Genetic Variation in the RNA Transcripts of Endogenous Virus Genes in Uninfected Chicken Cells

SAN Y. WANG, WILLIAM S. HAYWARD,* AND HIDESABURO HANAFUSA

The Rockefeller University, New York, New York 10021

Received for publication 23 May 1977

Uninfected cells from two different phenotypes of chicken embryos express significant amounts of endogenous viral information, though they do not produce virus particles. Cells of the phenotype gs^+chf^+ are positive for both group-specific (*gs*) antigens and chicken helper factor (*chf*) activity, whereas cells of a second phenotype, $gs_Lchf^+(h_E)$, demonstrate noncoordinate expression of these two viral activities (very low amounts of *gs* antigens, but extremely high helper activity). RNA from these cells was analyzed to determine the size, genetic content, and relative abundance of virus-specific RNAs in cells of each phenotype. Two major size classes of polyadenylic acid-containing RNA, homologous to the avian leukosis virus genome, were detectable in cells of both types. The larger RNA, which contained most of the sequences of the leukosis virus genome, was of different sizes in the two phenotypes, 31S in gs^+chf^+ cells but 35S in the noncoordinate cell type. Analysis of the viral RNA with gene-specific complementary DNA probes revealed the following characteristics. (i) The 31S RNA appeared to lack portions of the *gag* and *pol* genes. (ii) A smaller RNA species, which sedimented at 21S in both cell types, was a transcript of the 3'-proximal portion of the viral genome, consisting of the *env* gene and the "common" sequences. (iii) The amount of *env*-specific RNA in the 21S region was more than six times higher in the noncoordinate cell type than in the gs^+chf^+ cells; this difference was concordant with the 5- to 10-fold higher *chf* activity in the noncoordinate cells. (iv) The endogenous viral RNA in uninfected cells and the RNA from Rous-associated virus-0 virions hybridized only partially with DNA complementary to the common region of the Rous-associated virus-2 genome, whereas the RNA of all exogenous viruses tested hybridized almost completely to this complementary DNA. Small amounts of *src*-specific polyadenylated RNA were also present in uninfected chicken cells. This RNA sedimented as a single peak at 26S and was not covalently linked to any other identifiable virus-specific RNA sequences. The amount of *src* RNA was the same in the above two types of expression-positive cells and also in cells that were gs^-chf^- , indicating that the transcription of the cellular sequences homologous to the *src* gene is independent of the transcription of the other endogenous viral genes.

Genetic differences in expression of many of the thousands of active genes in vertebrate cells are known, but the transcriptional activities of most of these genes cannot be analyzed because of the lack of specific molecular probes. Among the handful that can be studied are the endogenous virus genes of chicken cells. Whereas they produce no detectable virus particles, uninfected chicken cells have in their chromosomal DNA at least part of the genetic information for an RNA tumor virus (3, 25, 28, 33, 41). The existence of these endogenous virus genes, the term used to distinguish them from exogenous virus genes acquired by infection, has been recognized by the expression of two virus-specific products, group-specific (*gs*) antigen and

chicken helper factor (*chf*), which complements glycoprotein-deficient strains of Rous sarcoma virus (RSV) (11, 17, 30, 43). Studies with many individual chicken embryos revealed that the levels of expression of these two products are genetically controlled. Payne and Chubb (30) and others (16, 44) showed that both *gs* antigen and *chf* activity in embryos of gs^+chf^+ phenotype are under the control of a single autosomal gene, with the expression-positive allele being dominant. Thus, embryos that are homozygous recessive for this gene are phenotypically gs^-chf^- . Later, another independent gene was postulated to explain the noncoordinate expression of *gs* antigen and *chf* in $gs_Lchf^+(h_E)$ embryos (L = low, h_E = helper extremely high;

characteristics of the different cell types are shown in Table 1) (1, 16, 32). The level of *chf* activity in these embryos is 5 to 10 times higher than that in gs^+chf^+ embryos, whereas the level of *gs* antigen is less than one-fifth of that in gs^+chf^+ embryos (5). For this gene, also, the allele for positive expression is dominant.

Our previous work showed that these three types of chicken embryos [gs^-chf^- , gs^+chf^+ , and $gs_{\text{L}}chf^+(h_E)$] contain essentially the same amounts of virus-specific DNA but varying amounts of virus-specific RNA, which are detectable by molecular hybridization of cellular nucleic acids to labeled DNA probes complementary to viral RNA (20). Thus, it appears that the expression of the endogenous virus genes is primarily regulated at the level of transcription. However, the details of the regulatory mechanisms are not known, particularly those involved in the differences between gs^+chf^+ and $gs_{\text{L}}chf^+(h_E)$ embryos and in the noncoordinate expression of *gs* antigen and *chf* in $gs_{\text{L}}chf^+(h_E)$ embryos. Data on the biological activities of endogenous viral genes (15, 35) suggest that some of the normal viral products, such as functional reverse transcriptase and one of the viral structural proteins, are not synthesized in uninfected chicken cells that are positive for other viral functions.

To better understand these phenomena, we have examined the size distribution and genetic content of virus-specific RNA in uninfected cells of these phenotypes, using DNA probes specific for known individual genes of avian leukosis-sarcoma viruses; preparation and characteristics of the gene-specific complementary DNA (cDNA) probes are described in the accompanying paper (19). As in the case of leukosis virus-infected chicken cells (19), two size classes of virus-specific RNA were resolved by rate zonal centrifugation of cellular RNA

extracted from gs^+chf^+ and $gs_{\text{L}}chf^+(h_E)$ cells, the smaller RNA representing only a portion of the viral genome. The sizes of the larger viral RNA transcripts in these two types of cells were not identical. Furthermore, a species of RNA homologous to the *src* gene of sarcoma viruses was demonstrated after velocity sedimentation of cellular RNA.

MATERIALS AND METHODS

General. Sepharose 6B was from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.); polyuridylic acid [poly(U)] was from Miles Laboratories, Inc. (Elkhart, Ind.); polyadenylic acid [poly(A)] was from General Biochemicals Div. (Mogul Corp., Chagrin Falls, Ohio); proteinase K was from EM Laboratories, Inc. (E. Merck, Elmsford, N.Y.); DNase I (ribonuclease-free) was from Worthington Biochemicals Corp. (Freehold, N.J.); and ^3H -labeled poly(A) (molecular weight, >100,000; specific activity, 3 Ci/mmol of AMP) was from New England Nuclear (Boston, Mass.). In some experiments, poly(U)-Sepharose from Pharmacia was used. TEN is 0.01 M Tris-hydrochloride (pH 7.4), 0.001 M EDTA, 0.1 M NaCl; 0.4 NETS is 0.01 M Tris-hydrochloride (pH 7.4), 0.01 M EDTA, 0.2% sodium dodecyl sulfate (SDS), 0.4 M NaCl; ETS is NETS without NaCl. Sucrose was ribonuclease-free (Schwarz/Mann, Orangeburg, N.Y.). Phenol (Mallinckrodt Chemical Works, St. Louis, Mo.) was distilled under nitrogen at about 22 mm of Hg (85°C) to remove impurities and stored at -20°C under nitrogen. Other chemicals were of reagent grade. Glassware was baked at 150°C for 3 h before use, and solutions were autoclaved.

Cells and viruses. Chicken embryo cells were cultured from individual embryos (SPAFAS, Inc., Norwich, Conn.) and typed into categories as previously described (22). Various properties of the three types of embryo cells used are summarized in Table 1. For brevity, $gs_{\text{L}}chf^+(h_E)$ cells will be designated h_E in this paper. None of these cell types spontaneously produces virus particles. Viruses used in this study, Rous-associated virus-0 (RAV-0), RAV-2, and Schmidt-Ruppin strain of RSV, subgroup B (SR-RSV-B), have been described (19, 21). Chicken embryos of line 7 × 15, which spontaneously release RAV-0, were generously provided by L. Crittenden (8).

Nucleic acid extraction. Total nucleic acid was extracted from cells using proteinase K and SDS to deproteinize nucleic acids, inhibit nucleases, and recover RNA quantitatively (12, 31, 46). Monolayers of cells in 100-mm plastic plates were lysed at room temperature with 2 ml of TEN containing 0.5% SDS and 1 mg of proteinase K per ml. Provided the cells were quickly lysed directly on the plate, by adding the lysing buffer and swirling it over the monolayer within 30 s, there was very little breakdown of high-molecular-weight RNA during lysis of the cells. At least 80% of marker ^{32}P -labeled 35S viral RNA, added to the plates before lysis, was recovered as intact 35S RNA after extraction. The lysate was passed three times through a 20-gauge needle to

TABLE 1. *Viral products and activities in various chicken cell types*

Type	<i>gs</i> antigen		<i>chf</i> activity ^c	Virus-specific RNA (ng/ml) ^d
	CF titer ^a	Radioimmunoassay of P19 (ng/ml) ^b		
gs^-chf^-	0	2-4	0-10	~1
gs^+chf^+	4-8	10-17	1,000-3,000	50-200
$gs_{\text{L}}chf^+(h_E)$	0-2	3-4	10,000-30,000	150-300

^a Reciprocal of the terminal dilution which gives more than a 3+ reaction in the complement fixation (CF) test (22).

^b Nanograms of P19 per milligram of cell protein (6).

^c Titer of RSV (*chf*) at day 5, determined by method A (18).

^d Nanograms of virus-specific RNA homologous to RAV-0 per milligram of total cell RNA (6).

reduce viscosity due to DNA and then extracted five times at room temperature with a mixture of equal volumes of phenol containing 0.1% 8-hydroxyquinoline and chloroform containing 1% isoamyl alcohol (30) and twice with chloroform containing 1% isoamyl alcohol. The aqueous phase was adjusted to 0.4 M NaCl and precipitated with 2 volumes of ethanol. For some experiments, as indicated in the figure legends, RNA was treated with DNase (50 μ g/ml, 30 min at 37°C, in a buffer containing 0.01 M Tris, pH 7.0, and 0.005 M $MgCl_2$) to remove host cell DNA and reextracted as described. Marker ^{32}P -labeled RNA from RAV-0 virions was produced as described (19) and used within a few days of preparation.

Poly(U)-Sephacrose fractionation of nucleic acid. Poly(U)-Sephacrose was prepared as described by Molloy et al. (27); after attachment of poly(U) to activated groups in the Sepharose, the redundant activated groups were blocked with ethanolamine. The capacity of each batch of poly(U)-Sephacrose to bind poly(A) was tested by loading a minute amount of 3H -labeled poly(A) (molecular weight, >100,000) in the presence of 10-fold dilutions of cold poly(A) and comparing the percentage of the label that came off in the 0.4 NETS wash-through with that in the 50% formamide eluate. Satisfactory batches had a capacity of at least 0.04 mg of poly(A) per ml of packed Sepharose. Poly(U)-Sephacrose was used rather than oligodeoxythymidylic acid-cellulose because the former probably has a superior capacity to bind small poly(A) segments (26). Poly(U)-Sephacrose purchased from Pharmacia gave equally satisfactory results.

For fractionation of cellular nucleic acid, approximately 8 mg of total cellular nucleic acid was loaded onto a 10-ml (packed volume) column and eluted with 20-ml steps of 0.4 NETS (wash-through), ETS, 50% formamide in ETS, and 90% formamide in ETS (27). The poly(A)-containing RNA, which eluted at the 50%-formamide step, was concentrated by precipitation with 2 volumes of ethanol after adjusting the concentration of NaCl to 0.4 M and adding 40 μ g of yeast 4S RNA per ml as carrier.

Size fractionation of poly(A)-containing RNA. Poly(A)-containing RNA was dissolved in 0.5 ml of ETS, heated to 85°C for 1 min, and quickly cooled to room temperature just before loading on a sucrose gradient. The RNA was centrifuged through a 15 to 30% (wt/wt) SDS-sucrose gradient (15 to 30% sucrose in 0.01 M Tris-hydrochloride, pH 7.4, 0.05 M NaCl, 0.01 M EDTA, and 0.2% SDS) in polyallomer tubes in a Beckman SW40 rotor at 24°C, 40,000 rpm, for 7 to 8 h. The gradient was fractionated by withdrawing the solution with a peristaltic pump into siliconized test tubes through a capillary tube inserted to the bottom of the gradient. The fractions were precipitated with 2 volumes of ethanol after adding 40 μ g of yeast 4S RNA and adjusting the NaCl concentration to 0.4 M. The precipitates were collected by centrifugation, residual ethanol was evaporated under vacuum, and the fractions were resuspended in 20 to 50 μ l of the reaction mixture for hybridization.

Nucleic acid hybridization. Synthesis of 3H -cDNA and isolation of gene-specific cDNA probes complementary to the *gag*, *pol*, *env*, and *c* ("com-

mon") regions of RAV-2 and to the *src* gene of SR-RSV-B are described in detail in the accompanying paper (19). Unselected RAV-0 cDNA, used in some experiments (see Fig. 1 and 4), protected at least 90% of the homologous ^{32}P -labeled RNA from RNase digestion after hybridization at a DNA/RNA ratio of 10, indicating that essentially all of the viral sequences were represented. Sequence representation in this particular probe, however, was not uniform. Hybridization of cellular RNA to 3H -cDNA was performed at 50°C in a reaction mixture containing 30% formamide, 0.45 M NaCl, 0.045 M sodium citrate, 0.1% SDS, 0.005 M EDTA, 3H -cDNA (200 to 600 cpm per reaction), and appropriate amounts of RNA (21). Hybridization was monitored by treatment with S-1 nuclease (20). Whenever possible, hybridization was carried out to 10 to 40% saturation (i.e., the maximal level of hybridization obtainable with the given RNA-cDNA pair), since in this range the extent of hybridization is roughly proportional to the concentration of the RNA sequences homologous to the cDNA, when RNA is in sufficient excess, and hence the amount of complementary RNA can be roughly compared between different size classes of RNA in the same cell type or in different cell types. The condition of sufficient RNA excess (>20-fold) was met in all experiments except those involving *gs*⁻*chf*⁻ embryos, which contain very small amounts of virus-specific RNA, and those involving endogenous *src*-specific RNA (see Fig. 4), in which an RNA excess of only 2- to 5-fold was present in the peak fractions. Thus, in the latter cases, the hybridization levels cannot be considered to reflect accurately the absolute concentration of virus-specific RNA, but they provide a reliable measurement of the size distribution of the RNAs.

RESULTS

Poly(A) in virus-specific RNA. Because normal chicken embryo cells contain only very low to modest amounts of virus-specific RNA, it was desirable to concentrate the virus-specific RNA before use in hybridization reactions. A sizable fraction of the virus-specific RNA in normal cells presumably serves as mRNA, and much of this fraction would be expected to contain a poly(A) segment at the 3'-OH end (4, 9). Passage of the total nucleic acid extracted from these cells through poly(U)-Sephacrose should concentrate the fraction of RNA that contains poly(A), separating it from the cellular DNA, rRNA, tRNA, and most of the mitochondrial nucleic acids (27).

First, the kinetics of hybridization of RAV-0 cDNA with poly(U)-Sephacrose-binding and nonbinding virus-specific RNA from *gs*⁻*chf*⁻, *gs*⁺*chf*⁺, and *h_e* embryos were determined. From *C_t* curves of several separate extractions, we calculated that at least 60 to 80% of the virus-specific RNA in *gs*⁻*chf*⁻, *gs*⁺*chf*⁺, and *h_e* cells contains poly(A). Using the same techniques, about 80 to 85% of viral 35 to 45S RNA

from virions of RAV-2 or SR-RSV or from cells infected with these viruses was shown to be polyadenylated (19). The lower recovery of poly(A)-containing virus-specific RNA from uninfected cells compared with that from infected cells may be due to slight overloading of the poly(U)-Sephadex with the former sample because of the large amounts of cellular RNA needed to recover sufficient amounts of virus-specific RNA. A recycling of the nonbinding RNA from uninfected cells on poly(U)-Sephadex generally gave some additional binding (5 to 10%). For $gs^{-}chf^{-}$ cells, it was necessary to treat the unbound material with DNase to remove endogenous DNA sequences before hybridization; the contribution of DNA to the hybridization was not significant with $gs^{+}chf^{+}$ or h_E cells.

Size of virus-specific RNA. The poly(A)-containing cellular RNA was subjected to velocity sedimentation through sucrose gradients after heating at 85°C in ETS for 1 min to break up aggregates. After centrifugation, the gradient was fractionated and aliquots of each fraction were hybridized with RAV-0 cDNA.

$gs^{+}chf^{+}$ cells contained two sizes of virus-specific RNA, one at approximately 31S and the other at 21 to 22S (Fig. 1). The 31S species was clearly smaller than ^{32}P -labeled RAV-0 35S RNA marker present in the same gradient (Fig. 1, top). In h_E cells, there were also two sizes of virus-specific RNA, but the larger species had a sedimentation value of 35S (Fig. 1, middle). The smaller species sedimented at 21 to 22S. The relative amounts of the two RNA species were quite different in the $gs^{+}chf^{+}$ and h_E cells; the more abundant species in the latter cells was the 21 to 22S RNA. There may be other minor species of viral RNA that are not present in high enough concentrations to give distinct peaks in the sucrose gradient. In the single $gs^{-}chf^{-}$ embryo tested, the levels of hybridization obtained were so low that the RNA could not be resolved into distinct peaks; the RNA sedimented in the range of 20 to 35S, with a possible peak at approximately 28 to 32S (Fig. 1, bottom). We have previously calculated that $gs^{-}chf^{-}$ cells contain less than 0.4 copies of virus-specific RNA per cell (22). The use of poly(A)-containing RNA from these cells facilitated detection of virus-specific RNA in the present experiments. It should be noted, however, that much higher RNA concentrations and longer annealing times were required to detect RNA in these cells, as compared with $gs^{+}chf^{+}$ and h_E cells (Fig. 1).

Poly(U)-Sephadex-nonbinding RNA from $gs^{+}chf^{+}$ and h_E cells was also fractionated through sucrose gradients and tested for virus-

FIG. 1. Size distribution of poly(A)-containing virus-specific RNA from uninfected cells. (Top) Poly(A) RNA from 3.6 mg of $gs^{+}chf^{+}$ cell total nucleic acid was fractionated through a 15 to 30% sucrose gradient, and equal portions (one-third) of each precipitated fraction were hybridized to 3H -labeled RAV-0 cDNA for 10 h; total 3H -cDNA per hybrid reaction was 480 cpm. (Middle) The poly(A) RNA was from 1.6 mg of h_E cell total nucleic acid, and hybridization to RAV-0 cDNA was for 11 h; total 3H -cDNA per hybrid reaction was 150 cpm. (Bottom) The poly(A) RNA was from 9.6 mg of $gs^{-}chf^{-}$ cell total nucleic acid, and hybridization was for 300 h at 50° C; total 3H -cDNA per hybrid reaction was 450 cpm. Centrifugation was for 8 h at 40,000 rpm, using conditions described in the text.

specific RNA in an effort to determine whether it contained a specific subpopulation of the viral RNAs. In both $gs^{+}chf^{+}$ and h_E cells, the non-binding viral RNA was heterogeneous in size, with no identifiable peaks of virus-specific RNA (data not shown). These data were inconclusive, however. The poor resolution could have been due to partial breakdown of non-poly(A)-containing viral RNA or to distortion of the loading of large amounts of total nonbinding RNA that was necessary to detect the low levels of viral RNA.

Hybridization of endogenous viral RNA to

different viral gene-specific cDNA's. Highly specific ^3H -cDNA probes, corresponding roughly to the *gag*, *pol*, *env*, *src*, and *c* regions of RSV RNA (19), were used to determine the genetic content of endogenous viral RNA. The relation of these cDNA probes to the genetic map of avian sarcoma virus is shown in the accompanying paper (19). The extent of homology between the specific cDNA probes and the endogenous viral RNA in gs^+chf^+ and h_E cells is shown in Table 2. Each of the probes hybridized with these cellular RNAs. However, the maximum levels of hybridization with each probe of cDNA were somewhat lower than those obtained with RNA from RAV-2 (the virus used for isolation of the probes). A striking difference was noted with cDNA_c , which hybridized extensively with RAV-2 RNA (95%) but poorly with RNA from RAV-0 (45%) or uninfected cells (37 to 39%), indicating significant differences in base sequences within the *c* region of the endogenous provirus and RAV-2. There also appeared to be some differences between the RNAs of gs^+chf^+ and h_E cells within the *gag* and *pol* genes. The possible implications of this will be discussed in a later section.

cDNA_{src} prepared with SR-RSV hybridizes approximately 90% with sarcoma virus RNA (19) but did not hybridize with RNA of the nontransforming viruses, RAV-0 and RAV-2, as expected (Table 2). However, cDNA_{src} hybridized to a significant extent (approximately 75%) with cellular RNA obtained from either gs^+chf^+ or gs^-chf^- embryos, when reactions were carried out to very high C_t values (Table

2). The presence in normal chicken cells of RNA sequences homologous to the *src* gene of sarcoma viruses has been demonstrated by Varus et al. (40).

Since the 21 to 22S RNA detected in gs^+chf^+ and h_E cells (Fig. 1) is significantly smaller than the viral genome, an attempt was made to examine whether this RNA species was a transcript of a selected portion of the viral genome or a mixture of various segments of the genome. The different cDNA probes were hybridized to poly(A)-containing cellular RNA that had been fractionated through sucrose gradients. The larger RNA at 30 to 35S in both cell types hybridized to all four leukosis virus-specific cDNA probes (Fig. 2). Smaller RNA species, sedimenting at 21 to 22S, were detectable with cDNA_{env} and cDNA_c in both cell types. The 21 to 22S peaks obtained with these two probes were almost superimposable, suggesting that both *env* and *c* sequences are present on a single molecule that is essentially a transcript of the 3' end of the viral genome. It was consistently noted that the ratios of the content of *env*-specific RNA in 30 to 35S and 21S RNAs were different in the two cell types. The predominant species in the h_E (noncoordinate) cells was the 21S RNA, whereas the higher-molecular-weight RNA was the major species in the gs^+chf^+ cell type.

Curiously, in gs^+chf^+ embryos there was also a smaller RNA, which hybridized with cDNA_{gag} . This RNA, however, was slightly smaller (19S) than the RNA containing *env* and *c* (21 to 22S), implying that the *gag* sequences are on a separate RNA transcript. This distinction of two different small RNA species was seen in three separate gs^+chf^+ embryos tested (see also Fig. 3), but with somewhat varying amounts of the 19S species. The 19S *gag*-specific RNA was found neither in h_E cells (Fig. 2) nor in cells infected with RAV-0, RAV-2, SR-RSV-B, or the Bryan high-titer strain of RSV-(BH-RSV) (19). In contrast, all of the cells infected with the leukosis viruses or expressing endogenous virus functions contained 21S RNAs with *env* and *c* sequences.

In our poly(A) RNA gradients, we consistently noted a lack of symmetry in the peaks of 31S or 35S virus-specific RNA, with some faster-sedimenting material near the bottom of the gradient. This material could represent larger RNA precursors of the major virus-specific RNA species, as described for adenovirus transcription (2), or it could simply represent aggregates of the poly(A) RNA species (10). The latter possibility was tested by sedimenting about 1/10 of the usual amount of poly(A)

TABLE 2. Percent ^3H -cDNA hybridized to RNA from virions or cells^a

Probe	Source of RNA				
	RAV-2 virion	RAV-0 virion	gs^+chf^+ cell	gs^-chf^- (h_E) cell	gs^-chf^- cell ^b
cDNA_{gag}	93	88	68	78	>35
cDNA_{pol}	89	79	49	75	NT ^c
cDNA_{env}	95	79	72	73	>55
cDNA_c	95	45	37	39	NT
cDNA_{src}	2	3	75 ^d	>50	74

^a RNA isolated from purified RAV-2 and RAV-0 and from uninfected cells was hybridized with ^3H -cDNA probes corresponding to different viral genes. Values represent the maximum levels of hybridization attained with each probe (C_t = 1 to 5 mol·s/liter for viral RNA and $\approx 3 \times 10^4$ mol·s/liter for cell RNA). Cell RNA was treated with DNase to remove host cell DNA sequences, as described in the text.

^b Because of the low concentration of virus-specific RNA, poly(A) RNA was used for gs^-chf^- cells to enrich for viral sequences. Reactions were incubated to effective C_t values of 5×10^5 mol·s/liter.

^c NT, Not tested.

^d Poly(A)-containing RNA. Reactions were incubated to an effective C_t value of 3×10^5 mol·s/liter.

FIG. 2. Hybridization of fractionated poly(A) RNA to gene-specific ^3H -cDNA probes. The poly(A) RNA from 6 to 9 mg of total cellular nucleic acid was fractionated through a sucrose gradient (7 h at 39,000 rpm). Equal portions of each precipitated fraction were hybridized to ^3H -cDNA specific for each of four genetic regions of RAV-2. A 200- to 600-cpm amount of ^3H -cDNA was present in each capillary, and hybridization was for 20 to 40 h at 50°C , the time of incubation being adjusted to give 20 to 50% maximal hybridization. In the top panels of each side; the profile of an RAV-2 35S ^{32}P -labeled RNA marker centrifuged in the same tube as the cellular RNA in these two runs is indicated; the amount of this marker RNA would not have contributed significantly to the hybridization with the cDNA. The positions of 28S and 18S RNA markers, centrifuged in parallel gradients, are also shown. Left, gs^+chf^+ cells; right, h_E cells.

RNA in the same gradient. The results (Fig. 3) show a definite improvement in the sharpness of the peaks. A small amount of hybridizable material was still found in the bottom fractions of the gradient, but no effort was made to further characterize this RNA.

Amount of gene-specific viral RNA in normal cells. The number of copies per cell of gene-specific RNA in the three different embryo types was calculated from C_t curves (Table 3). For comparison, cells derived from a line of chicken embryos (cross 7×15) that spontaneously produce RAV-0 and cells infected with

RAV-2 were also analyzed. The amount of virus-specific RNA in gs^-chf^- cells was extremely low, but the isolation of poly(A)-containing

FIG. 3. Fractionation of poly(A) RNA by rate zonal centrifugation, using lower RNA concentrations. The experimental procedure was as described in the legend to Fig. 2, except that a smaller amount of poly(A) RNA (obtained from 720 μg of gs^+chf^+ cell nucleic acid) was applied to the gradient to decrease the chances of aggregation. One-quarter of a fraction was used for hybridization with each probe, and hybridization was for 64 h (cDNA_{env}) and 90 h (cDNA_{gag}).

TABLE 3. Gene-specific RNA content of various cell types (copies per cell)^a

Gene analyzed	Cell type				
	gs^-chf^-	gs^+chf^+	$\text{gs}_\text{L}\text{chf}^+(\text{h}_\text{E})$	7×15	RAV-2-infected gs^-chf^-
<i>gag</i>	0.15	88	90	1,600	9,800
<i>pol</i>	NT ^b	73	94	1,700	9,400
<i>env</i>	0.4	109	255	3,300	16,000
<i>src</i>	5	6	8	NT	7

^a Cell RNA was hybridized to ^3H -cDNA probes specific for different viral genes, and RNA content (copies per cell) was determined from C_t curves as described in the accompanying paper (19). With the exception of the gs^-chf^- cells, total cell RNA (DNase treated) was used for annealing reactions. Because of the low concentration of viral RNA in gs^-chf^- cells, poly(A) RNA was first purified from these cells to enrich for viral sequences. Poly(A) RNA was also used for determination of *src*-specific RNA content in all cells. Values shown for *src*-specific RNA and for *gag*- and *env*-specific RNA in gs^-chf^- cells are subject to some error due to the long incubation times required and the relatively low RNA/DNA ratios. However, these values represent clear positive results, with hybridization levels of greater than 35% being attained in all cases (Table 2).

^b NT, Not tested.

RNA, to enrich for virus-specific sequences, allowed us to positively identify both *env*-specific and *gag*-specific RNA in these cells and to estimate their content. Even with this enrichment we were unable to achieve saturation in the annealing reactions, but hybridization levels of 55% (cDNA_{env}) and 35% (cDNA_{gag}) were attained at C_{t} values equivalent to 5×10^5 mol·s/liter (Table 2).

It can be seen that all three types of uninfected cells contained much less virus-specific RNA than did RAV-0- and RAV-2-infected cells (Table 3). The relative amounts of each gene-specific RNA were different in the various cell types, but in all cases *env*-specific RNA was more abundant than *gag*- and *pol*-specific RNA. This unequal representation can be attributed to the fact that only a portion of the viral genetic information is present in the smaller RNA transcripts. Whether this is due to independent transcription of different segments of the genome or to selective degradation of portions of the larger RNA transcripts has not been determined.

***src*-specific RNA in normal cells.** Stehelin et al. (38) demonstrated that normal chicken cell DNA contains sequences homologous to the *src* gene of avian sarcoma viruses, and Varmus et al. (40) later showed that these sequences are also present in small amounts in the RNA of uninfected cells. RNA homologous to the viral *src* gene was present in all three embryo types examined here (Table 2). The amount of this cellular *src*-specific RNA was essentially the same in all three types of chicken embryo, including *gs*⁺*chf*⁺ cells, in which very little RNA homologous to other viral genes was present (Table 3). Thus, the cellular *src* gene is apparently transcribed independently of all other viral genes. If this is the case, the *src*-specific sequences should not be covalently linked to other virus-specific RNA species. We looked at the size of *src*-specific poly(A) RNA in *gs*⁺*chf*⁺ and *gs*⁺*chf*⁺ cells by fractionating cellular poly(A) RNA on sucrose gradients and hybridizing gradient fractions with cDNA_{src} . The *src*-specific RNA in both cell types formed a definite peak, sedimenting at approximately 26S (Fig. 4). This species was different from the 31S and 21 to 22S virus-specific RNA in *gs*⁺*chf*⁺ cells and was present in much lower amounts than *env*-specific RNA (note reaction times required for hybridization with cDNA_{src} and cDNA_{env} [legend to Fig. 4]), which is consistent with the data in Table 3. In *gs*⁺*chf*⁺ cells the *src*-specific RNA was more abundant than other virus-specific RNAs. RNA homologous with RAV-0 cDNA was barely detectable under conditions that gave significant levels of hybridization

FIG. 4. Size of *src*-specific poly(A) RNA in uninfected cells. Poly(A) RNA from 3.8 mg of *gs*⁺*chf*⁺ total nucleic acid and 4 mg of *gs*⁺*chf*⁺ total nucleic acid was fractionated as described in the legend to Fig. 1. One-quarter of each fraction was hybridized to the following probes for the indicated times: for *gs*⁺*chf*⁺ RNA, cDNA_{src} (64 h) and cDNA_{env} (2 h); for *gs*⁺*chf*⁺ RNA, cDNA_{src} (144 h) and RAV-0 cDNA (168 h). (Top) RNA from *gs*⁺*chf*⁺ cells; (bottom) RNA from *gs*⁺*chf*⁺ cells. Symbols: cDNA_{src} (□), cDNA_{env} (×), cDNA_{RAV-0} (●).

with cDNA_{src} (Fig. 4, bottom). Since the 26S *src*-specific RNA is far larger than expected for a transcript of the viral *src* gene (which would sediment at 16 to 18S), this RNA species presumably also contains cellular genetic information.

DISCUSSION

In general, the properties of the RNA of endogenous provirus in uninfected chicken cells are very similar to those of viral RNA in infected chicken cells (19). The bulk (60 to 80%) of the RNA is polyadenylated and is present in two major size classes, 30 to 35S and 19 to 22S. The larger RNA contains most or all of the leukosis virus genetic information, whereas the smaller RNA represents only a portion of the viral genome.

However, further analysis, using cDNA

probes corresponding to the five individual genes of avian leukosis-sarcoma viruses, revealed several unique features of the endogenous viral RNA transcripts in uninfected cells. First of all, cDNA homologous to the *c* region, which hybridizes more than 80% with RNA from most viruses tested (19), hybridizes only about 40% with the RNA of $gs^{+}chf^{+}$ or h_E cells. This limited homology is also found with RAV-0, supporting the idea of the endogenous origin of this particular virus.

Another unexpected finding was the difference in the sizes of the higher-molecular-weight RNA in $gs^{+}chf^{-}$ and h_E -type cells. The larger RNA of h_E cells sediments at 35S, corresponding to approximately 8,400 nucleotides (36), whereas the larger RNA of $gs^{+}chf^{+}$ cells sediments at approximately 31S (~6,500 nucleotides). Thus, approximately 1,900 nucleotides would appear to be absent in the 31S RNA species. Hybridization of sequence-specific cDNA probes to the RNA from these two cell types indicates that portions of the *pol* and *gag* regions may be missing in the $gs^{+}chf^{+}$ cell RNA. This observation is consistent with the failure to detect functional reverse transcriptase in normal $gs^{+}chf^{+}$ chicken cells (15, 45) and with the absence of one of the *gag* proteins, p15, reported by Smith et al. (35). (In an earlier communication [6] we reported the presence of p15 in uninfected chicken cells. However, a reexamination of these cells, using more highly purified p15, confirmed the observation by Smith et al. [35] that p15 is not detectable in $gs^{-}chf^{-}$ or $gs^{+}chf^{+}$ cells [J. H. Chen, personal communication]; h_E cells have not yet been tested.) In addition, Eisenman et al. (R. Eisenman, R. Shaikh, and W. Mason, personal communication) have found that the $gs^{+}chf^{+}$ cells lack the *gag* precursor protein, Pr76, but contain a novel polypeptide, p92, which migrates in SDS gels with an apparent molecular weight of 92,000. This polypeptide contains the tryptic peptides of p27, p19, and p12 and also appears to contain a portion of the *pol* gene product, but it lacks the p15 tryptic peptides. Since p15 is located at the carboxyl terminus of the *gag* precursor protein (42), it seems likely that the 31S RNA in $gs^{+}chf^{+}$ cells contains a single deletion that encompasses the 3' portion of the *gag* gene, coding for p15, and a significant part of the *pol* gene. The RNA in h_E cells, on the other hand, appears to contain most of the sequences present in RAV-0 RNA, and the larger RNA species in these cells sediments at 35S, as does virion RNA of leukosis viruses.

The 21S RNA containing *env* and *c* sequences was found in both $gs^{+}chf^{-}$ and h_E -type cells. As shown by Stacey et al. (37), this appears to be

the major species of RNA serving as RNA for the *env* gene in RAV-2-infected cells. Therefore, it is reasonable to assume that the 21S RNA in these cells acts as a messenger for the synthesis of the subgroup E-specific envelope glycoprotein, which biologically functions as *chf*. Although the total amount of *env* RNA in the noncoordinate h_E cells is only 2.5 times higher than that in $gs^{+}chf^{+}$ cells (Table 3), the amount of *env*-specific 21S RNA is at least 6 times higher, as calculated from the distribution of 21S and 31S or 35S RNAs in these two cell types (Fig. 2). This value is in good agreement with the ratio of *chf* activities in h_E and $gs^{+}chf^{+}$ cells, which is generally between 5 and 10 (16).

An additional RNA species, sedimenting at 19S and containing only *gag* sequences, was detected in $gs^{+}chf^{+}$ cells. It should be emphasized that this species of RNA has been found only in $gs^{+}chf^{+}$ cells, and not in h_E cells or cells infected with RAV-0, RAV-2, SR-RSV-B, or BH-RSV. Whether this species of RNA is a processed product of the 31S RNA, an independent transcript of the *gag* gene of the same provirus, or a transcript from a different provirus is not known. The level of *gs* antigen in $gs^{+}chf^{+}$ cells is higher than that in h_E cells, but the total amount of *gag*-specific RNA in these two cell types is essentially the same. It is possible that the 19S *gag*-specific RNA, present only in the $gs^{+}chf^{+}$ cell type, is responsible for this difference, perhaps functioning as a more efficient messenger than the 31 to 35S RNA.

Other laboratories have examined the size of endogenous virus-specific RNA in mammalian cells. Fan and his collaborators (13, 14) have reported the presence of two size classes (38S and 27S) of virus-specific RNA in uninfected, non-virus-producing JLS-V9 cells. Interestingly, only RNA homologous to the xenotropic endogenous virus was found; RNA specific to the N-tropic endogenous BALB/c virus was not detectable. Tsuchida et al. (39) have identified a 30S RNA specific for Kirsten sarcoma virus in uninfected rat cells, but no smaller RNA species was detected.

We have confirmed the report of Varmus et al. (40) that uninfected chicken cells contain small amounts of RNA homologous to the *src* gene of RSV. The function of this RNA in the normal cell is unknown. The cellular *src*-specific RNA is present as a 26S species in both $gs^{-}chf^{-}$ and $gs^{+}chf^{+}$ cells and is distinct from either the 31S or the 21S viral RNAs in the $gs^{+}chf^{+}$ cells. The 26S *src* RNA in either cell type does not appear to be linked with any other viral sequences. This is consistent with the report of Padgett et al. (29) that the genome

sequences for the replicative functions of avian oncoviruses are present in the large macrochromosomes of chicken cells, whereas the *src* sequences are in the microchromosomes. The independent transcription of this cellular *src* gene is further substantiated by the fact that the amount of cellular *src*-specific RNA is essentially the same in $gs^{-}chf^{-}$, $gs^{+}chf^{+}$, and h_E cells. Infection of $gs^{-}chf^{-}$ cells with RAV-2 did not alter the expression of the endogenous *src* gene (Table 3).

The data shown in Table 3 demonstrate the great variation in virus gene expression in uninfected and infected cells and even among different types of infected or uninfected cells. In infected cells, as much as 0.5 to 1% of the total RNA is virus specific; hence, the proviral DNA must be a particularly favored site for transcription. Yet this partiality does not extend to the closely homologous genome of the endogenous avian virus. We have previously shown that infection of all three types of uninfected cells with BH-RSV(-) does not significantly alter the low levels of endogenous virus gene expression in these cells, whereas the BH-RSV genome is transcribed with high efficiency (22, 23). Thus, the expression of each integrated provirus is apparently controlled independently. One possibility is that the transcription of each provirus is controlled by some adjacent regulatory gene, such as the "cis-acting control element" postulated by Cooper and Temin (7). Such a regulatory gene could be located on the provirus itself or could be an adjacent cellular genetic element. In the latter case, the expression of each provirus would be governed by the transcriptional activity of the adjacent cellular genes. This explanation would be less likely, however, if the provirus of BH-RSV is integrated in tandem with the endogenous provirus, as reported by Shoyab et al. for avian myeloblastosis virus (34). Another possibility is that the differences in RNA levels reflect a differential breakdown of the RNA transcribed from each provirus. The RNA levels reported here represent steady-state values and thus reflect both the rate of synthesis and the rate of degradation. Humphries and Coffin (24), however, have presented evidence that viral RNA is synthesized much more rapidly in infected cells than in uninfected cells. This would favor the suggestion that transcriptional control plays an important role in determining the levels of expression of the endogenous and exogenous proviral genes.

The differences in viral RNA content in $gs^{-}chf^{-}$ and $gs^{+}chf^{+}$ cells can be explained by variations in regulatory elements, as discussed above. However, our observation that the

higher-molecular-weight viral RNAs in $gs^{+}chf^{+}$ and h_E cells are of different sizes suggests that structural as well as regulatory alterations may be responsible for some of the differences in endogenous virus gene expression. The 31S RNA in $gs^{+}chf^{+}$ cells appears to lack portions of the *pol* and *gag* genes. Since this deletion would be located internally, it would be most easily explained by a deletion in the provirus from which the RNA is transcribed. Otherwise, a very complicated scheme of transcription or processing must be assumed. Other differences—the presence of 19S *gag*-specific RNA in $gs^{+}chf^{+}$ cells and the sixfold-higher content of *env*-specific 21S RNA in h_E cells—could result from variations in provirus structure, transcriptional control, or processing of RNA. Further analysis of the proviral DNA in these cells and the processes involved in viral RNA metabolism should be extremely interesting.

ACKNOWLEDGMENTS

We thank Susan Braverman for excellent research assistance, Joseph Cozzitorto for much useful advice on preparing poly(U)-Sepharose, and Randie Davidson for her help in the preparation of the manuscript.

This work was supported by Public Health Service grants CA14935, CA16668, and CA18213 from the National Cancer Institute.

LITERATURE CITED

1. Ando, T., and K. Toyoshima. 1976. Genetic control of chick helper factor in cells which lack natural group-specific antigen of avian leukosis. *Virology* 73:521-527.
2. Bachenheimer, S., and J. E. Darnell. 1975. Adenovirus-2 mRNA is transcribed as part of a high molecular-weight precursor RNA. *Proc. Natl. Acad. Sci. U.S.A.* 72:4445-4449.
3. Baluda, M. A. 1972. Widespread presence in chickens of DNA complementary to the RNA genome of avian leukosis virus. *Proc. Natl. Acad. Sci. U.S.A.* 69:576-580.
4. Braverman, G. 1974. Eukaryotic messenger RNA. *Annu. Rev. Biochem.* 43:621-642.
5. 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.
6. 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.
7. Cooper, G. M., and H. M. Temin. 1976. Lack of infectivity of the endogenous avian leukosis virus-related genes in the DNA of uninfected chicken cells. *J. Virol.* 17:422-430.
8. Crittenden, L. B., J. V. Motta, and E. J. Smith. 1977. Genetic control of RAV-0 production in chickens. *Virology* 76:90-97.
9. Darnell, J. E., W. R. Jelinek, and A. R. Molloy. 1973. Biogenesis of mRNA. Genetic regulation in mammalian cells. *Science* 181:1215-1221.
10. Derman, E., and J. E. Darnell. 1974. Relationship of chain transcription to poly(A) addition and processing of hnRNA in HeLa cells. *Cell* 3:255-264.
11. Dougherty, R. M., and H. S. DiStefano. 1966. Lack of relationship between infection and avian leukosis virus and the presence of COFAL antigen in chick embryos. *Virology* 29:586-595.

12. 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.
13. Fan, H., and F. Besmer. 1975. RNA metabolism of murine leukemia virus. II. Endogenous virus-specific RNA in the uninfected BALB/c cell line JLS-V9. *J. Virol.* 15:836-842.
14. Fan, H., and N. Mueller-Lantzsch. 1976. RNA metabolism of murine leukemia virus. III. Identification and quantitation of endogenous virus-specific mRNA in the uninfected BALB/c cell line JLS-V9. *J. Virol.* 18:401-410.
15. Hanafusa, H., and T. Hanafusa. 1971. Noninfectious RSV deficient in DNA polymerase. *Virology* 43:313-316.
16. 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.
17. Hanafusa, H., T. Miyamoto, and T. Hanafusa. 1970. A cell-associated factor essential for formation of an infectious form of Rous sarcoma virus. *Proc. Natl. Acad. Sci. U.S.A.* 66:314-321.
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. Hayward, W. S. 1977. Size and genetic content of viral RNAs in avian oncovirus-infected cells. *J. Virol.* 24:47-63.
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 uninfected and infected cells, p. 21-35. *In* D. Baltimore, A. S. Huang, and C. F. Fox (ed.), *Animal virology*. Academic Press Inc., New York.
24. Humphries, E. H., and J. M. Coffin. 1976. Rate of virus-specific RNA synthesis in synchronized chicken embryo fibroblasts infected with avian leukosis virus. *J. Virol.* 17:393-401.
25. 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.
26. Lindberg, V., and T. Persson. 1974. Messenger RNA. Isolation with poly(U) agarose. *Methods Enzymol.* 34B:496-499.
27. Molloy, A. R., W. Jelinek, M. Salditt, and J. E. Darnell. 1974. Arrangement of specific oligonucleotides within poly(A) terminated hnRNA molecules. *Cell* 1:43-53.
28. Neiman, P. E. 1973. Measurement of endogenous leukosis virus nucleotide sequences in the DNA of normal avian embryos by RNA-DNA hybridization. *Virology* 53:196-204.
29. Padgett, T. G., E. Stubblefield, and H. E. Varmus. 1977. Chicken macrochromosomes contain an endogenous provirus and microchromosomes contain sequences related to the transforming gene of ASV. *Cell* 10:649-657.
30. 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.
31. 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.
32. Robinson, H. L., and W. F. Lamoreux. 1976. Expression of endogenous ALV antigens and susceptibility to subgroup E ALV in three strains of chickens (endogenous avian C-type virus). *Virology* 69:50-62.
33. Rosenthal, P. N., H. L. Robinson, W. S. Robinson, T. Hanafusa, and H. Hanafusa. 1971. DNA in uninfected and virus-infected cells complementary to avian tumor virus RNA. *Proc. Natl. Acad. Sci. U.S.A.* 68:2336-2340.
34. Shoyab, M., M. N. Dastoor, and M. A. Baluda. 1976. Evidence for tandem integration of avian myeloblastosis virus DNA with endogenous provirus in leukemic chicken cells. *Proc. Natl. Acad. Sci. U.S.A.* 73:1749-1753.
35. Smith, E. J., J. R. Stephenson, L. B. Crittenden, and S. A. Aaronson. 1976. Avian leukosis-sarcoma virus gene expression. Noncoordinate control of group-specific antigens in virus-negative avian cells. *Virology* 70:493-501.
36. Spirin, A. S. 1963. Some problems concerning macromolecular structure of RNA. *Prog. Nucleic Acid Res. Mol. Biol.* 1:301-345.
37. 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.
38. 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.
39. Tsuchida, N., R. V. Gilden, and M. Hatanaka. 1974. Sarcoma-virus-related RNA sequences in normal rat cells. *Proc. Natl. Acad. Sci. U.S.A.* 71:4503-4507.
40. Varmus, H. E., D. Stehelin, D. Spector, J. Tal, F. Fujita, T. Padgett, D. Roulland-Dussoix, H.-J. Kung, and J. M. Bishop. 1976. Distribution and function of defined regions of avian tumor virus genomes in viruses and uninfected cells, p. 339-358. *In* D. Baltimore, A. S. Huang, and C. F. Fox (ed.), *Animal virology*. Academic Press Inc., New York.
41. Varmus, H. E., R. A. Weiss, R. R. Friis, W. E. Levinson, and J. M. Bishop. 1972. Detection of avian tumor virus-specific nucleotide sequences in avian cell DNA. *Proc. Natl. Acad. Sci. U.S.A.* 69:20-24.
42. Vogt, V. M., R. Eisenman, and H. Diggelmann. 1975. Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor polypeptide. *J. Mol. Biol.* 96:471-493.
43. 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.
44. 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.
45. Weissbach, A., A. Bolden, R. Muller, H. Hanafusa, and T. Hanafusa. 1972. Deoxyribonucleic acid polymerase activities in normal and leukovirus-infected chicken embryo cells. *J. Virol.* 10:321-327.
46. Wieggers, U., and H. Hilz. 1971. A new method using proteinase K to prevent mRNA degradation during isolation from HeLa cells. *Biochem. Biophys. Res. Commun.* 44:513-519.