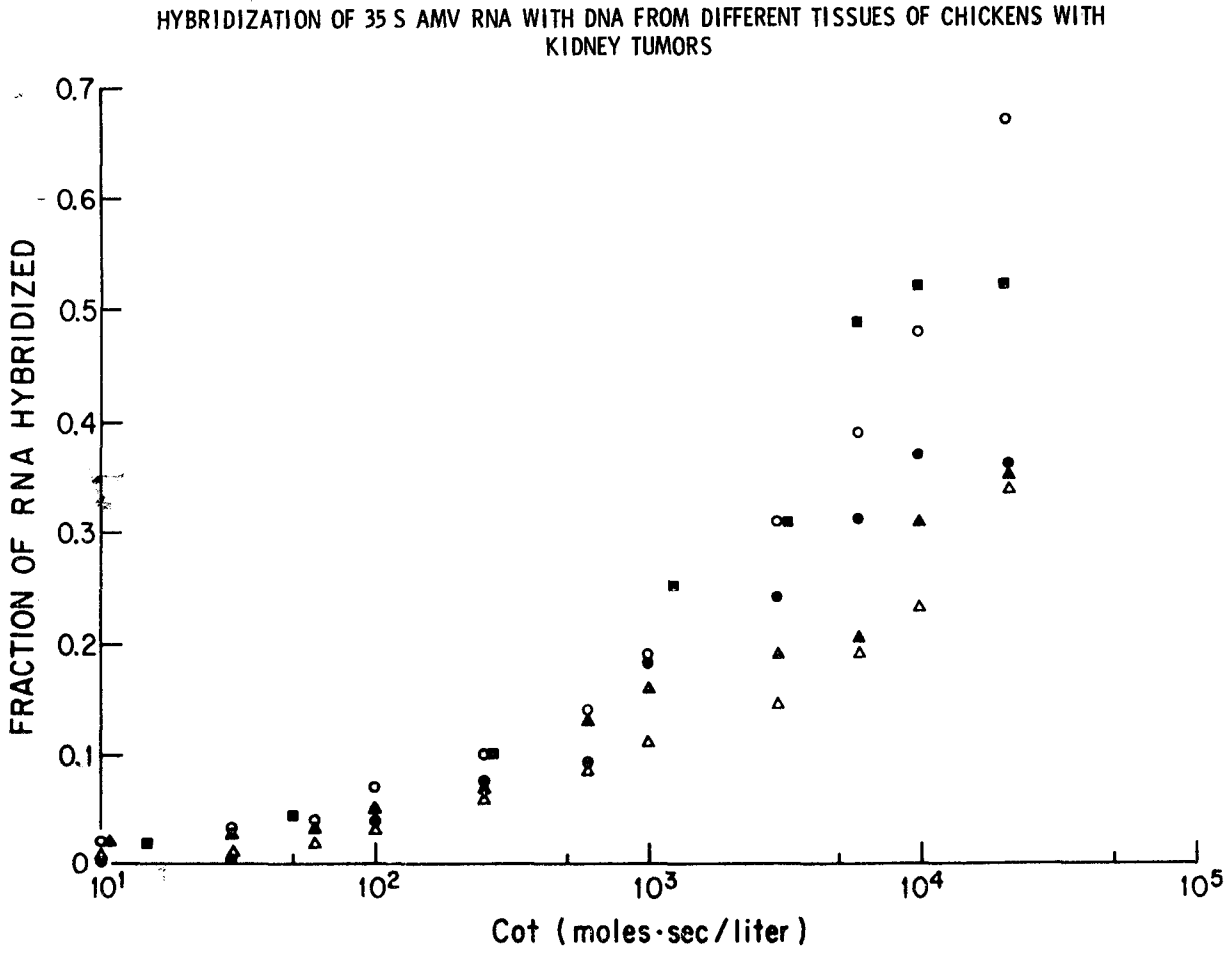

Fig. 5: Hybridization kinetics of ^{3}H-labeled 35S AMV RNA with excess DNA from various tissues of chickens with kidney tumors: The conditions of hybridization were identical to those in Figure 1.

○ Kidney Tumor DNA
△ Liver DNA
■ Apparently normal kidney DNA
● Spleen DNA
▲ Lung DNA

The intermediate increase of viral DNA sequences in some tissues, e. g., heart, liver, lung, and spleen, may reflect either a mixture of noninfected and infected cells, infiltration of the organ by leukemic myeloblasts and RBC, or addition of fewer or incomplete DNA copies of the AMV genome in certain types of cells.

The data in Table IV show that the cellular concentration of AMV DNA increases as much in kidney tumor cells as it did in leukemic myeloblasts or in RBC of leukemic chicks but that there is no increase in non-target organs such as muscle or liver. To determine whether the increase in AMV DNA content represented the acquisition of new complete DNA copies of the AMV genome or amplification of preexisting endogenous sequences, ^{3}H-AMV RNA was hybridized in an excess of cellular DNA from various tissues of kidney tumor-bearing chickens. The results (Figure 5) show that DNA from kidney tumors hybridizes approximately twice as much viral RNA (67 %) as does DNA from spleen, lung or liver. DNA from the latter tissues hybridizes with AMV RNA to the same extent (33–36 %) as DNA from the tissues of uninfected chickens. The kinetics of hybridization indicate that entire DNA copies of AMV RNA have been acquired by kidney tumor cells and that there are 4–6 copies of viral DNA per diploid cell genome.

To demonstrate more directly the presence of viral DNA sequences in neo-

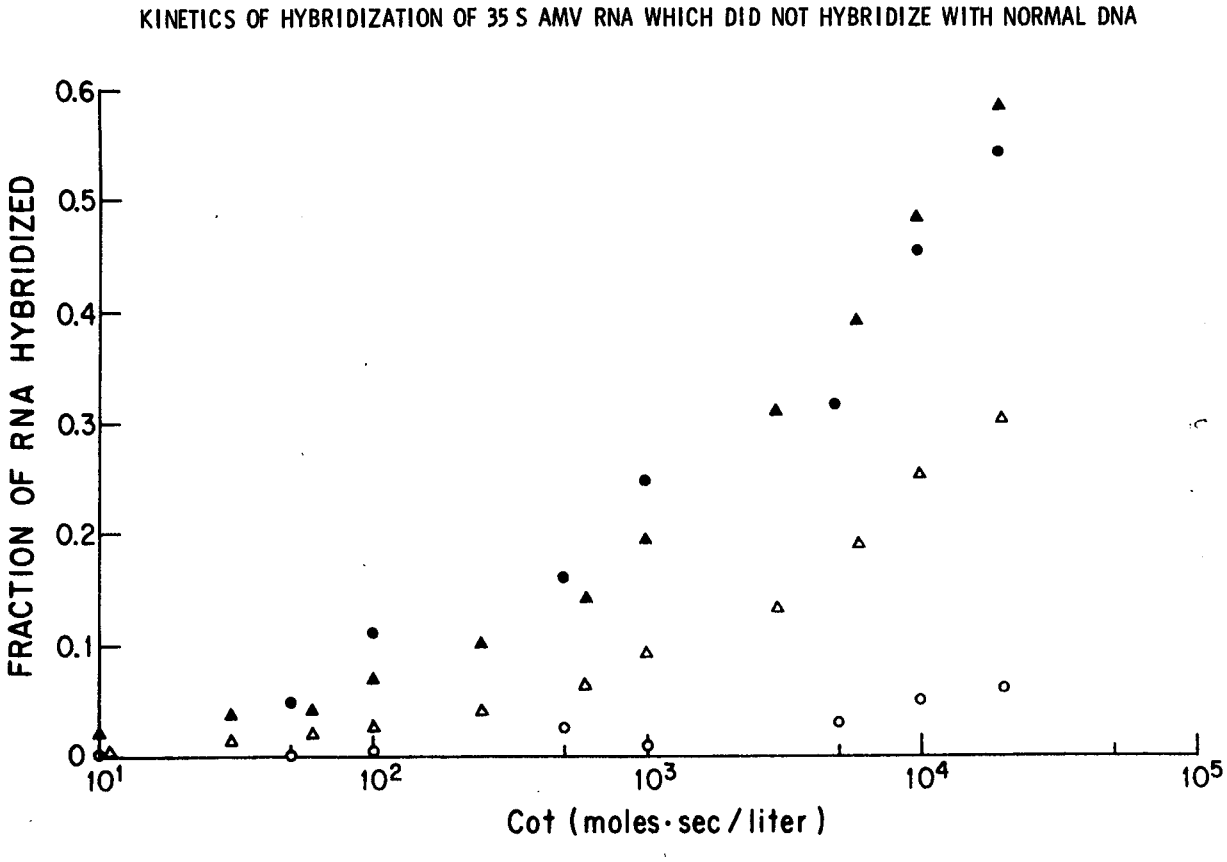

Fig. 6: Kinetics of hybridization in excess of DNA of ^{3}H-labeled 35S AMV RNA and 35S AMV RNA which failed to hybridize with normal chicken embryonic DNA. The conditions of hybridization were the same as those described in Figure 1.

△ Hybridization of 35S AMV RNA with normal chicken embryo DNA
▲ Hybridization of 35S AMV RNA with chicken kidney tumor DNA
○ Hybridization of residual AMV RNA with normal chicken embryo DNA
● Hybridization of residual AMV RNA with chicken kidney tumor DNA

plastic tissues, e. g., leukemic myeloblasts and kidney tumor cells that are not present in normal tissue, sonicated 35S AMV RNA was hybridized exhaustively with an excess of DNA from normal chicken cells to remove all viral RNA sequences homologous to endogenous viral DNA in normal cells. The unhybridized AMV RNA was then isolated and rehybridized with excess DNA from normal or neoplastic kidney tissues. The kinetics of the second hybridization are shown in Figure 6, which also includes for comparison hybridization of untreated 35S AMV RNA with DNA from the same tissues. At Cot 20,000 kidney tumor DNA hybridized 59 % of untreated 35S AMV RNA whereas normal embryonic chicken DNA hybridized only 30 % of the viral RNA. The residual AMV RNA fraction that failed to hybridize with DNA from normal chickens hybridized only 6 % with DNA from normal cells. In contrast, the kinetics of hybridization of the residual AMV RNA with the kidney tumor DNA was similar to the kinetics of untreated 35S RNA and 54 % of the residual RNA became RNase resistant. These results provide direct evidence that kidney tumor DNA contains new AMV DNA sequences acquired after infection with AMV. Similar results were obtained with DNA from leukemic myeloblasts.

Conclusions

Proviral DNA is synthesized within an hour after infection of chicken cells with an avian oncornavirus and is integrated into nuclear cellular DNA within a short time. The viral DNA appears to be synthesized as double-stranded molecules of approximately 6×10^6 daltons some of which are converted into supercoiled circles perhaps as a requisite for integration.

The endogenous v-DNA in normal chicken cells and both the endogenous and AMV v-DNA in leukemic chicken myeloblasts are covalently linked with chromosomal DNA. There is no detectable free DNA either circular or linear present in leukemic cells several weeks after infection. The endogenous v-DNA which is transmitted vertically from parents to offspring is uniformly and stably distributed in all chicken organs. There are about 1–2 copies of endogenous provirus per haploid genome of all normal cells. This DNA is very closely related to RAV-0 RNA. After infection with AMV it seems that target cells such as leukemic myeloblasts, RBC and nephroblasts acquire complete copies of AMV DNA. Interestingly, only these target cells can be converted to neoplastic cells in the chicken as well as *in vitro*. The target cells acquire 1–2 copies of AMV specific DNA per haploid genome in addition to the endogenous v-DNA.

All the available evidence shows that leukemic and kidney tumor cells have acquired AMV v-DNA. It remains to be elucidated whether the newly added viral DNA is alone responsible for neoplastic changes or does so in conjunction with endogenous viral information.

Acknowledgements

The work described here was supported by USPHS Grant CA-10197 from the National Cancer Institute and by contract CP-33283 within the Virus Cancer Program of the National Cancer Institute.

References

Ali, M. and M. A. Baluda (1974) Synthesis of avian oncornavirus DNA in infected chicken cells. J. Virol. *13*: 1005–1013.

Bader, J. P. (1964) The role of deoxyribonucleic acid in the synthesis of Rous sarcoma virus. Virology *22*: 462–468.

Bader, J. P. (1965) The requirement for DNA synthesis in the growth of Rous sarcoma and Rous-associated viruses. Virology *26*: 253–261.

Baltimore, D. (1970) Viral RNA-dependent DNA polymerase. Nature *226*: 1209–1211.

Baluda, M. A., I. E. Goetz and S. Ohno (1963) Induction of differentiation in certain target cells by avian myeloblastosis virus: an *in vitro* study. In 17th Annual Symposium on Fundamental Cancer Research, M. D. Anderson Hospital. Viruses, Nucleic Acids and Cancer. Baltimore, William & Wilkins Co., pp. 387–400.

Baluda, M. A. and P. P. Jamieson. (1961) *In vivo* infectivity studies with avian myeloblastosis virus. Virology *14*: 33–45.

Baluda, M. A. and D. P. Nayak (1970) DNA complementary to viral RNA in leukemic cells induced by avian myeloblastosis virus. Proc. Nat. Acad. Sci. USA *66*: 329–336.

Bentvelzen, P., J. H. Daams, P. Hageman and J. Calafat (1970) Genetic transmission of viruses that incite mammary tumors in mice. Proc. Nat. Acad. Sci. USA *67*: 377–384.

Borek, E. and A. Ryan (1973) Lysogenic induction. Prog. Nuc. Acid. Res. & Mol. Biol. *13*: 249–300.

Echols, H. (1971) Lysogeny: viral repression and site-specific recombination. Ann. Rev. Biochem. *40*: 827–857.

Hanafusa, H. and T. Hanafusa (1971) Noninfectious RSV deficient in DNA polymerase. Virology *43*: 313–316.

Harel, L., J. Harel and G. Frezouls (1972) DNA copies of RNA in rat cells transformed by Rous sarcoma virus. Biochem. Biophys. Res. Comm. *48*: 796–801.

Hill, M. and J. Hillova (1972a) Virus recovery in chicken cells tested with Rous sarcoma cell DNA. Nature New Biology *237*: 35–39.

Hill, M. and J. Hillova (1972) Recovery of the temperature-sensitive mutant of Rous sarcoma virus from chicken cells exposed to DNA extracted from hamster cells transformed by the mutant. Virology *49*: 309–313.

Hirt, B. (1967) Selective extraction of polyoma DNA from infected mouse cell culture. J. Mol. Biol. *26*: 365–369.

Markham, P. D. and M. A. Baluda (1973) Integrated state of oncornavirus DNA in normal chicken cells and in cells transformed by avian myeloblastosis virus. J. Virol. *12*: 721–732.

May, J. T., K. D. Somers and S. Kit (1972) Defective mouse sarcoma virus deficient in DNA polymerase activity. J. Gen. Virol. *16*: 223–226.

Neiman, P. E. (1972) Rous sarcoma virus nucleotide sequences in cellular DNA: measurement by RNA-DNA hybridization. Science *178*: 750–753.

Peebles, P. T., D. K. Haapala and A. F. Gazdar (1972) Deficiency of viral ribo-

nucleic acid-dependent deoxyribonucleic acid polymerase in noninfectious virus-like particles released from murine sarcoma virus-transformed hamster cells. J. Virol. *9*: 488–493.

Sambrook, J., H. Westphal, P. R. Srinivasan and R. Dulbecco (1968) The integrated state of viral DNA in SV-40 transformed cells. Proc. Nat. Acad. Sci. USA *60*: 1288–1295.

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.

Shoyab, M., M. A. Baluda and R. M. Evans (1974) Acquisition of new DNA sequences after infection of chicken cells with avian myeloblastosis virus. J. Virol. *13*: 331–339.

Shoyab, M., P. D. Markham and M. A. Baluda (1974) Reliability of the RNA-DNA filter hybridization for the detection of oncornavirus-specific DNA sequences. J. Virol. *14*: 225–230.

Shoyab, M., R. M. Evans and M. A. Baluda (1974) Presence in leukemic cells of avian myeloblastosis virus-specific DNA sequences absent in normal chicken cells. J. Virol. *14*: 47–49.

Shoyab, M., P. D. Markham and M. A. Baluda (1975) Host induced alteration of avian sarcoma virus B-77 genome. Proc. Nat. Acad. Sci. USA *72*: 1031–1035.

Svoboda, J., I. Hlozanek and O. Mach (1972) Detection of chicken sarcoma virus after transfection of chicken fibroblasts with DNA isolated from mammalian cells transformed with Rous virus. Folia Biologia *18*: 149–153.

Temin, H. M. (1962) Separation of morphological conversion and virus production in Rous sarcoma virus infection. Cold Spring Harbor Sym. Quant. Biol. XXVII, 407–414.

Temin, H. M. (1963) The effect of actinomycin D on growth of Rous sarcoma virus *in vitro*. Virology *20*: 577–582.

Temin, H. M. (1964) Nature of the provirus of Rous sarcoma. Nat. Cancer Inst. Monogr. *17*: 557–570.

Temin, H. M. and S. Mizutani (1970) RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature *226*: 1211–1213.

Temin, H. M. (1971) Mechanism of cell transformation by RNA tumor viruses. Ann. Rev. Microbiol. *25*: 609–648.

Varmus, H. E., P. K. Vogt and J. M. Bishop (1973) Integration of deoxyribonucleic acid specific for Rous sarcoma virus after infection of permissive and nonpermissive hosts. Proc. Nat. Acad. Sci. USA *69*: 576–580.

Varmus, H. E., H. Suzanne and J. M. Bishop (1974) Use of DNA-DNA annealing to detect new virus specific DNA sequences in chicken embryo fi-roblast after infection by avian sarcoma virus. J. Virol. *14*: 895–903.

Walter, W. G., B. R. Burmester and C. H. Cunningham (1962) Studies on the transmission and pathology of a viral-induced avian nephroblastoma (embryonal nephroma). Avian Diseases *6*: 455–477.