Avian Tumor Virus Proteins and RNA in Uninfected Chicken Embryo Cells

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The content of proteins P19 and P15 (mol wt 19,000 and 15,000, respectively) of avian leukovirus in various types of uninfected chicken embryos has been determined by radioimmunoassay. All chicken embryos examined, including embryos which have thus far been classified as group specific (gs) antigen negative by complement fixation tests, contained these viral proteins as well as P27 as previously reported. The embryos known as "gs antigen-positive" type contained about five times as much of these viral proteins as did the "gs antigen-negative" type. The ratio of the three viral proteins was similar for all types of embryos, suggesting that the genes for these proteins are coordinately controlled. In contrast to the relatively high levels of viral internal proteins in gs antigen-negative cells, the amounts of virus-specific RNA detectable by molecular hybridization were extremely low. The levels of helper activity, which presumably reflect the level of viral envelope glycoprotein, were also generally low or undetectable in these cells. Thus, the expression of the gene for envelope glycoprotein does not appear to be controlled coordinately with the genes for viral internal proteins.

Both virological and molecular biological studies have demonstrated that chicken cells contain viral genes specific to RNA tumor viruses in the form of provirus DNA, and that the expression of these genes is under certain genetic controls (7, 10, 18, 22, 23). Two major groups of chickens have been described which are positive or negative for the avian leukosis virus group-specific antigens (gs antigens), and the presence of these viral proteins is generally correlated with the presence of another phenotypic expression detectable as a helper function (7, 23).

Previously published data, however, showed that a major viral protein known as gsl or P27 is present in both gs antigen-positive and gs antigen-negative chicken cells in different quantities (2). We have extended the radioimmunoassay to two other viral proteins, P19 and P15. The levels of these viral proteins have been compared with viral RNA content and helper activity in a variety of chicken cell types in order to gain further insight into the control of the expression of endogenous viral genes.

MATERIALS AND METHODS

Cells and viruses. Viruses used in these studies were Rous-associated virus 2 (RAV-2), B77 strain of avian sarcoma virus and Bryan strain of Rous sarcoma virus with the envelope antigen specified by

chicken helper factor [B-RSV(*chf*)]. The source of the viruses, preparation of the chicken and quail embryo cell cultures, and the conditions for the infection of cells have been described previously (5-8). RAV-2 and B77 viruses were prepared in cells negative for both gs antigen by complement fixation and helper activity (gs⁻h⁻ cells). The RSV(*chf*) was prepared in cells positive for gs antigen and helper activity (gs⁺h⁺) as previously described (8).

Infected cells were prepared by infecting chick cells with RAV-2 at a multiplicity of infection (MOI) of 10 or B77 at an MOI of 1. Quail cells were infected with B-RSV(*chf*) at an MOI of 1. All infected cells were transferred three times before use.

The preparation and purification of [³H]amino acid-labeled RAV-2 and large quantities of RAV-2 from infected roller bottle cultures followed the previously published procedures (2, 20).

Classification of chicken embryo cells. The methods for determinations of gs antigen by complement fixation test and helper activity of each embryo were described previously (3, 8, 12, 18). Based on their gs antigen level by complement fixation test and helper activity (8), the chicken embryos used were classified into three distinct classes: gs⁻h⁻ (negative for both gs antigen and helper activity), gs_Lh_E (low gs titer and extremely high helper activity), and gs⁺h_H (high gs titer and high helper activity) (also see Table 4) as described previously (11).

Separation and purification of individual viral structural proteins. Structural proteins of RAV-2 were separated by chromatography on guanidine-hydrochloride (Heico, Inc., Delaware Gap, Pa.) aga-

rose columns as previously described (2, 3). Each viral protein was rechromatographed on a similar column, and was occasionally further purified by isoelectric focusing (14). The purity of viral proteins was examined by polyacrylamide gel electrophoresis. The purified viral proteins were lyophilized and stored at -20°C before use for immunization to produce monospecific antibody or for iodination with ^{125}I .

Polyacrylamide gel electrophoresis. Electrophoretic analysis of purified viral proteins in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) has been described previously (20). [^3H]leucine-labeled RAV-2 was used as a marker.

Iodination. Purified viral proteins of apparent molecular weights of 19,000 and 15,000 (P19 and P15) of RAV-2 were coupled with ^{125}I by the method of Greenwood et al. (4). Iodinated P19 (^{125}I P19) and P15 (^{125}I P15) had specific activities in the range of 20 to 30 $\mu\text{Ci}/\mu\text{g}$. ^{125}I P19 was 71% precipitable by rat anti P19 serum, whereas ^{125}I P15 was 79% precipitable by rat anti P15 serum.

Preparation of antibodies against purified viral proteins. Anti P27, P19, and P15 sera were prepared in (W/FuXBN)F hybrid rats as described previously (2). The appearance of antibody was monitored by the Ouchterlony technique. Rabbit anti rat immunoglobulin G (IgG) serum was prepared by immunizing rabbits with purified rat IgG. Some rabbit anti rat IgG serum was purchased from Miles Laboratories, Inc., Kankakee, Ill.

Preparation of viral and cellular extracts for radioimmunoassay (RIA). The procedures for preparation of viral extracts have been described (2). Briefly, the purified viruses were dissolved in 0.05 M potassium phosphate buffer, pH 7.5, containing 1% Triton X-100 and 0.2% 2-mercaptoethanol and incubated at 60°C for 15 min. The solubilized virus was then extracted with ether. After removal of residual ether with nitrogen gas, the viral extract was stored at -20°C before use. Cell extracts prepared by the previously described method (2) gave satisfactory results for P19 and P15. However, incomplete recovery has frequently been noticed for P27, particularly when cells are in the resting state. Therefore, in this study whole cell extracts prepared in the following way were used for the analysis of all viral proteins. Cells were suspended in 1% Triton X-100 in 0.05 M potassium phosphate buffer and sonically treated in a Raytheon sonic oscillator (Raytheon Co., Waltham, Mass.) for 3 min at 10 kc. The resulting sonic extract was centrifuged at $7,000 \times g$ for 10 min and extracted once with ether. The aqueous phase after ether extraction had protein contents equivalent to 0.24 to 0.33 ng per cell, which represents 40 to 50% of the total cell protein. The aqueous solution was used for quantitation of viral protein after removal of ether by flushing with nitrogen.

Protein was determined by the procedure of Lowry et al. (17) with bovine serum albumin as standard.

RIA procedure. The titrations of rat anti P19 and anti P15 sera were similar to that of rat anti P27 serum described previously (2).

For the assay of P19 and P15 a competitive RIA method similar to that for the assay of P27 (2) was used. A standard dose response curve for each viral

protein was constructed as follows: the reaction mixture (0.5 ml) containing a known quantity of P19 (or P15) and rat anti P19 (or anti P15) serum which would bind 70% of the precipitable ^{125}I P19 (or ^{125}I P15) was incubated at 37°C for 2 h, and then 0.2 ng of ^{125}I P19 (or ^{125}I P15) was added and further incubated at 37°C for 1 h followed by incubation at 4°C overnight. The rabbit anti rat IgG serum was then added and the assay proceeded as described previously (2).

Preparation of cell cultures and synchronization of cell growth. Secondary chicken embryo cells were plated at 1.5×10^6 per 100-mm petri dish in minimal essential media (MEM) containing 5% calf serum. Eighteen to 20 h later the media were changed to MEM without serum. The cultures were maintained in serum-free medium for 1 week with a daily change of the serum-free medium. Cell growth was then initiated by adding MEM containing 5% calf serum. After addition of the growth medium the cultures were harvested every 4 h during a 24-h period and were processed for viral protein and viral RNA determinations.

DNA synthesis was monitored by pulse labeling the culture with [^3H]thymidine. A 4- μCi amount of [^3H]thymidine in 2 ml of medium was added to chicken embryo cell cultures in 60-mm petri dishes and was incubated for 30 min. The cells were then washed twice with cold TEN buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.001 M EDTA, and 0.1 M NaCl) and solubilized with 0.5 ml of 0.5% SDS in 1 mM EDTA. After addition of 2.5 ml of TEN buffer and 100 μg of bovine serum albumin to the solubilized cell sample, an equal volume of 10% trichloroacetic acid was added. The precipitate was collected on a glass fiber filter, washed with 5% acid, and dried, and acid-precipitable radioactivity was determined.

Extraction of viral RNA. The procedure for RNA extraction from chicken embryo cells was described previously (11).

Hybridization of nucleic acids. ^3H -labeled DNA complementary to the viral RNA was synthesized using detergent-treated RAV-0 in a reaction mixture containing 5 mM MgCl_2 , 20 mM Tris (pH 8.1), 15 mM dithiothreitol, 0.03% Nonidet P-40, 50 μM each dATP, dCTP, and dGTP, 10 μM [^3H]TTP (50 Ci/mmol), 150 μg of actinomycin D per ml, and purified virus (0.5 to 1.0 mg of viral protein per ml). The reaction mixture was incubated for 3 h at 39°C , followed by addition of EDTA to 10 mM, predigested Pronase to 200 $\mu\text{g}/\text{ml}$, and SDS to 0.1%. After further incubation for 1 h, the mixture was extracted twice with phenol at room temperature, and nucleic acid was precipitated with ethanol. The pellet was resuspended in a small volume of 10^{-3} M EDTA and treated for 15 min at 37°C with 20 μg of RNase A per ml (previously boiled 10 min), followed by incubation with NaOH (final concentration of 0.1 M) for 20 min at 100°C . The mixture was then neutralized with 0.5 M HCl, phenol extracted two more times, and ethanol precipitated. The [^3H]DNA was then self-annealed at 60°C in 0.45 M NaCl plus 0.045 M sodium citrate (pH 7.3) to a C_{ot} (product of DNA concentration $\times$ time in mol/s per liter) of 0.2 and passed through hydroxylapatite as previously described (11) to re-

move any double-stranded DNA (usually less than 25% of the total radioactive material). Single-stranded DNA was then passed through Sephadex G-75 (medium) to remove sodium phosphate and low molecular weight ^3H -labeled material, and was concentrated by ethanol precipitation. Parallel samples of [^3H]DNA were prepared under identical conditions, but with a specific activity which was 1/500th of that in the probe DNA, and tested for its ability to protect ^3H -labeled 70S viral RNA from digestion with RNase. The DNA preparations used in this study protected at least 90% of the labeled viral RNA at a DNA/RNA ratio of 10, and approximately 95% at a ratio of 100. Hybridization was performed at 50 C using a reaction mixture which contained 50% formamide, 0.45 M NaCl, 0.045 M sodium citrate (pH 7.3), 0.005 M EDTA, 0.1% SDS, 0.001 to 0.003 μg of [^3H]DNA per ml and 5 to 30 mg of cell RNA per ml. Concentrations of viral RNA within the cell were determined by comparing C_{r} (product of RNA con-

centration $\times$ time in mol/s per liter) values at which 50% hybridization is obtained ($1/2 C_{\text{r}}$) with that for purified viral 70S RNA preparations as described previously (11, 16).

RESULTS

Isolation and characterization of P19 and P15. Viral proteins P19 and P15 were purified from RAV-2 and labeled with ^{125}I as described in Materials and Methods. The purity of the iodinated proteins was examined by SDS-polyacrylamide gel electrophoresis by using [^3H]leucine-labeled RAV-2 as a marker. As shown in Fig. 1 and 2, both ^{125}I -labeled P19 and P15 migrated as single peaks in gel electrophoresis. However, the radioimmunoassay showed a slight contamination of the P15 and P27 preparations with P19 (see next section). Seven-

FIG. 1. Polyacrylamide gel electrophoresis of [^{125}I]P19 in the presence of SDS. [^{125}I]P19 was mixed with [^3H]leucine-labeled RAV-2 solubilized in 1% SDS and subjected to electrophoresis in a 7% gel as described (20).

ty-one per cent of the [125 I]P19 and 79% of the [125 I]P15 could be precipitated by the respective monospecific antibodies prepared in rats. These figures were used as the maximum precipitation of these proteins by their antibodies in competitive RIA.

Competitive RIA of P19 and P15. To quantitate P19 and P15, competitive RIA was used for each individual protein. Anti P19 and anti P15 were used at 1:2,000 and 1:40,960, respectively; at these concentrations the antisera gave about 70% of their respective maximal binding. As shown in Fig. 3 and 4, P19 was detectable in amounts as low as 0.3 ng, whereas P15 was

detectable at 0.6 ng. The purity of each reagent was tested by measuring the degree of cross-reaction with heterologous proteins. When high concentrations of unlabeled P27 or P15 were used to compete with [125 I]P19 for binding with anti P19 some cross reaction was observed (Fig. 3). This indicated that the P27 and P15 preparations contain small amounts of P19. The content of P19 in the P27 and P15 preparations was estimated by RIA to be about 0.5 and 1%, respectively, of the P27 and P15 preparations. Cross reaction was not observed when unlabeled P27 or P19 was used to compete with [125 I]-P15 binding with anti P15 (Fig. 4).

FIG. 2. Polyacrylamide gel electrophoresis of [125 I]P15 in the presence of SDS. [125 I]P15 was mixed with [3 H]leucine-labeled RAV-2 solubilized in 1% SDS and subjected to electrophoresis in a 7% gel as described (20).

FIG. 3. Competitive radioimmunoassay for P19. Different amounts of P27, P19, or P15 were mixed with rat anti P19 at a 1:2,000 dilution, which binds 71% of the precipitable [125 I]P19. The input of [125 I]P19 was 15,000 counts/min (0.3 ng).

FIG. 4. Competitive radioimmunoassay for P15. The procedure is the same as for Fig. 3 except that [125 I]P15 was used at an input of 15,000 counts/min. Rat anti P15 was used at a 1:40,960 dilution.

Content of P27, P19, and P15 in virus and various chicken cells. Purified RAV-2 and B77 virus were disrupted with Triton X-100 as described in Materials and Methods. The amounts of the three viral proteins in the extracts were assayed by RIA. The three viral proteins P27, P19, and P15 represent 75% of the total RAV-2 protein and 83% of the B77 virus protein (Table 1). The molar ratios of these three proteins were similar in these two viruses (P27:P19:P15 = 2:1:2.4). These values are comparable to those estimated from the results of polyacrylamide gel electrophoresis and agarose chromatography of another RNA tumor virus, MC29 (3).

TABLE 1. Content of viral proteins in viral particles^a

Virus	P27 (μ g)	P19 (μ g)	P15 (μ g)	Molar ratio (P27:P19:P15)
RAV-2 ^b	45	16	30	2:1:2.4
B77 ^c	40	14	27	2:1:2.4

^a Determined by RIA.

^b Total RAV-2 proteins: 120 μ g.

^c Total B77 proteins: 100 μ g.

All of these viral proteins were found in virus-infected cells (Table 2). However, the molar ratios of the viral proteins were variable

in different samples of infected cells. No detectable P19 and P15 were found in uninfected quail and BALB 3T3 cells.

Three types of uninfected chicken embryos, gs^+h_H , gs^-h^- , gs_Lh_E , were examined for the content of P19 and P15, as well as P27. The results are shown in Table 3. All three types of embryos contained the three viral proteins. The amounts of these viral proteins in gs^+h_H embryos were about four to five times higher than in gs^-h^- embryos. In gs_Lh_E embryos the levels of these proteins were within the same range as those for gs^-h^- embryos.

The representative binding curves of viral proteins from purified virus, virus-infected cells, and uninfected cells toward their respective antisera are shown in Fig. 5 to 7. It can be seen that the binding curves have similar slopes, except for small deviations with some gs^-h^- and gs_Lh_E embryos. (These analyses were not carried to high enough cell protein concentrations to determine whether or not complete competition would occur.) The significance of these deviations is not clear, but this may be due to the high concentrations of cellular proteins in the reaction mixture required to detect these low levels of viral protein. A reconstruc-

tion experiment with various concentrations of uninfected gs^-h^- cell proteins mixed with a known amount of RAV-2 protein (P27) showed that an inhibitory effect by cell protein on the quantitation of RAV-2 P27 became significant only when more than 5 mg of cellular protein were present in the reaction mixture. Thus, under the conditions used (the maximum amounts of protein used in the experiments shown in Fig. 5 to 7 were about 1 mg), the inhibitory effect would not appear to be a major factor.

From these results we conclude that P27, P19, and P15 are present in practically all chicken cells. It can be seen in Table 3 that these proteins are present not only in gs^+ cells, but also in cells classified as gs^- by the complement fixation test. The difference in the two types of cells is thus quantitative rather than qualitative. These proteins are present in all three types of uninfected cells at a fairly constant molar ratio of 2:1:4, which is very similar to that in the virion (2:1:2.4). This fact seems to confirm the reliability of the determination of each protein. Whereas the molar ratios of P19 and P15 found in virus-infected cells were similar to those found in uninfected cells, the

TABLE 2. Viral protein content (nanograms per milligram of cell protein) in different cells

Cell	P27	P19	P15	Ratio ^a
B77-infected chick cells	1,430	338	1126	3.0:1:4.2
RAV-2-infected chick cells	6,700	800	2666	5.9:1:4.2
Hamster cells transformed by RSV ^b	240	144	320	1.2:1:2.8
Quail cells transformed by RSV ^c	910	136	364	4.7:1:3.4
Quail cell				
No. 961	<0.3	<0.3	<0.6	
No. 967	<0.5	<0.5	<1.0	
No. 978	<0.3	<0.3	<0.6	
No. 980	<0.9	<0.3	<0.6	
BALB 3T3	<0.9	<0.3	<0.6	

^a The molar ratio is calculated as follows. The content of each viral protein in the cell extract is divided by its molecular weight. Values thus obtained are compared by setting the value for P19 equal to one.

^b H-RSV(BH) cell line (19).

^c Infected with B-RSV(*chf*).

TABLE 3. Content of viral proteins in uninfected chicken cells^a

Type of embryo	No of samples	P27	P19	P15	Ratio ^b
gs^+h_H	8	37.6 ± 11.3	13.0 ± 2.4	35.1 ± 8.9	2:1:3.3
gs^-h^-	9	7.3 ± 2.1	2.8 ± 0.7	8.4 ± 1.9	1.8:1:3.8
gs_Lh_E	7	8.6 ± 1.4	3.4 ± 0.4	9.6 ± 2.4	1.8:1:3.6

^a The content of viral proteins is expressed as the mean ± standard deviation in nanograms per milligram of cell protein.

^b Molar ratio, computed as described in Table 2.

relative levels of P27 were quite variable in the virus-infected cells. The reason for this difference is not immediately clear.

Viral RNA in normal and infected cells.

The presence of viral RNA in uninfected gs^+ cells has been reported previously by this and other laboratories (1, 11, 16, 21), although no viral RNA was detected in gs^- cells (1, 11, 21).

FIG. 5. Binding curves of viral protein P27 from purified viral particles, infected cells, and different types of uninfected chick cells. Different amounts of proteins were used to compete with $[^{125}I]P27$ for binding with rat anti P27 serum. $[^{125}I]P27$ input was 12,000 counts/min, and rat anti P27 was used at 1:5,000. Symbols: ●, purified P27 from RAV-2; Δ, RAV-2-infected chick cells; ○, B77-infected chick cells; ×, gs^+h_H cells; □, gs^+h_E cells; ▽, gs^-h^- cells.

FIG. 6. Binding curves of viral protein P19 from purified viral particles, infected cells, and different types of uninfected chick cells. The competitive radioimmunoassay is similar to that described in Fig. 5. $[^{125}I]P19$ was used at 15,000 counts/min, and rat anti P19 serum was used at a 1:2,000 dilution. Purified P19 from RAV-2 (●); the other symbols are the same as in Fig. 5.

In more recent experiments (see below), low but probably significant amounts of viral RNA have been detected in gs^- cells, utilizing somewhat more favorable annealing conditions (increased incubation temperature, higher RNA/DNA ratios) than previously reported (11), and using [3H]DNA synthesized from RAV-0 virions. The RAV-0 [3H]DNA has a greater degree of sequence homology with the endogenous viral

nucleic acids than does the RAV-2 probe used in the previous analyses (W. S. Hayward, unpublished data; see also ref. 24).

The RNA from uninfected gs^+h_H cells hybridizes with approximately 80% of the RAV-0 [3H]DNA probe (Fig. 8). The content of viral RNA in these cells, estimated from $1/2 C_{ot}$ values (see 11, 16), was approximately 1/40th of that in RAV-2-infected cells. By contrast, viral

FIG. 7. Binding curves of viral protein P15 from purified viral particles, infected cells, and different types of uninfected chick cells. The competitive radioimmunoassay is similar to that described in Fig. 5. [^{125}I]P15 was used at 15,000 counts/min, and rat anti P15 serum was used at a 1:40,960 dilution. Purified P15 from RAV-2 (●); the other symbols are the same as in Fig. 5.

FIG. 8. Hybridization of cellular RNAs with viral [3H]DNA probe. Hybridization was performed as described in Materials and Methods using [3H]DNA synthesized from RAV-2 (Δ , $\blacktriangle$) or RAV-0 (all others). The specific activity of the DNAs was 2.0×10^7 counts/min per μg . Sources of RNA and concentrations are as follows: RAV-0, 6 $\mu g/ml$ ($\circ$); RAV-2, 6 $\mu g/ml$ (Δ); RAV-2-infected gs^-h^- cells, 4 mg/ml ($\blacktriangle$); gs_Lh_H cells, 6 mg/ml ($\bullet$); gs^+h_H cells, 6 mg/ml ($\square$); gs^-h^- cells, 20 mg/ml ($\blacksquare$); turkey cells, 20 mg/ml ($\times$).

RNA was barely detectable in $gs^{-}h^{-}$ cells. Only 15% of the [3H]DNA probe was hybridized at the highest C_{t} values attained (2×10^5 mol/s per liter), although this does not appear to represent a plateau value. This low level of hybridization could conceivably represent hybridization of contaminating host sequences in the DNA probe. However, it seems more likely that these sequences are viral specific, since more than 90% of this probe DNA hybridizes with purified viral 70S RNA. Furthermore, no hybridization was detected with RNA from related host cells such as turkey (see Fig. 8), quail, and duck (data not shown).

The amounts of viral RNA in cells of various types, calculated from the $1/2 C_{\text{t}}$ values, are shown in Table 4, along with other pertinent information about these cells. (The value shown for $gs^{-}h^{-}$ cells is only approximate, since the reaction was not complete at the highest C_{t} attained.) A comparison of the viral RNA content with values for internal proteins (represented in Table 4 by P19) indicates that there is very little correspondence between RNA and protein levels in the various cell types. However, a reasonable correlation was found between helper activity and viral RNA content.

Change in viral protein and RNA content with respect to cell cycle. Since leukosis virus replication is dependent on cell cycle (13), we investigated the content of viral proteins during the growth cycle of uninfected gs^{+} cells. Cells were accumulated in G1 phase by maintaining them in serum-free medium (15). Addition of serum-containing medium resulted in the semi-synchronous synthesis of DNA and subsequent cell division. The content of P27 and P15 per culture of $gs^{+}h_{\text{H}}$ cells increased linearly with time after addition of growth medium (Fig. 9).

This increase in viral proteins was parallel to the increase in total cellular protein. Similar phenomena were also observed in $gs^{-}h^{-}$ cells. These results indicate that the synthesis of P27 and P15 in uninfected chick cells is not dependent on any particular phase in the cell cycle.

Parallel cultures were analyzed for content of viral-specific RNA at various intervals after addition of serum. As can be seen in Fig. 9, the serum-starved cells contain significant amounts of viral RNA. During the 24-h period after addition of serum, the amount of viral RNA per culture increased approximately threefold. The majority of this increase occurred between 8 and 12 h, just prior to the time of maximal cell DNA synthesis.

DISCUSSION

A summary of the various viral activities and products which have been analyzed in different cell types is presented in Table 4. Perhaps the most unexpected result of these analyses is the relatively high levels of the viral internal proteins in cells which were classified as gs^{-} by the complement fixation test. The difference in levels between " gs^{+} " and " gs^{-} " cells was only five- to 10-fold. By contrast, the helper activity in these two cell types differed by approximately 100-fold. Furthermore, the level of viral specific RNA in the gs^{-} cells was extremely low, probably less than 1% of that in gs^{+} cells.

$Gs^{-}h^{-}$ cells contain viral DNA sequences which anneal to at least 80% of the RAV-0 [3H]DNA probe (H. Hanafusa, W. S. Hayward, J. H. Chen, and T. Hanafusa, Cold Spring Harbor Symp. Quant. Biol., in press). The viral DNA in the $gs^{-}h^{-}$ cells is similar both in amount and in sequence representation to that in the $gs^{+}h_{\text{H}}$ cells. Thus the difference in the

TABLE 4. Viral products and activities in various cell types^a

Cell type	Helper act ^b	CF titer ^c	P19 (ng/mg) ^d	RNA (ng/mg) ^e
$gs^{-}h^{-}$	0-10	0	2.1-3.7	~1
$gs^{+}h_{\text{H}}$	1,000-3,000	4-8	10-17	50-200
$gs_{\text{L}}h_{\text{E}}$	10,000-30,000	0-2	2.8-3.7	150-300
$gs^{-}h^{-}$ infected with RAV-2		64-128	800	5,000-10,000

^a The numbers shown represent the range of values obtained with at least four different embryos of each type.

^b Titer of RSV(*chf*) at day 5, determined by method A (8).

^c Reciprocal of the terminal antigen dilution which gives more than a +3 reaction in the complement fixation test (8).

^d Determined by RIA.

^e Nanograms of viral specific RNA per milligram of total cell RNA, calculated from C_{t} curves (11, 16). Calculations assume that the complexity of the viral RNA within the cells is the same as that of the purified viral RNA used as a standard.

FIG. 9. Content of viral proteins P27 and P15 and viral RNA during one cycle of cell growth. Uninfected gs^+h_H cells were partially synchronized by serum depletion and then initiated into the growth cycle by addition of serum-containing media. P27 and P15 were determined by competitive RIA. Viral RNA levels were determined by hybridization with RAV-0 [3H]DNA probe. The pulse labeling of cells with [3H]thymidine is described in Materials and Methods.

viral RNA levels in these two cell types appears to be due to differential rates of transcription of proviral DNA, rather than to differences in viral DNA content. However, no clear correlation was found between the levels of viral RNA and internal proteins within the various cell types. This is particularly evident when comparing cells of type gs_{1hE} and gs^-h^- , which contain similar amounts of viral proteins, but in which the viral RNA levels differ by a factor of more than 100 (see Table 4). It seems possible, therefore, that post-transcriptional controls may also play a role in determining the level of viral protein synthesis. However, it is also possible that the viral RNA sequences coding for the internal proteins are transcribed at a

different rate from that of the bulk viral RNA. These specific gene sequences might remain undetected in the hybrid analysis if they are represented in relatively low amounts in the viral probe. It should be stressed that, although all or essentially all of the sequences are present in the probe, the distribution of sequences is not uniform.

The relative amounts of P27, P19, and P15 were the same in the three types of normal cells tested, and were similar to that found in virions. Even in infected cells, where more variability was observed in the P27 levels, the ratios of these three proteins did not differ greatly from that of the virion. These results strongly suggest that the genes for the three viral proteins are

linked or coordinately controlled.

The analysis of the envelope glycoprotein has not been performed by radioimmunoassay, although its presence in gs^+h_H cells has been demonstrated by the capacity of cell extracts to block the neutralizing antibody against subgroup E virus (9). It is reasonable to assume that *chf* level is determined by the extent of synthesis of the glycoprotein. Even though a low level of *chf* is detectable in some gs^-h^- type embryos, the majority of gs^-h^- embryos exhibit no helper activity (less than 10^{-6} of gs^+h_H type). Therefore, in gs^-h^- cells, it appears that significant amounts of P27, P19, and P15 are synthesized but no envelope glycoprotein is formed. A good correlation has been reported for the synthesis of *gs*-antigen detectable by the complement fixation test and the expression of *chf* activity (7, 10, 23), and their expression is believed to be determined largely by a single cellular gene (10, 23). However, the results shown in this paper demonstrate that the suppression of viral internal protein synthesis is not complete in gs^- cells, contrary to previous notions based on analyses using the less sensitive complement fixation test.

The noncoordinate control of the expression of viral internal proteins and envelope glycoprotein has been shown distinctly in gs_Lh_E type embryos (10). These embryos manifest an extremely high level of helper activity. The level of the viral internal proteins in these cells was, however, only slightly higher than that in the gs^-h^- type, supporting data obtained by complement fixation tests in which extracts of gs_Lh_E type embryos give barely positive reactions. The genetics of h_E type expression is currently being examined in breeding experiments involving various types of chickens.

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LITERATURE CITED

- Bishop, J. M., N. Jackson, N. Quintrell, and H. E. Varmus. 1973. Transcription of RNA tumor virus genes in normal and infected cells, p. 61-73. *In* Possible episomes in eukaryotes: 4th Lepetit colloquium. North Holland Publishing Co., Amsterdam.
- Chen, J. H., and H. Hanafusa. 1974. Detection of a protein of avian leukoviruses in uninfected chick cells by radioimmunoassay. *J. Virol.* **13**:340-346.
- Fleissner, E. 1971. Chromatographic separation and antigenic analysis of proteins of oncornoviruses. I. Avian leukosis-sarcoma viruses. *J. Virol.* **8**:778-785.
- Greenwood, R. W., W. M. Hunter, and J. S. Glover. 1963. The separation of ^{125}I -labeled human growth hormone of high specific activity. *Biochem. J.* **89**:114-123.
- Hanafusa, H. 1965. Analysis of the defectiveness of Rous sarcoma virus. III. Determining influence of a new helper virus on the host range and susceptibility to interference of RSV. *Virology* **25**:248-255.
- Hanafusa, H., and T. Hanafusa. 1966. Determining factor in the capacity of Rous sarcoma virus to induce tumors in mammals. *Proc. Nat. Acad. Sci. U. S. A.* **55**:532-538.
- Hanafusa, H., T. Miyamoto, and T. Hanafusa. 1970. A cell associated factor essential for formation of an infectious form of Rous sarcoma virus. *Proc. Nat. Acad. Sci. U. S. A.* **66**:314-321.
- 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.
- 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.
- Hanafusa, H., T. Hanafusa, S. Kawai, and R. E. Luginbuhl. 1974. Genetic control of expression of endogenous virus genes in chicken cells. *Virology* **58**:439-448.
- Hayward, W. S., and H. Hanafusa. 1973. Detection of avian tumor virus RNA in uninfected chicken embryo cells. *J. Virol.* **11**:157-167.
- Huebner, R. J., D. Armstrong, M. Okuyan, P. S. Sarma, and H. C. Turner. 1964. Specific complement fixing viral antigens in hamster and guinea pig tumors induced by the Schmidt-Ruppin strain of avian sarcoma virus. *Proc. Nat. Acad. Sci. U. S. A.* **51**:741-750.
- Humphries, E. H., and H. M. Temin. 1972. Cell cycle-dependent activation of Rous sarcoma virus-infected stationary chicken cells: Avian leukosis virus group specific antigens and ribonucleic acid. *J. Virol.* **10**:82-87.
- Hung, P. P., H. L. Robinson, and W. S. Robinson. 1971. Isolation and characterization of proteins from Rous sarcoma virus. *Virology* **43**:251-266.
- Johnson, L. F., H. T. Abelson, H. Green, and S. Penman. 1974. Changes in RNA in relation to growth of the fibroblast. I. Amounts of mRNA, rRNA, and tRNA in resting and growing cells. *Cell* **1**:95-100.
- Leong, J., A. 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.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **192**:265-275.
- Payne, L. N., and R. C. Chubb. 1968. Studies on the nature and genetic control of an antigen in normal chick embryos which reacts in the COFAL test. *J. Gen. Virol.* **3**:379-391.
- Sarma, P. S., W. Vass, and R. J. Huebner. 1966. Evidence for the in vitro transfer of defective Rous sarcoma virus genome from hamster tumor cells to chick cells. *Proc. Nat. Acad. Sci. U. S. A.* **55**:1435-1442.
- Scheele, C. M., and H. Hanafusa. 1971. Proteins of helper dependent Rous sarcoma virus. *Virology* **45**:401-410.
- 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.
- Weiss, R. A. 1969. The host range of Bryan strain Rous sarcoma virus synthesized in the absence of helper virus. *J. Gen. Virol.* **5**:511-528.
- Weiss, R. A., and L. N. Payne. 1971. The heritable nature of the factor in chicken cells which acts as a helper virus for Rous sarcoma virus. *Virology* **45**:508-515.
- Wright, S. E., and P. E. Neiman. 1974. Base sequence relationships between avian RNAs: endogenous and sarcoma virus assayed by competitive RNA-DNA hybridization. *Biochemistry* **13**:1549-1554.