Expression of Endogenous Retroviral Genes in Leukemic Guinea Pig Cells

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The expression of guinea pig retrovirus (5-bromodeoxyuridine[BUDR]-induced GPV) was studied in guinea pig L₂C leukemic lymphoblasts by use of molecular hybridization of viral complementary DNA (cDNA) to cellular RNA. It was found that L₂C leukemic lymphoblasts, leukemic spleen, and BUDR-induced virus-producing cells contain virus-specific RNA: 0.05% (800 to 960 copies per cell), 0.02% (360 copies per cell), and 0.3% (5,120 copies per cell), respectively. Adult normal liver and spleen, on the other hand, contain less than 0.2 copy of viral RNA per cell. Both BUDR-induced cells and L₂C leukemic lymphoblasts contained 14S, 22S, 35S, and 70S RNA species of total and cytoplasmic virus-specific RNA as determined by sucrose velocity gradient analysis and hybridization of sucrose gradient fractions to cDNA. Virus-specific mRNA was identified in both BUDR-induced cells and L₂C leukemic lymphoblasts by the criterion that it cosedimented with purified polyribosomes in a sucrose gradient and that it changed to a lower sedimentation value if polyribosomes were disaggregated with EDTA prior to centrifugation. Virus-specific mRNA obtained from either the polyribosome region of purified polyribosomes or the released messenger region of EDTA-disaggregated purified polyribosomes consisted of 14S, 20S, and 35S species in both BUDR-induced cells and L₂C leukemic lymphoblasts. Hybridization of cDNA to the RNA of L₂C leukemic lymphoblasts and BUDR-induced cells was essentially complete. Additionally, leukemic lymphoblast RNA could displace 95% of the hybridization of BUDR-induced GPV 70S RNA to guinea pig DNA. The midpoints of thermal denaturation of hybrids formed between GPV cDNA and the RNA of either L₂C leukemic lymphoblasts or the 70S RNA of BUDR-induced GPV were both 89°C in 2× concentrated 0.15 M NaCl plus 0.015 M sodium citrate. These results show that BUDR-induced GPV genes are essentially completely expressed in L₂C leukemic lymphoblasts and that virus-specific mRNA is present, although fewer copies of RNA are present in L₂C leukemic lymphoblasts than in BUDR-induced cells.

L₂C guinea pig leukemia, a transplantable leukemia of spontaneous origin (4), has been reported to have a pathological and clinical syndrome resembling that of acute human lymphoblastic leukemia (S. Murphy and A. LoBuglio, *Fed. Proc.*, in press) and has been extensively used in studies involving immunotherapy and chemotherapy. In spite of some early reports of transmissibility, there is no infectious virus. The leukemic cells are always B lymphoblast and of female origin irrespective of their passage in male or female guinea pigs (28, 31). Viruslike particles found in L₂C leukemic lymphoblasts appear to bud from the endoplasmic reticulum into cisternae. Similar particles have been observed in the germinal centers of normal animals (16), guinea pig hepatoma (7), and fetal germ cells (2).

Endogenous retroviral particles (BUDR-induced GPV) can be activated from any cultured guinea pig cells by treatment with 5-bromodeoxyuridine (BUDR) or 5-iododeoxyuridine (12, 24, 26). These particles are noninfectious for both guinea pig cells and all other cells tested (20). The amount and copy number of the endogenous proviral DNA is the same in uninduced, BUDR-induced, and leukemic guinea pig cells (21). BUDR-induced GPV has been reported to have the morphogenesis and morphology of a type B virus (5) (i.e., preformed nucleoids bud from the plasma membrane and virions have spikes on the outer surface and eccentrically located nucleoids). Thus, BUDR-induced GPV appears to be different in morphology and morphogenesis from viruslike particles observed in L₂C leukemic lymphoblasts

(5). BUdR-induced GPV also has a reverse transcriptase which, like mouse mammary tumor virus (MMTV), prefers Mg^{2+} over Mn^{2+} (17). However, GPV shares no sequence homology with murine type B (MMTV) or with other type C viruses (17).

The question whether BUdR-induced endogenous retroviral genes are expressed either fully or partially in leukemic cells has not previously been answered. Although BUdR-induced GPV reverse transcriptase can use exogenous template and primer efficiently, we and others (17) have been unable to synthesize sufficient complementary DNA (cDNA) using the endogenous reverse transcriptase reaction of detergent-disrupted virions. In this report, we have synthesized cDNA by using purified GPV RNA and reverse transcriptase of avian myeloblastosis virus. We have studied the expression of the GPV genome in L_2C leukemic guinea pig cells and tissues both by direct hybridization of this GPV cDNA to cellular RNA as well as by the competition with cellular RNA of the hybridization of labeled viral RNA to guinea pig DNA. We describe the essentially complete expression of BUdR-induced guinea pig virus, a non-infectious and potential type B virus, in L_2C leukemic guinea pig lymphoblasts. We also find that all virus-specific mRNA species present in BUdR-induced cells are also present in L_2C leukemic lymphoblasts.

MATERIALS AND METHODS

Animals. Leukemia in strain 2 animals was developed by inoculating 10^6 to 2×10^6 L_2C leukemic lymphoblasts into healthy strain 2 animals (24). The animals developed leukemia in 14 to 16 days when a white cell count in peripheral blood reached approximately 250,000/ μ l. Either circulating lymphoblasts, spleen, or other organs were used. Organs of similar size from healthy strain 2 animals were used for comparative analysis.

BUdR induction of virus and preparation of viral 70S RNA. Guinea pig retroviruses were activated by treating nearly confluent cultures of guinea pig embryo fibroblasts with BUdR (40 μ g/ml) and dexamethasone (10^{-6} M) for 48 h (17). Cultures were thereafter switched to complete medium with 10^{-6} M dexamethasone only. Harvests were then made at 24-h intervals for 5 days. Virus was purified from culture supernatant fluid on a 20 to 60% (wt/vol) linear sucrose gradient by use of a continuous-flow CF 32 rotor (Beckman Instruments, Fullerton, Calif.). The region of density 1.14 to 1.20 g/ml was pooled and precipitated with polyethylene glycol 6000 (23); virus was purified on a linear 20 to 65% sucrose gradient (24). Viral RNA (vRNA) was extracted (25), and 70S RNA was isolated in a linear sucrose gradient (24). Approximately 10 μ g of 70S vRNA was obtained from 10 liters of culture super-

natant fluid, indicating that guinea pig cells after BUdR and dexamethasone treatment produce an average of 10^8 virus particles/ml in 24 h or about 200 particles/cell per 24 h. BUdR and dexamethasone in combination produced two- to threefold more virus particles than BUdR alone.

^{32}P -labeled GPV 70S RNA was prepared by labeling GPV with carrier-free [^{32}P]orthophosphate according to the procedure of Dahlberg et al. (6). ^{32}P -labeled GPV was obtained by replacing the standard medium at 5 days after BUdR induction with phosphate-free medium containing 5% dialyzed inactivated fetal bovine serum and 10^{-6} M dexamethasone. After 2 h, the medium was replaced with fresh medium of the same composition containing 100 μ Ci of carrier-free [^{32}P]orthophosphate per ml. The medium containing ^{32}P was harvested and replaced three times at 12-h intervals. ^{32}P -labeled virus was purified (24), and ^{32}P -labeled 70S RNA (specific activity, 1.3×10^6 cpm/ μ g) was isolated.

Preparation and characterization of cDNA probes. (i) Oligodeoxythymidylic acid [oligo(dT)] as primer. Transcription of GPV 70S RNA was carried out essentially according to the procedure of Kacian and Myers (13) for 2 h at 37°C in a reaction containing Tris-hydrochloride (pH 8.3), 50 mM; $MgCl_2$, 8 mM; KCl, 50 mM; dithiothreitol, 0.4 mM; oligo(dT)₁₂₋₁₈ (Collaborative Research, Waltham, Mass.), 5 μ g/ml; sodium pyrophosphate, 4 mM; dATP, dGTP, and dCTP, 0.2 mM; [3H]dTTP, 0.2 mM (46 Ci/mmol); BUdR-induced GPV 70S RNA, 35 μ g/ml; and avian myeloblastosis virus reverse transcriptase (obtained from Life Sciences Inc., St. Petersburg, Fla.; lot G-1176, specific activity, 39,216; isolated according to the procedure of Kacian and Spiegelman [14]), 45 U/ml. The reaction was adjusted to 0.2 M NaCl and 1% sodium dodecyl sulfate (SDS) and was deproteinized by phenol extraction; nucleic acid was recovered by ethanol precipitation. This was dissolved in 0.01 M Tris-hydrochloride (pH 7.4)-0.001 M EDTA and was passed through a column (1.5 by 26 cm) of Sephadex G-50 fine (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.01 M Tris-hydrochloride (pH 7.4)-0.4 M NaCl-0.001 M EDTA. The material eluting in the void volume was adjusted to 0.3 N KOH and incubated for 18 h at 37°C. After exhaustive dialysis against distilled water, the GPV [3H]cDNA was lyophilized and redissolved in a small volume of distilled water. This 3H -labeled cDNA protects 75% of ^{32}P -labeled 70S vRNA at a cDNA-to-vRNA ratio of 1.9. Sedimentation of the cDNA as described by Studier (29), by use of a 15 to 30% linear sucrose gradient containing 0.9 M NaCl and 0.1 N NaOH in an SW60 rotor for 16 h at 42,000 rpm and 2°C, revealed peaks, with distribution at both sides, sedimenting at 8S and 14S relative to markers of simian virus 40 (SV40) form II DNA (16 and 18S). Molecular weights of 2.8×10^5 and 1.14×10^6 , respectively, were determined by use of the expression derived by Studier (29) for these conditions. However, a considerable amount of [3H]cDNA in our preparation (5 to 10%) sedimented at a position greater than 14S, indicating that some molecules may represent a nearly complete copy of the vRNA. A portion of the DNA transcript synthesized by Leis (15), using AMV polymerase and 34S

AMV RNA with oligo(dT) as primer, was found to have a sedimentation coefficient of 13S, corresponding to a molecular weight of 1.05×10^6 .

(ii) DNase-digestion end product of calf thymus DNA as primer. Transcription of GPV 70S RNA was carried out for 2.5 h at 37°C in a reaction containing Tris-hydrochloride (pH 8.3), 50 mM; $MgCl_2$, 8 mM; KCl, 50 mM; dithiothreitol, 0.4 mM; DNase-digestion end product of calf thymus DNA (30), 2 mg/ml; actinomycin D, 100 μ g/ml; dATP, dGTP, and dCTP, 0.2 mM; [3H]dTTP, 0.2 mM (46 Ci/mmol); BUdR-induced GPV 70S RNA, 25 μ g/ml; and AMV reverse transcriptase, 45 U/ml. The [3H]cDNA was isolated as described above. The specific activity was 2.4×10^7 cpm/ μ g. This cDNA protects 86% of ^{32}P -labeled 70S vRNA at a cDNA-to-vRNA ratio of 1.0. Sedimentation of this cDNA as described above revealed peaks sedimenting at 5S and 7S relative to the SV40 DNA markers. Molecular weights of 8.7×10^4 and 2.0×10^5 , respectively, were determined.

Preparation of cellular and tissue RNA and DNA. Total RNA was prepared from leukemic and normal strain 2 guinea pig tissues and cells according to a phenol-sodium dodecyl sulfate procedure (27), followed by treatment with DNase I for 30 min at 37°C. Total cellular RNA from guinea pig embryo fibroblasts treated for 48 h with BUdR as described above was prepared according to the method of Fan and Baltimore (8). Cytoplasmic RNA from leukemic lymphoblasts was prepared by extraction of cytoplasmic lysates (22) by a phenol-sodium dodecyl sulfate procedure (27). DNA was isolated from pooled organs of strain 2 animals as previously described (23).

Isolation of polyribosomes and polyribosome-associated RNA. Cytoplasmic lysates were prepared as previously described (22) by treating cells with sodium deoxycholate (0.5%) and Tween 40 (1%) in TKM buffer (10 mM Tris-hydrochloride [pH 7.4], 100 mM KCl, 5 mM $MgCl_2$) containing 0.01% diethylpyrocarbonate. Polyribosomes were pelleted through sucrose layers consisting of 2.0 ml of 1.0 M sucrose in TKM containing 0.01% diethylpyrocarbonate over 1.0 ml of 2.0 M sucrose in TKM containing 0.01% diethylpyrocarbonate in an SW50.1 rotor for 105 min at 49,000 rpm and 2°C. The pellet was resuspended in TKM buffer containing 0.01% diethylpyrocarbonate. The pelleted polyribosomes were divided into two equal portions; one portion was adjusted to 0.02 M EDTA and treated for 15 min at 0°C to dissociate polyribosomes into ribosomal subunits and free mRNA, and the other portion remained untreated. Each was then fractionated on a 5.0-ml 0.5 to 1.5 M linear sucrose gradient containing 0.01% diethylpyrocarbonate and either TKM buffer (untreated polyribosomes) or buffer containing 10 mM Tris-hydrochloride (pH 7.4), 100 mM KCl, 1 mM $MgCl_2$ (EDTA-treated polyribosomes) in an SW50.1 rotor for 40 min at 49,000 rpm and 2°C.

For RNA extraction of polyribosome fractions, samples were brought to 0.4 M NaCl, 0.01 M EDTA, 1% SDS, and 40 μ g of yeast RNA/ml. They were extracted three times with an equal volume of phenol and chloroform and precipitated with ethanol.

Hybridization techniques. Hybridization of 3H -

labeled cDNA to viral, cellular, or tissue RNA was performed in $5 \times$ SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C. Approximately 1,000 trichloroacetic acid-precipitable counts per minute were used for each 20- μ l sample. Hybridization was assayed by incubation for 30 min at 37°C in 1 ml of a reaction mixture (11) containing sodium acetate buffer (pH 4.5), 0.03 M; $ZnSO_4$, 5×10^{-3} M; NaCl, 0.3 M; salmon sperm DNA, 20 μ g/ml; and S-1 nuclease (Calbiochem, La Jolla, Calif.), 120 μ g/ml. Hybridization values were plotted as a function of Crt (1) corrected for standard conditions (3).

Virus-specific RNA in sucrose gradients of cellular or polyribosome-associated RNA was detected by layering RNA samples on a 12.6-ml linear 5 to 20% sucrose gradient in NTE buffer (23) followed by centrifugation for 150 min (total and cytoplasmic RNA) or 240 min (polyribosome-associated RNA) at 39,000 rpm and 4°C in an SW40 rotor. cDNA (approximately 1,500 trichloroacetic acid-precipitable cpm) was added to samples (100 μ l) of gradient fractions, and samples were adjusted to $5 \times$ SSC and annealed at 68°C.

For direct hybridization of polyribosomal fractions, each gradient fraction was diluted with 5 volumes of NTE buffer containing 0.2% SDS, 40 μ g of yeast RNA was added, and the fraction was precipitated with ethanol. Precipitates were pelleted by centrifugation and dissolved in 75 μ l of NTE buffer containing 0.15% SDS. cDNA prepared by use of the DNase-digestion end product of calf thymus DNA (approximately 1,500 trichloroacetic acid-precipitable cpm) was added to the concentrated fractions, and samples were adjusted to $5 \times$ SSC and 0.1% SDS. Virus-specific RNA was detected by annealing at 68°C. Percentage of cDNA hybridized was measured by treatment with S-1 nuclease, and the relative concentrations of virus-specific RNA were determined from the relationship: relative virus-specific RNA = $-\ln(1 - \text{fraction hybridized})/0.693$ (8).

Hybridization of ^{32}P -labeled 70S vRNA to cellular DNA or 3H -labeled cDNA was performed at 68°C in 0.5 M sodium phosphate buffer, and acquisition of resistance to RNase was assayed as previously described (23).

RESULTS

Expression of viral RNA in leukemic tissues and cells. To determine the extent of transcription of GPV RNA in leukemic and normal tissues and cells, the hybridization of GPV [3H]cDNA to guinea pig RNA from various sources was studied as a function of Crt. cDNA prepared with oligo(dT) as primer hybridized to the RNA of L₂C leukemic lymphoblasts, leukemic spleen, BUdR-induced cells, and 70S vRNA, but did not hybridize appreciably to the RNA of normal guinea pig liver or spleen at the highest Crt values obtained (Fig. 1). The ratio of Crt_i of 70S vRNA to the Crt_i of cellular or tissue RNA yields the percentage of cellular or tissue RNA that is homologous to 70S vRNA. based on these Crt_i values, we have calculated that approximately 0.05% of L₂C leukemic lymphoblast

FIG. 1. Hybridization of GPV ^3H -labeled cDNA to RNA from leukemic and normal guinea pig tissues and cells. [^3H]cDNA (specific activity, 2.4×10^7 cpm/ μg) prepared with oligo(dT) as primer was hybridized to guinea pig RNA from various sources. Details of the hybridization are as follows. (A) ∇ , 70S RNA—RNA concentration, 1.93 $\mu\text{g}/\text{ml}$; RNA-to-[^3H]DNA ratio, 928; $\bullet$, total leukemic lymphoblast RNA—RNA concentration, 3.2 mg/ml; RNA-to-[^3H]DNA ratio, 1.5×10^6 ; $\blacktriangle$, BUdR-induced cell RNA—RNA concentration, 2.2 mg/ml; RNA-to-[^3H]DNA ratio, 1.1×10^6 ; $\blacksquare$, normal liver RNA—RNA concentration, 19.2 mg/ml; RNA-to-[^3H]DNA ratio, 9.3×10^6 . (B) $\blacksquare$, normal spleen RNA—RNA concentration, 2.8 mg/ml; RNA-to-[^3H]DNA ratio, 1.4×10^6 ; $\bullet$, leukemic spleen RNA—RNA concentration, 2.4 mg/ml; RNA-to-[^3H]DNA ratio, 1.2×10^6 . (---) Theoretical plots for RNA excess hybridization of DNA to RNA with Crt_t values of 0.18 for 70S vRNA, 56 for BUdR-induced cell RNA, 360 for leukemic lymphoblast RNA, 800 for leukemic spleen RNA, and maximum hybridization of 100% (70S vRNA) and 75% (leukemic lymphoblast RNA, leukemic spleen RNA, and BUdR-induced cell RNA).

phoblast RNA is homologous to BUdR-induced GPV RNA, 0.02% of leukemic spleen RNA is similarly homologous, and 0.3% of BUdR-induced cell RNA is virus specific (Table 1). These values correspond to values of 360, 800 to 960, and 5,120 copies of viral RNA/cell in leukemic spleen, leukemic lymphoblasts, and BUdR-induced cells, respectively, based on an estimate of 5×10^{-12} μg of viral RNA per virion and 8×10^{-6} μg of total RNA per cell (11). The estimate for the percentage of virus-specific RNA in BUdR-induced cells is in good agreement with other animal cells producing retroviruses (8, 11). In this experiment, although the maximum hybridization for both BUdR-induced and L_2C leukemic lymphoblast RNA was slightly lower (75%) than with GPV 70S RNA (95 to 100%), we

have found in other experiments approximately the same hybridization level (90 to 95%) with RNA from both BUdR-induced and leukemic cells using the same cDNA as well as cDNA prepared with calf thymus DNA as primer (30). Leukemic liver RNA hybridized 23% at a Crt of 4×10^4 (Table 1). This low level of hybridization, most likely caused by either a relatively low viral RNA-to-cDNA ratio or by failure to achieve a sufficiently high Crt , may reflect a low ratio of L_2C leukemic lymphoblasts to liver tissue compared to the ratio of L_2C leukemic lymphoblasts to spleen tissue in leukemic spleen. Leukemic spleen becomes 5 to 10 times enlarged in leukemic animals as a result of accumulation of leukemic lymphoblasts.

[^3H]cDNA was also synthesized from GPV 70S RNA with DNase-treated calf thymus DNA as primer (30). Unlike oligo(dT) (used to prepare cDNA for the experiments shown in Fig. 1), which binds to the 3' end of vRNA containing polyadenylic acid, priming with calf thymus DNA occurs throughout the vRNA. As reported by Taylor et al. (30), we found that this procedure yields a very efficient transcription with a relatively uniform representation of vRNA. (This cDNA protected 86% of ^{32}P -labeled 70S vRNA at a cDNA-to-vRNA ratio of 1.0. Yields of cDNA were as high as 50% of the input RNA.) When this cDNA was used to repeat the hybridizations to leukemic lymphoblast total cellular RNA and normal spleen RNA of Fig. 1, a similar Crt_t (300) and a similar plateau level of hybridization (90%) for leukemic total cell RNA (data not shown) were obtained. The cDNA did not hybridize appreciably to normal spleen (7%) at a Crt of 9×10^4 .

Competitive molecular hybridization. The percentage of competition of the annealing reaction between ^{32}P -labeled 70S RNA of BUdR-induced GPV and guinea pig DNA by leukemic lymphoblast cytoplasmic RNA was determined. The addition of increasing amounts of leukemic lymphoblast cytoplasmic RNA displaced 95% of hybrid formation between ^{32}P -labeled 70S RNA and guinea pig DNA (Fig. 2). The addition of RNA (205 μg) from normal guinea pig spleen did not displace ^{32}P -labeled 70S RNA, and 97% of the control hybridization was obtained.

Thermal denaturation of hybrids. The thermal denaturation profiles of hybrids formed between leukemic lymphoblast total cellular RNA and [^3H]cDNA prepared with oligo(dT) as primer and hybrids formed between 70S vRNA and this cDNA were compared.

[^3H]cDNA prepared with oligo(dT) as primer was hybridized to 70S vRNA to a Crt of 0.95 or to total leukemic lymphoblast cellular RNA to a Crt of 4,066. Hybridization values obtained

TABLE 1. Estimation of amounts of viral RNA present in leukemic, BUdR-induced, and normal guinea pig cells

Type of RNA	Crt _{1/2}	% vRNA	Copies of vRNA/cell
70S vRNA ^a	0.18		
BUdR-induced cell RNA ^a	56	0.32	5,120
Leukemic spleen RNA ^a	800	0.02	360
Leukemic lymphoblast RNA ^a	350	0.05	800
Leukemic lymphoblast RNA ^b	300	0.06	960
Leukemic liver RNA ^a	23% hybridization at Crt of 4×10^4		
Normal liver RNA ^a	<5% hybridization at Crt of 10^5		
Normal spleen RNA ^a	<5% hybridization at Crt of 10^5		
Normal spleen RNA ^b	<7% hybridization at Crt of 9×10^4		

^a Hybridization using [³H]cDNA prepared with oligo(dT) as primer.

^b Hybridization using [³H]cDNA prepared with the DNase digestion end product of calf thymus DNA as primer.

were 95% and 96%, respectively. Samples containing 800 trichloroacetic acid-precipitable cpm were heated for 10 min in $2 \times$ SSC at temperatures from 65 to 100°C. The amount of hybrid remaining was determined with S-1 nuclease. The midpoint of thermal denaturation of either of the hybrids was 89°C in $2 \times$ SSC, further substantiating that the virus-specific RNA found in leukemic cells is homologous to BUdR-induced GPV 70S RNA.

Size distribution of virus-specific RNA. The species of virus-specific RNA present in L₂C leukemic lymphoblasts and BUdR-induced cells were compared. Total and cytoplasmic RNA was extracted from L₂C leukemic lymphoblasts, and total RNA was extracted from BUdR-induced cells. After centrifugation of the extracted RNA through a sucrose gradient, the fractions were assayed for virus-specific RNA by hybridization with GPV cDNA. Figure 3 shows that major species of 11 to 14S, 22 to 24S, 35S, and 70S are present in both the total and cytoplasmic RNA of L₂C leukemic lymphoblasts and the total RNA of BUdR-induced cells. Duplicate hybridization of samples of the gradients shown in Fig. 3A-3C for 24, 40, and 65 h, respectively, yielded essentially the same profile of virus-specific RNA. To minimize aggregation, total RNA extracted from BUdR-induced cells was denatured by heating for 5 min at 100°C. Gradient analysis of the heat-denatured RNA again showed species of virus-specific RNA sedimenting at 14S, 22 to 24S, and 35S (data not shown). No 70S virus-specific RNA was observed in gradients of heat-denatured RNA. To determine whether virus-specific RNA was degraded or aggregated during extraction, ³²P-labeled GPV 35S RNA was mixed with L₂C leukemic lymphoblasts prior to extraction of cytoplasmic RNA. Comparison of gradients of ³²P-labeled GPV 35S RNA ex-

FIG. 2. Competition hybridization of the GPV genome with leukemic lymphoblast cellular RNA. DNA (237 μ g) from pooled organs of normal strain 2 guinea pigs was hybridized to ³²P-labeled 70S RNA (1,000 cpm). To this reaction increasing amounts of leukemic lymphoblast cytoplasmic RNA were added. The samples were incubated at 68°C in 0.5 M sodium phosphate buffer to a Cot of 10,700 and then assayed for RNase resistance. Control hybridization in the absence of competitor yielded 67% hybrid.

tracted along with L₂C leukemic lymphoblasts and ³²P-labeled GPV 35S RNA extracted alone revealed no difference in sedimentation profile.

Virus-specific RNA species similar to those described above have been found in JLS-V9 cells and Moloney murine leukemia virus (M-MuLV)-infected cells where 14S, 20 to 27S, and 35S were found to serve as mRNA (8-10, 19). The 70S RNA is the virion structural RNA. It appears, therefore, that all GPV-specific RNA species, as well as 70S virion RNA, found in BUdR-induced cells are also present in L₂C leukemic lymphoblasts. As has been previously shown for M-MuLV-producing cells (8, 9), these species are found in the cytoplasm of the leukemic lymphoblasts.

Virus-specific RNA associated with polyribosomes. To find whether the virus-specific

FIG. 3. Distribution of virus-specific RNA in leukemic lymphoblasts and BUdR-induced cells. Total cellular RNA from BUdR-induced cells 48 h after BUdR induction and total and cytoplasmic RNA from leukemic lymphoblasts were prepared and fractionated on a sucrose gradient as described in Materials and Methods. The relative amount of virus-specific RNA in each fraction was determined by hybridization to [3 H]cDNA prepared with oligo(dT) as primer. (A) BUdR-induced cell RNA, 445 μ g of RNA layered on gradient, 18 h of hybridization. (B) Leukemic lymphoblast total cell RNA, 426 μ g of RNA layered on gradient, 14 h of hybridization. (C) Leukemic lymphoblast cytoplasmic RNA, 551 μ g of RNA layered on gradient, 12 h of hybridization.

RNA present in the cytoplasm was also associated with polyribosomes and served as mRNA, the presence of virus-specific RNA in polyribosomes and its dissociation with EDTA was determined. Since viral RNA complexed with protein or other materials may have a sedimentation coefficient similar to polyribosomes, the latter criterion that virus-specific mRNA must change to a lower sedimentation value if polyri-

bosomes are disrupted was used. Additionally, polyribosomes were purified by sedimentation through layers of sucrose (9, 10). It has been determined that such purification results in efficient removal of virus-specific RNA present in ribonucleoprotein structures that are precursors to virus particles (9). Cytoplasmic extracts of both BUdR-induced cells and L₂C leukemic lymphoblasts were prepared and pelleted through layers of 1.0 and 2.0 M sucrose. Before lysis, cells were treated with 1 μ g of cycloheximide/ml for 30 min to increase the yield of polyribosomes (22). The pelleted polyribosomes were resuspended in buffer, and one half was treated with EDTA and the other half was directly layered on a sucrose gradient. As can be seen from the absorbance profile (Fig. 4A), there are relatively few monosomes and small polyribosomes present in the untreated preparation, indicating that degradation of the polyribosomes was minimal. Disaggregation of polyribosomes with EDTA before sedimentation resulted in a nearly complete conversion to 40S and 60S ribosomal subunits (Fig. 4A).

Portions of each of the gradient fractions were concentrated by ethanol precipitation and assayed for the amount of virus-specific RNA present by the addition of cDNA and annealing. Virus-specific RNA was found in a broad distribution throughout the polyribosome region of gradients of purified polyribosomes of both BUdR-induced cells and L₂C leukemic lymphoblasts with peaks in the area of the fastest sedimenting polyribosomes (Fig. 4B and 4C). Disaggregation of purified polyribosomes with EDTA resulted in a clear shift of the sedimentation pattern of virus-specific RNA with the majority of virus-specific RNA sedimenting between 60S and 80S (Fig. 4B and 4C). We conclude that the majority of virus-specific RNA in the purified polyribosomes of both BUdR-induced cells and L₂C leukemic lymphoblasts, as defined by the association of this RNA with purified polyribosomes and its release with EDTA, is mRNA.

Size distribution of virus-specific mRNA. The size of virus-specific RNA present in purified polyribosomes of both BUdR-induced cells and L₂C leukemic lymphoblasts was determined. Regions I (polyribosome region) and II (released messenger) were pooled from gradients of untreated and EDTA-treated purified polyribosomes, respectively. RNA was isolated from each of the pooled fractions. After centrifugation of the extracted RNA through sucrose gradients, the fractions were concentrated by ethanol precipitation and analyzed for virus-specific RNA. Figures 5A and 5B show that, in

FIG. 4. Virus-specific RNA in purified polyribosomes of BUdR-induced cells and L_2C leukemic lymphoblasts. (A) Purified polyribosomes were prepared from BUdR-induced cells 72 h after induction. Fresh growth medium containing 10^{-6} M dexamethasone and cycloheximide ($1 \mu\text{g}/\text{ml}$) were added 150 and 30 min, respectively, before cells were harvested. (—) Absorbance at 254 nm; (---), absorbance at 254 nm after EDTA release. A similar absorbance profile was obtained when purified polyribosomes of L_2C leukemic lymphoblasts were analyzed (data not shown). (B) Fractions of gradients of purified polyribosomes of BUdR-induced cells were analyzed for virus-specific RNA as described in Materials and Methods. Fractions were annealed for 45 h. (Δ) Untreated control; ($\bullet$) EDTA released. (C) Heparin-treated whole blood from a leukemic strain 2 animal (white blood cell count, $245,000/\mu\text{l}$) was adjusted to $1 \mu\text{g}$ of cycloheximide per ml and incubated for 30 min at 37°C . Fractions of gradients of purified polyribosomes of the L_2C leukemic lymphoblasts were analyzed for virus-specific RNA. Fractions were annealed for 44 h. (Δ) Untreated control; ($\bullet$) EDTA released.

FIG. 5. Size distribution of virus-specific mRNA in purified polyribosomes of BUdR-induced cells and L_2C leukemic lymphoblasts. Fractions corresponding to region I for the untreated controls and region II for the EDTA-dissociated polyribosomes (see Fig. 4A) from both BUdR-induced cells and L_2C leukemic lymphoblasts were pooled and extracted with phenol-chloroform. Each purified RNA sample was fractionated on a sucrose gradient as described in Materials and Methods. RNA in each fraction was concentrated by ethanol precipitation in the presence of $40 \mu\text{g}$ of yeast RNA and resuspended in distilled water. Hybridization of each fraction was performed as described in Materials and Methods to cDNA prepared with the DNase digestion end product of calf thymus DNA as primer. (A) BUdR-induced cell RNA from region I, 43 h of hybridization. (B) BUdR-induced cell RNA from region II, 43 h of hybridization. (C) L_2C leukemic lymphoblast RNA from region I, 68 h of hybridization. (D) L_2C leukemic lymphoblast RNA from region II, 68 h of hybridization.

BUdR-induced cells, major species of 14S, 20S, and 35S were present as virus-specific mRNA in the RNA extracted from the polyribosome region of untreated purified polyribosomes as well as the RNA released from purified polyribosomes upon treatment with EDTA. Figures 5C and 5D similarly show that 14S, 20S, and 35S virus-specific RNA were also present as mRNA in L_2C leukemic lymphoblasts. As expected, the 70S RNA (virion structural RNA) found in cytoplasmic and total RNA (Fig. 3) was not associated with polyribosomes.

DISCUSSION

We have reported here that RNA homologous to BUdR-induced GPV is present in leukemic cells. Using cDNA produced with the DNase digestion end product of calf thymus DNA as primer, which provides an efficient transcript of vRNA, we found 90% hybridization to the RNA of leukemic cells. Furthermore, leukemic

cell RNA can displace 95% of the hybridization of BUdR-induced GPV 70S RNA to guinea pig DNA, and the midpoints of thermal denaturation for hybrids formed between cDNA and either L₂C leukemic lymphoblast RNA or BUdR-induced GPV 70S RNA are similar. These results lead us to conclude that the BUdR-induced GPV genome is essentially completely expressed (90 to 95%) in leukemic guinea pig cells.

Analysis of total and cytoplasmic RNA shows that all species of virus-specific RNA found in BUdR-induced cells are also produced in L₂C leukemic lymphoblasts. Certain of the species (14S, 20S, and 35S) are associated with purified polyribosomes in both BUdR-induced cells and L₂C leukemic lymphoblasts and are released by EDTA. The latter criterion strongly suggests their messenger function in L₂C leukemic lymphoblasts and their possible involvement in protein synthesis. Direct demonstration of viral protein synthesis must await direct analysis by radioimmune assay or isolation and analysis of virus-specific proteins by immunoprecipitation.

Although the expression of the BUdR-induced GPV genome appears complete, we cannot conclude that an exact similarity exists between the virus-specific RNA present in leukemic cells and the BUdR-induced GPV RNA. These experiments do not discount the possibility that virus-specific RNA in leukemic cells contains additional nonhomologous sequences. Any deletion of BUdR-induced GPV RNA would, however, be less than 10%. Since we detected little or no hybridization using normal adult tissue RNA (liver, spleen) at relatively high values of *C*_{rt}, we conclude that the expression is either greatly reduced or absent in the normal adult tissues studied. Increased expression of endogenous viral genes has also been observed in the natural lymphomas of cats, where the endogenous xenotropic virus RD-114 is found associated with the disease (24a).

Since guinea pig virus has been reported to be a type B virus, this may represent the occurrence of the expression of type B viral genes in leukemic cells. The presence of 70S RNA and the presence of virus-specific mRNA species in polyribosomes in leukemic cells suggests the formation of mature virus particles in L₂C leukemic lymphoblasts. The only particles observed in L₂C leukemic lymphoblasts are those found budding from endoplasmic reticulum into cisternae. If these particles represent a type B virus (i.e., same as BUdR-induced GPV), then BUdR-induced GPV may have more than one form of morphogenesis. Alternatively, although BUdR-induced GPV is completely expressed in leukemic cells, these particles are so

rare that they have not yet been observed, and the particles observed in L₂C leukemic lymphoblasts are different from BUdR-induced GPV.

Using competition hybridization, Michalides et al. (18) reported the homology between the genome of BUdR-induced GPV and virus particles in the plasma pellet of L₂C leukemic guinea pigs. However, two types of particles were found in leukemic plasma. The majority of particles resembled those found in L₂C leukemic lymphoblasts, and only a minority resembled BUdR-induced GPV. The origin of plasma particles containing RNA homologous to BUdR-induced GPV RNA was not determined. The results presented here, that leukemic lymphoblasts express BUdR-induced GPV genes, taken together with those presented by Michalides et al., may indicate that these particles come from L₂C leukemic lymphoblasts. However, experiments using radio-labeled probes (cDNA or vRNA) from purified particles found in leukemic plasma and leukemic lymphoblasts will be necessary to determine whether viruslike particles observed in leukemic lymphoblasts and leukemic plasma are identical to BUdR-induced GPV. We and others have found the preparation of such probes difficult, using either leukemic plasma (18) or leukemic cells (24).

Although we describe in this report the essentially complete expression of endogenous viral genes in L₂C leukemic lymphoblasts, it is not possible to associate a causal relationship of the expression of these viral genes to the malignancy. First, these viral particles are noninfectious and, therefore, cannot produce disease in guinea pigs upon inoculation. Second, the expression of endogenous viral genes may have occurred during serial passage of leukemic cells. Third, viral genes may be involved in other cellular functions such as growth or differentiation and may be expressed in leukemic cells as a result of the malignant transformation. Among these alternatives, the association of viral gene expression and cell division can be experimentally tested. Such experiments are currently underway.

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