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Integrated State of Oncornavirus DNA in Normal Chicken Cells and in Cells Transformed by Avian Myeloblastosis Virus

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The covalent linkage of oncornavirus-specific DNA to chicken DNA was investigated in normal chicken embryo fibroblasts (CEF) and in virus-producing leukemic cells transformed by avian myeloblastosis virus (AMV). The virus-specific sequences present in cellular DNA fractionated by different methods were detected by DNA-RNA hybridization by using 70S AMV RNA as a probe. In CEF and in leukemic cells, the viral DNA appeared to be present only in the nucleus. After cesium chloride-ethidium bromide density equilibrium sedimentation, the viral DNA was present as linear, double-stranded molecules not separable from linear chicken DNA. After extraction by the Hirt procedure, the viral DNA precipitated with the high-molecular-weight DNA. After alkaline sucrose velocity sedimentation, the viral DNA cosedimented with the high-molecular-weight cellular DNA. The results indicate that in both types of cells studied, the oncornavirus-specific DNA sequences were linked by alkali stable bonds to nuclear cellular DNA of high molecular weight and did not appear to be present in free form of any size.

It is generally acknowledged that the RNA of RNA tumor viruses replicates by synthesis and transcription of a DNA intermediate (3, 5, 11, 24, 47, 48, M. A. Baluda, in press). The integration i.e., covalent linkage to cellular DNA, of viral DNA has been postulated to explain the persistence of the viral genome in the RNA tumor virus-cell system (12, 48, 49). Such a model is supported by the presence in virions of enzymes necessary to accomplish integration (31, 48). Also, genetic analysis has shown that the information for virus-specific products is closely associated with the cell genome. The expression and transmission of information specifying the viral group-specific antigen, chicken helper factor for Rous sarcoma virus (O), and oncogenic functions have been shown to follow Mendelian genetics (2, 29, 38, 51, 54). Spontaneous synthesis and induction of RNA tumor viruses from nonproducing cells have also been reported (12, 16, 17, 22, 23, 43, 53). The persistence of viral information and the ability to rescue, or induce, viral gene products resembles some properties of lysogenic bacteria (13, 18).

Similar evidence for the persistence of viral information in non-virus-producing cells has also been reported in cells transformed by SV-40, polyoma virus, or adenovirus (15, 19, 21, 52, R. Wall, J. Weber, Z. Gage, and J. E. Darnell, Proc. Nat. Acad. Sci. U.S.A., in press). Covalent linkage of the viral DNA to the host cell DNA has been demonstrated in these systems (1, 14, 25, 44). Persistence of herpes simplex virus-2 genetic information in a cervical carcinoma and integration of part of the viral DNA in the host cell DNA has also been reported (20).

However, oncornavirus information might persist in a plasmid state, as with the temperate bacteriophage P1 (27). The persistence of the Epstein-Barr virus DNA in a nonintegrated state has been reported in non-virus-producing Raji cells (36, 55).

The present study was undertaken to determine the intracellular location of oncornavirus-specific DNA and whether it is convalently linked to cellular DNA by using techniques described by Sambrook et al. (44). The state of the viral DNA was investigated in non-virus-producing, apparently normal, chicken embryo fibroblasts and in virus-producing, leukemic myeloblasts in which a stable relationship between viral genetic information and host cell genome might be expected. In both types of

cells the virus-specific DNA sequences were found in the cell nucleus and appeared to be covalently linked to cellular DNA.

(This work was submitted in partial fulfillment of the requirements of the Ph.D. degree.)

MATERIALS AND METHODS

Viral RNA. Avian myeloblastosis virus (AMV) BAI strain A, subgroup B, was used. Procedures for the production of virus and purification of 70S viral RNA have been published (9, 11, 41).

Cells and cell cultures. Chicken embryo fibroblast (CEF) cultures were prepared from individual embryos of White Leghorn cross K-137 chickens or of its subline SPF-137 chickens purchased from Kimber Farms of California (5). Leukemic myeloblasts (MB) were isolated from 2 to 4-week-old leukemic chickens injected with AMV on the day after hatching (6, 9). All of the cell cultures were tested for virus production by looking for labeled avian leukosis virions after exposure to ^{3}H-labeled uridine for 10 h (5, 6). Only non-virus-producing CEF were used.

Marker DNA. Tritium-labeled, single-stranded, circular DNA from bacteriophage M-13 was a gift from Dan Ray (Department of Biology, UCLA), tritiated SV-40 DNAs, forms I and II, were a gift from John Jordan (Department of Chemistry, UCLA) and tritium-labeled adenovirus type 5 was a gift from Wendell Winters (Department of Surgery, UCLA).

Radioactively-labeled AMV RNA. Tritium-labeled AMV 70S RNA was prepared with uridine-5,6-^3H (10^{-6} M, 51 Ci/mmol; Amersham/Searle Corp.) and cytidine-5-^{3}H (10^{-6} M, 27 Ci/mmol; Amersham/Searle Corp.) (9, 41). ^{32}P-labeled 70S RNA was prepared with phosphate-free medium supplemented with 2.5% heat-inactivated fetal calf serum, 2.5% heat-inactivated chick serum, 10^{-6} M each of thymidine, uridine, cytidine, guanosine, and adenosine, 0.08 mmol glutamine, 0.02% sodium bicarbonate, and carrier-free ^{32}P orthophosphoric acid (100 μCi/ml, ICN). After a 10- to 12-h prelabeling period, labeled virus was harvested at 6- to 8-h intervals.

^{3}H or ^{14}C labeling of cellular DNA or protein. For the ^{3}H or ^{14}C labeling of DNA, freshly cultured MB or CEF were incubated in medium supplemented with 5% each of dialyzed fetal calf and dialyzed chicken sera, 10^{-6} M uridine, and either thymidine (methyl-^{3}H) (2 μCi/ml, 21.4 Ci/mmol; Schwarz/Mann) or thymidine (methyl-^{14}C) (0.2 μCi/ml, 37 mCi/mmol; Schwarz/Mann). After an 8-h labeling period the cells were washed twice with warm culture medium and incubated in nonlabeled medium for an additional 10 h.

For the dual labeling of DNA and protein, two labeling procedures were used. (i) The cells were incubated for 8 h in normal culture medium containing one-tenth the normal concentration of amino acids supplemented with 5% dialyzed fetal calf serum, 5% dialyzed chick serum, 10^{-6} M uridine, thymidine (methyl-^{14}C, 0.2 μCi/ml), and ^{3}H amino acid mixture (10 μCi/ml, 1 mCi/0.053 μg, New England Nuclear Corp). The cells were then washed with warm culture medium and incubated for an additional 16 h in low amino acid medium containing dialyzed serum, 10^{-6} M thymidine, 10^{-6} M uridine, and the ^{3}H amino acid mixture, or (ii) the same procedure was carried out with thymidine (methyl-^{3}H, 2 μCi/ml) and both L-leucine-^{14}C (0.2 μCi/ml, 240 mCi/mmol; Schwarz/Mann) and L-valine-^{14}C (0.2 μCi/ml, 160 mCi/mmol, Schwarz/Mann).

DNA extraction. For hybridization to AMV RNA, DNA from MB and CEF was extracted, purified, denatured, and trapped on filters according to published methods (3, 10, 11).

The MB DNA to be analyzed by cesium chloride-ethidium bromide (CsCl-EthBr) density sedimentation was extracted as usual but was dissolved in 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) and treated with RNase A (pancreatic fraction A, Worthington Biochemical Corp; 50 μg/ml) and RNase T1 (Sigma; 25 U/ml) at 37 C for 30 min. The DNA was then re-extracted twice with phenol, dialyzed against 0.1 × SSC and fractionated in a 5 to 20% neutral linear sucrose gradient (7, 9). DNA fractions having a sedimentation coefficient of 24S or greater (the maximum was 40S) were pooled, precipitated with ethanol, dissolved in 0.1 × SSC, and dialyzed against TE 8.5 buffer (0.01 M EDTA and 0.02 M Tris, pH 8.5).

Cell fractionation. Nuclei were isolated from whole cells by detergent fractionation (39). All stock detergent solutions were filter-sterilized before use. Each step of the fractionation procedure was monitored by light and phase-contrast microscopy. Care was taken to obtain nuclei free from observable cytoplasmic contamination. All procedures were carried out at 0 to 4 C. After rinsing with phosphate-buffered saline (PBS), CEF were released from culture plates by mild trypsinization (7), washed twice with cold PBS, and suspended at 5 × 10^7 cells/ml in an isotonic buffer (0.14 M NaCl, 0.02 M MgCl, and 0.01 M Tris, pH 8.5). The cells were lysed by slowly adding an equal volume of the same buffer containing 2% Nonidet P-40 (a gift from Shell Chemical Co.), mixing gently with a blunt-nosed pipette, and storing at 0 C for 10 min. Nuclei were pelleted by centrifugation at 600 × *g* for 3 min. After removing the supernatant fluid, the nuclear pellet was rinsed once with a small volume of buffer containing 1% Nonidet P-40 and the rinse was added to the supernatant fluid. The nuclei were resuspended in a hypotonic buffer (RSB) containing 0.01 M NaCl, 0.0015 M $MgCl_2$, and 0.01 M Tris, pH 8.5. At this stage, essentially all of the cells were disrupted. The nuclei were further freed from cytoplasmic contamination by the addition of one-seventh volume of a double detergent mixture (two parts of 10% Tween 40 [Nutritional Biochemical Corp.] to one part of 10% sodium deoxycholate [Mann Research Lab]) and mixed for 3 to 5 s on a Vortex mixer. The nuclei were again pelleted and rinsed with RSB and all supernatant fluids and rinsings were pooled. Before extraction of DNA, the supernatant fluid was adjusted to 0.02 M EDTA, and the nuclei were suspended in extraction buffer, NTE 8.5 (0.1 M NaCl, 0.001 M EDTA, and 0.02 M Tris, pH 8.5).

The MB were fractionated in a similar manner except that the cells were initially suspended in RSB, and an equal volume of RSB containing 1% Nonidet P-40 was added to lyse the cells. Pooled supernatant

fractions were adjusted to 0.1 M NaCl and 0.02 M EDTA before extraction of DNA.

Hirt fractionation. The procedure described by Hirt (26) was followed directly for CEF. However, MB were first suspended in TE 7.4 buffer (0.01 M EDTA and 0.01 M Tris, pH 7.4) at a concentration of 10^8 cells/ml. An equal volume of TE 7.4 buffer containing 1.2% of recrystallized, filtered SDS (sodium lauryl sulfate, Sigma Chemical Co.) was added and mixed by slowly inverting the tube. The rest of the procedure was that described by Hirt. Before the extraction of DNA from the separated fractions, the supernatant fluid was concentrated by ethanol precipitation, pelleted at 24,000 rpm for 1 h in a Beckman SW 25.2 rotor, and suspended in NTE 8.5 buffer. The Hirt pellet was dissolved in TE 8.5 buffer at 37 C.

Mitochondrial DNA. Mitochondria were purified and DNA was extracted as previously described (33, 34) with a slight modification. Hearts taken from six 2- to 3-week-old chicks were washed three times in an isotonic sucrose buffer (0.25 M sucrose, 0.002 M EDTA, and 0.025 M Tris, pH 7.4), cut into small pieces, disrupted for 10 s in a Waring blender, and homogenized in a dounce homogenizer (6). Nuclei were separated by pelleting twice at 600 × *g* for 10 min. The mitochondria were pelleted from the remaining supernatant fluid by centrifugation at 10,000 × *g* for 30 min, rinsed twice, resuspended in isotonic sucrose buffer, and banded in a 30 to 58% linear sucrose gradient (0.002 M EDTA, 0.0025 M Tris, pH 7.4) by centrifugation for 90 min at 22,000 rpm and 4 C in a Beckman SW 25.1 rotor. The mitochondrial band was collected from the bottom of the tube, diluted with isotonic sucrose buffer to give a final sucrose concentration of 0.5 M, and pelleted at 30,000 × *g* for 30 min. Supercoiled mitochondrial DNA was extracted from the pellet by a modified Hirt procedure. To mitochondria suspended in buffer (0.1 M NaCl, 0.1 M EDTA, and 0.02 M Tris, pH 8.5) 20% SDS was added to give a final concentration of 0.8% SDS, followed by the normal Hirt procedure. The supernatant fluid from the Hirt fractionation was dialyzed against TE 8.5 buffer before CsCl-EthBr centrifugation.

Alkaline sucrose velocity gradients. The release of DNA by lysing whole cells on top of preformed alkaline sucrose gradients has been described (1, 14, 36, 44). Linear alkaline sucrose (15–30% Harshaw Chemical Co.) gradients containing 0.3 N NaOH, 0.01 M EDTA, 0.5 M NaCl, and either 0.05% sodium deoxycholate for CEF or 0.05% Sarkosyl-NL 30 (Ciba-Geigy) for MB were prepared on top of a 2-ml cushion of 70% sucrose in tubes for the Beckman SW 27 rotor. Lysing solution (1.5 ml) (0.5 N NaOH, 0.1 M EDTA, and either 1% Nonidet P-40 and 0.5% sodium deoxycholate for CEF or 1% Sarkosyl for MB) was carefully layered on top of the gradient. The radioactively labeled cells were washed three times with PBS, suspended in 0.2 ml of cold PBS, and placed at a concentration of 2×10^6 to 3×10^6 cells per gradient on top of the lysing solution for 12 h at 4 C. The DNA was fractionated directly by centrifugation for 7 h at 22,000 rpm and 4 C. From 22 to 24 fractions were collected from the top of each gradient by pumping 70% sucrose into the bottom of each tube. The DNA was located by determination of radioactivity in samples of each fraction which had been neutralized with one to two drops of glacial acetic acid. Fractions were pooled according to the desired range of sedimentation coefficients. The pooled fractions from 60 to 72 gradients were neutralized with 2 N HCl in 0.4 M Tris, pH 7.4, and yeast-soluble RNA (5 μg/ml, Calbiochem) previously treated with DNase and phenol extracted, was added. The DNA fractions were concentrated by ethanol precipitation and centrifugation for 1 h at 10,000 rpm. The pelleted DNA was suspended in 20 to 30 ml of 0.1 × SSC, dialyzed against NTE 8.5, and extracted with phenol.

Density sedimentation of DNA in CsCl-EthBr. A volume of 0.4 ml of EthBr (Calbiochem) solution (3,000 μg/ml in TE 8.5 buffer) was added to DNA dissolved in TE 8.5 buffer, and the volume was adjusted to 3.4 ml with TE 8.5 buffer. Solid CsCl (Harshaw Chemical Co.) (3.2 g) was added to give a density of 1.58 g/cm^3 and an EthBr concentration of 300 μg/ml. The gradients were centrifuged to equilibrium in a Beckman SW 50.1 rotor (55 h at 33,000 rpm and 20 C). Forty fractions were collected from the bottom of each tube and the density was determined from the refractive index (44). The DNA was located either by counting 25-μliter samples from each fraction in Bray scintillation fluid or by diluting 25- or 50-μliter samples in 0.5 ml of water and reading the absorbancy at 260 nm. Fractions of appropriate densities to be used for DNA-RNA hybridization were pooled and dialyzed against 1 × SSC containing 0.001 M EDTA for 3 days at 4 C (34). The DNA was then further dialyzed against 0.1 × SSC, exposed to fluorescent light at a distance of 3 to 4 in (approximately 7.5–10 cm) for 16 h at room temperature, and boiled for 10 min to cleave the circular molecules (28, C. Shipman Jr., personal communication).

DNA-RNA hybridization. The DNA was purified, denatured, and trapped on nitrocellulose membrane filters (Millipore Corp.) and hybridized to either ^{3}H- or ^{32}P-labeled 70*S* AMV RNA as published (3, 10, 11). DNA isolated from mouse embryos was used as a control to measure nonspecific binding of 70*S* RNA to the filters during the hybridization procedure. The background radioactivity was in the range of 30 counts per min per filter when ^{3}H-labeled 70*S* RNA was used and 100 counts per min per filter with ^{32}P-labeled 70*S* RNA. The number of viral genome equivalents per chicken cell genome in a given DNA preparation was determined by using a reference DNA preparation (5, 6).

RESULTS

Nuclear location of DNA hybridizable to AMV RNA. After fractionation of CEF and MB into nuclear and cytoplasmic components, 89% of the total CEF DNA and 96 to 97% of the MB DNA was recovered in the nuclear fraction (Table 1). This represents a 10- to 30-fold enrichment of any DNA originating in the cytoplasm when compared with the same DNA extracted from whole cells. The relatively large amount of DNA in the cytoplasmic fraction

resulted from leakage of nuclear DNA during the extraction procedures, since only a very small amount (less than 1%) of the total cell DNA, i.e., mitochondrial DNA, is cytoplasmic (32).

Despite the enrichment of cytoplasmic DNA in the cytoplasmic fraction, the concentration of virus-specific DNA measured by hybridization with ^{3}H 70*S* AMV RNA and expressed as hybridized counts per minute per 100 μg of DNA was the same in the DNA from either cellular fraction and in the DNA from unfractionated cells (Table 1). This was true for the normal, non-virus-producing CEF and for the transformed virus-producing MB. Therefore, the results indicate a nuclear origin of all virus-specific DNA sequences. If even a small portion of virus-specific DNA originated in the cytoplasm it could have been detected. For example, in experiment 3 of Table 1, less than an average of two-tenths of one viral subunit per cell could have been detected in the cytoplasm of the leukemic cells. This can be calculated assuming that 95% of the determinations fall within $\pm$ two standard deviations of 60 counts/min with a mean of 909 counts/min hybridized per 100 μg of DNA, and from the knowledge that there are 22.2 viral subunit equivalents per cell genome and a 25-fold enrichment for cytoplasmic DNA in the cytoplasmic fraction.

Alkaline sucrose velocity sedimentation of unsheared cellular DNA and proteins. A typical alkaline sucrose velocity sedimentation profile of ^{3}H-labeled DNA from cells lysed directly on top of the gradient is illustrated in Fig. 1. The bulk of the cellular DNA sedimented between 100 and 120*S* by comparison with the M-13 DNA marker, which had a sedimentation coefficient of 20*S* under the conditions used in our gradients (30). Similar *S* values for the cellular DNA were obtained with either SV-40 form I DNA (53*S*) or adenovirus type 5 DNA (33*S*).

Figure 2 shows the alkaline sucrose velocity sedimentation profile of ^{3}H and ^{14}C radioactivity after lysing the cells directly on top of the alkaline sucrose gradients as in Fig. 1. A ^{3}H amino acid mixture and ^{14}C-thymidine were used to label cellular proteins and DNA, respectively, as described in Materials and Methods. There was no cosedimentation of labeled cellular proteins with the high-molecular-weight cellular DNA. Identical results were obtained when ^{14}C-leucine, ^{14}C-valine, and ^{3}H-thymidine were used to label the cellular macromolecules.

Presence of virus-specific DNA in high-

TABLE 1. *Hybridization of ^{3}H-labeled 70S AMV RNA with DNA from nuclear or cytoplasmic fractions*[a]

Cells	Cell fraction	Amount of DNA (μg) per fraction[b]	Counts per min hybridized	
			Per filter[c]	Per 100 μg of DNA[c]
Expt 1				
CEF	Nuclear	308.7	128 ± 10	766 ± 48
	Cytoplasmic	38.5	53 ± 2	731 ± 44
	Whole cell		125 ± 11	781 ± 59 (17.4)[d]
	Reference DNA		260 ± 28	1,142 ± 70 (25.5)
Expt 2				
MB	Nuclear	5,637.6	416 ± 33	1,487 ± 122
	Cytoplasmic	166.9	216 ± 17	1,613 ± 168
	Whole cell		446 ± 78	1,583 ± 170
Expt 3				
MB	Nuclear	6,763.5	292 ± 25	883 ± 54
	Cytoplasmic	267.9	97 ± 4	897 ± 68
	Whole cell		266 ± 18	947 ± 57 (22.2)
	Reference DNA		297 ± 53	1,087 ± 52 (25.5)

[a] In experiments 1 and 3, DNA from CEF or MB was hybridized with ^{3}H-labeled 70*S* AMV RNA (10^6 counts per min per ml; specific activity, 3.6×10^5 counts per min per μg). In experiment 2, DNA from MB was hybridized with ^{3}H-labeled 70*S* AMV RNA (8×10^5 counts per min per ml; specific activity, 1.4×10^6 counts per min per μg).

[b] Total DNA recovered from each cellular fraction measured by absorbancy at 260 nm (30 μg of DNA per 1.0 absorbancy unit).

[c] Mean of three to five filters $\pm$ standard deviation; background counts per minute hybridized to mouse DNA filters have been deducted.

[d] Number per cell genome of DNA equivalents of the 3×10^6 daltons viral RNA subunit calculated from the reference DNA.

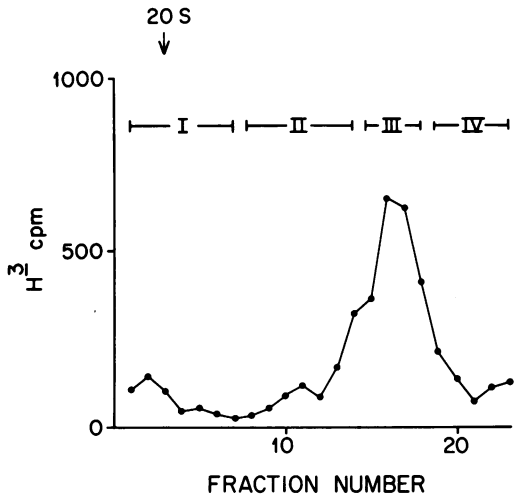

FIG. 1. *Alkaline sucrose velocity sedimentation of lysed MB labeled with ^{3}H-thymidine. MB were labeled, lysed, and centrifuged as described in Materials and Methods. Fractions were collected from the top of each gradient by pumping 70% sucrose into the bottom of the centrifuge tube. Samples from each fraction were precipitated with 5% trichloroacetic acid, filtered, washed, dried, and counted in toluene scintillation fluid. Direction of sedimentation is from left to right.*

molecular-weight cellular DNA. ^{3}H-labeled DNA from K-137 CEF, SPF-K-137 CEF, or leukemic MB was fractionated by alkaline sucrose velocity sedimentation as in Fig. 1. For each type of cell, 72 gradients were run. Each gradient was divided into four parts according to a specified range of sedimentation coefficients (see Fig. 1): I, 0 to 47*S*, II, 53 to 93*S*, III, 100 to 120*S*, and IV, greater than 120*S*, i.e., DNA that sedimented to the top of the heavy sucrose cushion. DNA fractions having a similar range of sedimentation coefficients were pooled from 60 to 72 gradients. After purification and denaturation, the DNA from each pool was hybridized to ^{32}P-labeled 70*S* AMV RNA to determine the concentration of virus-specific DNA sequences in each pool.

Except for pool I of K-137 CEF and MB, at least 60% of the ^{3}H-radioactivity in each DNA pool was retained by the nitrocellulose filters after boiling and gravity filtration. The nature of the ^{3}H-labeled compounds in pool I from K-137 CEF and MB has not been analyzed in detail but it appears that nearly all of the counts per minute were either in free thymidine or in DNA molecules too small to stick to filters. Most of the counts per minute were acid soluble. Also, most of the cellular proteins were present in that part of the gradient (Fig. 2). For unknown reasons pool I from the SPF-K 137 CEF contained fewer counts per minute but more low-molecular-weight cellular DNA.

Table 2 shows that the virus-specific DNA cosedimented in alkaline sucrose with the high-molecular-weight cellular DNA and that the ^{32}P viral RNA hybridized to the same extent with DNA from each pool and with unfractionated DNA from whole cells. This was true for the three types of cells that were studied. It appears, therefore, that the viral DNA was covalently linked to cellular DNA of high molecular weight and that the viral DNA present in DNA pools I and II was due to partial degradation of cellular DNA. If the viral DNA had existed in a free intracellular state of lower molecular weight, it would have become more concentrated in pools I and II. More ^{32}P viral RNA would have hybridized per 100 μg of DNA from these pools than to the DNA in pools III and IV, since there was a three- to fourfold enrichment of lower-molecular-weight DNA in pools I and II.

Evidence to rule out the nonspecific linkage of viral DNA to cellular DNA in the alkaline sucrose gradients was obtained in the following experiments. Double-stranded linear adenovirus DNA with a molecular weight of 22×10^6, double-stranded circular or linear SV-40 DNA with a molecular weight of 3×10^6, single-stranded circular M-13 DNA with a molecular

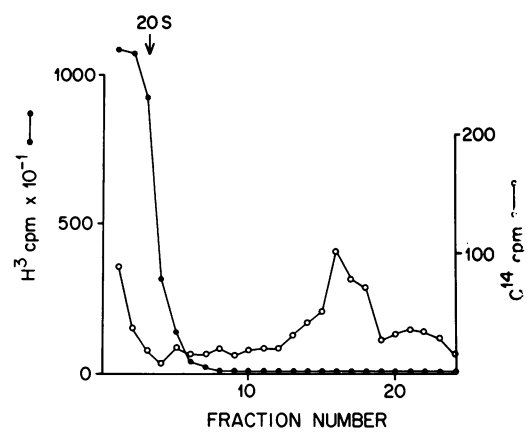

FIG. 2. *Alkaline sucrose velocity sedimentation of lysed leukemic cells labeled with ^{14}C-thymidine and ^{3}H-amino acids. Cultured MB were labeled for 8 h with ^{14}C-thymidine (0.2 μCi/ml) and a ^{3}H-amino acid mixture (10 μCi/ml) in medium containing one-tenth the normal concentration of amino acids and incubated an additional 16 h in similar medium without ^{14}C-thymidine. After labeling, the cells were lysed and centrifuged as in Fig. 1. Samples from each fraction were neutralized with one to two drops of glacial acetic acid and counted directly in Bray scintillation fluid. Direction of sedimentation is from left to right. ^{3}H radioactivity, ●; ^{14}C radioactivity, O.*

TABLE 2. *Hybridization of ^{32}P-labeled 70S AMV RNA with ^{3}H-labeled DNA from cells fractionated by alkaline sucrose velocity sedimentation*[a]

Cells	DNA pool	Sedimentation coefficient	^{3}H counts per min per pool[b]	^{3}H counts per min per filter[c] (after hybridization)	^{32}P counts per min hybridized	
					Per filter[d]	Per 100 μg of DNA[d]
CEF K-137	I	0-47	1,252,480	7,452 ± 2,507	4 ± 13	
	II	53-93	220,800	100,779 ± 10,934	92 ± 6	836 ± 82
	III	100-120	1,297,440	199,195 ± 12,476	176 ± 15	928 ± 88
	IV	>127	934,080	205,212 ± 13,520	123 ± 3	617 ± 13
	Whole cell				218 ± 43	728 ± 6 (12.6)[e]
	Reference DNA				419 ± 30	1,191 ± 75 (20.7)
CEF SPF-K-137	I	0-47	345,328	104,243 ± 5,919	103 ± 13	988 ± 149
	II	53-93	783,216	98,201 ± 15,017	90 ± 18	1,028 ± 130
	III	100-120	3,562,380	494,503 ± 20,965	488 ± 112	990 ± 110
	IV	>127	365,052	102,892 ± 25,139	100 ± 15	971 ± 42
	Whole cell				446 ± 140	1,048 ± 43 (10.4)
	Reference DNA				730 ± 69	2,088 ± 138 (20.7)
MB	I	0-47	976,000	8,735 ± 7,794	0.3 ± 14	
	II	53-93	283,680	57,303 ± 2,468	120 ± 20	2,106 ± 174
	III	100-120	1,983,520	319,337 ± 34,947	743 ± 112	2,322 ± 51
	IV	>127	250,900	73,745 ± 1,255	156 ± 17	2,084 ± 46
	Whole cell				454 ± 32	2,137 ± 143 (31.5)
	Reference DNA				503 ± 27	1,406 ± 162 (20.73)

[a] Cultured cells from three sources (CEF from K-137 embryos, CEF from SPF-K-137 embryos, and MB from leukemic K-137 chicks) were labeled for 8 h with ^{3}H-thymidine (2 μCi/ml), washed, and incubated in normal medium for an additional 16 h. Cells (2-3 × 10^{6}) were layered on the top of each alkaline sucrose gradient. The cells were lysed at 4 C for at least 12 h, then the gradients were centrifuged for 7 h at 22,000 rpm at 4 C in a Beckman SW-27 rotor. For each cell type, 72 gradients were run, and the DNA was pooled from 60 to 72 gradients according to specified sedimentation values and neutralized with 2 N HCl in 0.4 M Tris, pH 7.4. Soluble yeast RNA was added as carrier (5 μg/ml), and the DNA was concentrated by ethanol precipitation, phenol extracted, treated with alkali (0.3 N KOH, 18 h at 37 C), dialyzed, denatured, and trapped on nitrocellulose membrane filters. CEF (K-137) DNA filters were hybridized with 10^{6} counts per min per ml of ^{32}P 70S AMV RNA (specific activity, 5.4 × 10^{5} counts per min per μg); MB DNA filters were hybridized with 1.2 × 10^{6} counts per min per ml of ^{32}P 70S RNA (specific activity, 5.4 × 10^{5} counts per min per μg) and CEF (SPF-K-137) DNA filters were hybridized with 1.4 × 10^{6} counts per min per ml of ^{32}P 70S RNA (specific activity, 6.5 × 10^{5} counts per min per μg).

[b] Total ^{3}H counts per minute recovered in each pool. The specific activity of the ^{3}H-DNA varied between 8 × 10^{3} to 10 × 10^{3} counts per min per μg.

[c] Mean of two to five filters ± standard deviation; measured after the hybridization procedure.

[d] Mean of two to five filters ± standard deviations. ^{32}P counts per minute hybridized to mouse DNA (approximately 100 counts per min per filter) have been deducted.

[e] Number per cell genome of DNA equivalents of 3 × 10^{6} daltons viral RNA subunits calculated by comparison with ^{32}P counts per minute hybridized to reference DNA.

weight of 1.7 × 10^{6}, or linear alkali denatured chicken DNA with a molecular weight of 16 × 10^{6} was mixed with the cells before the lysis treatment at 4 C or at the time of centrifugation. All of these ^{3}H-labeled exogenous DNAs became completely separated from the bulk of cellular DNA under the conditions used to obtain cellular DNA of high molecular weight. In addition, the high-molecular-weight cellular DNA appeared to be free of proteins (Fig. 2).

Evidence that the high-molecular-weight DNA does not contain supercoiled viral DNA. Assuming that the number per cell genome of DNA equivalents of the 36S viral RNA subunit is correct, the sedimentation coefficient of the viral DNA would be very high if all the subunits were present as a single, supercoiled, circular molecule. For instance, in leukemic cells with 31.5 viral DNA equivalents per cell, the viral DNA could be a double-stranded, circular molecule of 190 × 10^{6} daltons. To investigate the possible existence of such giant supercoiled, circular DNA molecules, DNA from leukemic cells was centrifuged to equilibrium in neutral CsCl containing ethidium bromide. Under these conditions, circular DNA binds less dye than does linear DNA and bands at a higher density (32, 40).

Figure 3A shows the separation of supercoiled SV-40 DNA (peak I) from linear SV-40 DNA

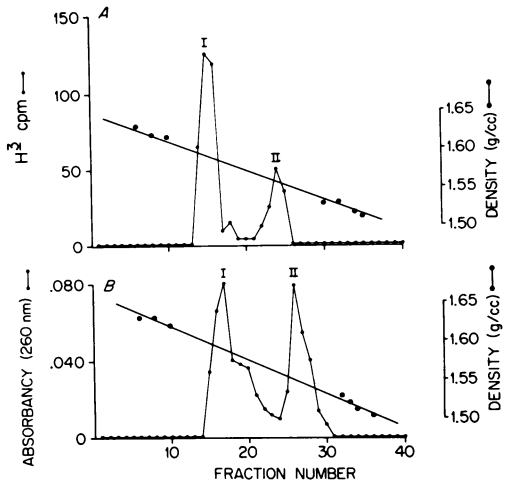

FIG. 3. *Density equilibrium centrifugation in CsCl-EthBr, of SV-40 and chick mitochondrial DNAs. DNA in TE 8.5 buffer was mixed with solid CsCl and an EthBr solution to give a density of 1.58 g/ml and an EthBr concentration of 300 μg/ml and centrifuged for 55 h at 30,000 rpm and 20 C in a Beckman SW-50.1 rotor. Fractions (0.1 ml) were collected from the bottom of the tube. The density was determined from the refractive index. A, SV-40 DNA. ^{3}H-labeled SV-40 DNA forms I and II were dialyzed against TE 8.5 buffer and fractionated in a CsCl-EthBr gradient as described above. The radioactivity in each fraction was determined by counting 25-μliter samples in Bray scintillation fluid. Peak I consists of SV-40 supercoiled DNA and peak II consists of linear DNA molecules. B, Chick heart mitochondrial DNA. Mitochondria were partially purified from the hearts of freshly sacrificed 2- to 3-week-old chicks by homogenization of tissues, differential centrifugation, and banding in a 30 to 58% linear sucrose gradient. DNA was extracted from the mitochondria by the Hirt procedure, dialyzed against TE 8.5 buffer, and centrifuged in a CsCl-EthBr density gradient. The DNA concentration in each fraction was determined by reading absorbancy at 260 nm. The background absorbancy determined from a gradient centrifuged without DNA was subtracted from each fraction. Peak I consists of mitochondrial supercoiled DNA and peak II consists of linear DNA molecules.*

(peak II), and Figure 3B shows the separation of chick mitochondrial supercoiled DNA (peak I) from chicken linear DNA (peak II).

DNA isolated from leukemic cells was fractionated by neutral sucrose velocity sedimentation, and only those DNA molecules sedimenting faster than 24S were used for banding in CsCl-EthBr gradients. Low-molecular-weight DNA was excluded to prevent linear viral DNA from banding at the density of supercoiled molecules. It was found that if chicken DNA fragments of 13S or less were banded in alkaline CsCl, the virus-specific DNA banded at a higher density than the bulk of chicken DNA because of its higher G + C content (Shoyab and Baluda, in press). Figure 4 shows the absorbancy (260 nm) profile of leukemic DNA after density equilibrium sedimentation in CsCl plus EthBr. The DNA region of the gradient was divided into three parts according to density as shown by the horizontal arrows. Fractions with densities from 1.605 to 1.582 correspond to the region where supercoiled circular DNA should band. Fractions with densities from 1.582 to 1.572 should contain a mixture of supercoiled and nicked circular molecules. Fractions with densities from 1.572 to 1.530 contained the bulk of linear chicken DNA. Two gradients were used and the fractions within the specified density range were pooled. The DNA from each pool was then tested for its capacity to hybridize with ^{3}H-70S AMV RNA.

Most of the DNA was recovered in the fraction which contained linear molecules, and all of the virus-specific DNA sequences were present in that fraction (Table 3). Also, there was no loss of viral DNA from the linear DNA which hybridized as many counts per minute of ^{3}H-viral RNA as did unfractionated DNA from whole cells.

The absence of viral DNA in the other fractions of higher densities did not result from the conversion of supercoiled molecules to linear

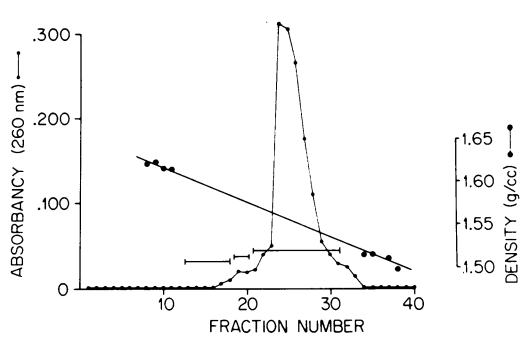

FIG. 4. *Density equilibrium sedimentation in CsCl-EthBr of leukemic chicken DNA. DNA was isolated from leukemic myeloblasts and centrifuged in a CsCl-EthBr gradient as described in Materials and Methods. Fractions were collected from the bottom of the centrifuge tube and the DNA concentration in each fraction was measured as in Fig. 3B. Fractions 13 through 18 which contained supercoiled DNA were pooled fractions 19 and 20 which contained opened circular DNA were pooled, and fractions 21 through 31 which contained linear DNA were also pooled. The pooled DNAs were dialyzed to remove EthBr and CsCl and then exposed to fluorescent light and boiled to generate linear molecules, trapped on filters, and hybridized to ^{3}H-labeled 70S AMV RNA (see Table 3).*

TABLE 3. *Hybridization of ^{3}H-labeled 70S AMV RNA with leukemic DNA fractionated by density sedimentation in CsCl-EB gradients*[a]

DNA			Counts per min hybridized	
Type	Density	Amount (μg) per fraction[b]	Per filter[c]	Per 100 μg of DNA[c]
Linear	1.572–1.530	63.4	396 ± 16	3,070 ± 155 (24.2)
Opened circles	1.582–1.572	Not detectable	1 ± 2	
Supercoiled circles	1.582–1.605	Not detectable	3 ± 2	
Whole cell			326 ± 31	3,225 ± 148 (25.4)[d]
Reference DNA			779 ± 38	2,630 ± 30 (20.7)

[a] DNA was phenol-extracted from leukemic myeloblasts, treated with RNases A and T1, re-extracted with phenol, dialyzed, and subjected to velocity sedimentation in linear sucrose gradients. The DNA which sedimented faster than 24S was ethanol-precipitated, dialyzed, and analyzed by density sedimentation in CsCl (ρ = 1.58 g/cm^3) and ethidium bromide (300 μg/ml) (see Fig. 4). Two gradients were run. DNA which banded at density regions corresponding to supercoiled circular, opened circular, and linear molecules was dialyzed, treated with fluorescent light, denatured, trapped on filters, and hybridized with ^{3}H-labeled 70S AMV RNA: 1.34×10^6 counts per min per ml; specific activity, 9×10^5 counts per min per μg.

[b] Determined by absorbancy at 260 nm; the initial amount of DNA added to the two gradients was 66 μg.

[c] Counts minus background counts per minute hybridized to mouse DNA filters. Average of three to five filters ± standard deviation.

[d] Number per cell genome of DNA equivalents of 3×10^6 daltons viral RNA subunits calculated from reference DNA.

molecules or from the loss of circular molecules during the extraction procedure. When a mixture of ^{3}H-labeled circular and linear SV-40 DNA molecules were added to approximately the same number of leukemic cells used for the experiments described in Table 3 before the DNA extraction procedure (50 μg of DNA recovered per gradient), there was no loss of circular or linear SV-40 DNA after density equilibrium centrifugation in CsCl-EthBr as compared with untreated circular and linear SV-40 DNA banded in a parallel CsCl-EthBr gradient.

Also, the absence of circular DNA capable of hybridizing to the viral RNA was not caused by the inability of supercoiled or circular DNA to become trapped on filters. Of ^{3}H-labeled mitochondrial DNA, 80% could be trapped on filters under conditions identical to those used with the leukemic cell DNA.

Fractionation of cellular DNA by the Hirt procedure. Additional evidence for the integration of virus-specific DNA into cellular DNA was obtained after DNA extraction by the Hirt method that separates low-molecular-weight DNA molecules, irrespective of their form, from bulk cellular DNA (26, 44). This procedure can be used to detect free intracellular virus-specific DNA molecules early after infection of CEF with AMV or Rous sarcoma virus (Ali and Baluda, unpublished data). When cellular DNA is extracted by the Hirt method under conditions of minimal shear, nearly 100% of the cellular DNA should be present in the precipitate. Only 70 to 80% of the total cellular DNA could be precipitated with the cells used due to shearing (Table 4). Nevertheless, this separation was sufficient for our purpose.

DNAs from the two Hirt fractions and from whole cells were compared for their ability to hybridize with 70S AMV RNA. DNA in the Hirt supernatant fluid, despite a three- to fourfold enrichment for native small-molecular-weight DNA, did not hybridize more to AMV RNA than did DNA from the pellet (Table 4).

Also, DNA in the pellet hybridized with AMV RNA to the same extent as did DNA from whole cells, i.e., there was no loss of virus-specific DNA in the large-molecular-weight cellular DNA after fractionation. From the results with DNA from MB-1 in Table 4 it can be calculated that there is less than one DNA equivalent of the viral 36S subunit in an intracellular free form. It appears, therefore, that the viral DNA was associated with the high-molecular-weight cellular DNA.

DISCUSSION

The integration into the host cell genome of DNA complementary to AMV RNA was studied in two types of cells: normal, non-virus-producing chicken embryo fibroblasts, and virus-producing leukemic myeloblasts transformed by AMV. It had been previously shown that both types of cells contain oncornavirus-specific DNA (5, 10, 35, 42, 50). The viral DNA in apparently normal chicken cells, even gs-antigen negative cells, is of unknown origin but represents, at least in some instances, the entire

genome of an endogenous oncornavirus since virus synthesis can be induced under appropriate conditions (43, 53). Leukemic cells contain up to six times as much AMV complementary DNA as do uninfected cells (5, 6, 10). This DNA represents the entire AMV genome as shown by hybridization of viral RNA with an excess of cellular DNA (35; Shoyab, Evans, and Baluda, unpublished data), and the RNA recovered from leukemic DNA-viral RNA hybrids had the same average base composition as that of viral RNA (8).

The hybridization of denatured cellular DNA trapped on filters to an excess of viral RNA seems to give a good estimate of the average cellular concentration of virus-specific DNA. The number per cell genome of DNA equivalents of the viral 36S RNA subunits was found to be essentially the same whether determined by the filter technique or by hybridization of viral RNA in vast DNA excess (Shoyab and Baluda, unpublished data). Also, the number of copies of 28 and 18S ribosomal RNA genes present per chicken cell genome was similar with either hybridization technique, i.e., 177 and 236 copies, respectively (Shoyab and Baluda, unpublished data). The number of Epstein-Barr virus genomes per cell has been found to be the same whether determined by filter hybridization or by DNA-DNA reassociation kinetics (37).

After cell fractionation that allowed the total recovery of cellular DNA, nuclei free from cytoplasm contained all the detectable viral DNA in CEF and in MB. There was no enrichment of virus-specific DNA in the cytoplasmic fraction nor a loss from the nuclear fraction when compared with DNA extracted from whole cells.

Results of the CsCl-EthBr experiments indicated that the viral DNA did not exist in MB as supercoiled circular molecules of any size. Reconstruction experiments ruled out the specific loss of supercoiled circular DNA during the experimental procedures. Furthermore, fractionation of DNA from CEF and MB by the Hirt method (26) indicated that viral DNA was not present in a free form but was associated with high-molecular-weight DNA molecules. The same ratio of virus-specific DNA to cellular DNA was detected in Hirt precipitable DNA, nonprecipitable DNA, and in DNA from unfractionated cells.

Alkaline sucrose velocity sedimentation ex-

TABLE 4. *Hybridization of ^3H- or ^{32}P-labeled 70S AMV RNA with cellular DNA fractionated by the Hirt procedure*[a]

Cells	DNA		Counts per min hybridized	
	Fraction	Amount (μg)[b]	Per filter[c]	Per 100 μg of DNA[c]
Expt 1				
CEF[d]	Precipitate	524.4	238 ± 17	992 ± 45
	Supernatant	160	113 ± 9	907 ± 35
	Whole cell		436 ± 16	1,064 ± 118 (10.4)[e]
MB	Precipitate	1,663.2	282 ± 21	1,878 ± 256
	Supernatant	411.6	380 ± 36	1,735 ± 101
	Whole cell		427 ± 100	1,780 ± 201 (17.4)
	Reference DNA		676 ± 100	2,117 ± 150 (20.7)
Expt 2				
MB[f]	Precipitate	209.6	438 ± 13	2,111 ± 57
	Supernatant	104.4	382 ± 56	2,164 ± 118
	Whole cell		460 ± 32	2,152 ± 110 (31.7)
	Reference DNA		503 ± 27	1,406 ± 162 (20.7)

[a] DNA was extracted from CEF and MB by the Hirt procedure. The DNA from each fraction was then purified, trapped on filters, and hybridized with 70S AMV-RNA. DNAs in experiment 1 were hybridized with ^3H-labeled 70S RNA (1.1×10^6 counts per min per ml; specific activity, 8.4×10^5 counts per min per μg). In experiment 2, MB DNA was hybridized with ^{32}P-labeled 70S RNA (1.2×10^6 counts per min per ml; specific activity, 5.4×10^5 counts per min per μg).

[b] Total amount of purified DNA recovered from each Hirt fraction.

[c] Mean of three to five filters ± standard deviation. Counts per minute hybridized to mouse DNA filters have been deducted.

[d] Same CEF as those used in experiment 2 of Table 2.

[e] Number per cell genome of DNA equivalents of the 3.0×10^6 daltons viral RNA subunit calculated with reference DNA.

[f] Same leukemic myeloblasts as those used in Table 2.

periments showed that the viral DNA was attached by alkali stable bonds to the high-molecular-weight cellular DNA which had a sedimentation coefficient greater than 120*S*. Linear denatured viral DNA of that size can be ruled out since the normal SPF-K-137 CEF studied contained, per cell genome, only an average of 10.4 viral DNA equivalents and the leukemic MB 31.5 equivalents. If present as a single molecule, the denatured viral DNA in the SPF-K-137 CEF would have had a molecular weight of 33×10^6 and an *S* value in alkali of less than 54 (45). The values for the viral DNA in MB would have been 96×10^6 and 83*S*. Viral DNA molecules of much larger size might exist if they were not uniformly distributed among all the cells. This is unlikely since, after alkaline velocity sedimentation, there was no enrichment or loss of viral DNA in DNA fractions of various sizes compared with DNA from whole cells. There was relatively little fluctuation in the average viral DNA content per cell in several hundred chicken embryos (5; Baluda, unpublished data), and different adult tissues from the same chicken, or from different chickens of the same species, contained the same average cellular concentration of viral DNA (36). Also, CEF and MB cultured in vitro for up to 2 months and tested at various time intervals did not show a fluctuation in their average cellular concentration of viral DNA (4).

The possibility that nonintegrated viral DNA could cosediment in alkali with high-molecular-weight cell DNA is unlikely. Entrapment or attachment of viral DNA by cell DNA was checked by the addition of exogenous DNAs of various sizes and configurations to cells before lysis and fractionation. In all cases these DNAs were completely separated from the main DNA band. Also, protein linker molecules are considered unlikely since cellular proteins labeled before cell fractionation did not appear to cosediment with the cellular DNA. The association of viral DNA with cellular components other than DNA can also be ruled out since the high-molecular-weight DNA that hybridized to AMV RNA banded in CsCl-EthBr at the correct density for DNA (1.56 g/cm^3). Therefore, it appears that a covalent linkage existed between the oncornavirus DNA sequences and high-molecular-weight cellular DNA.

Integration may be a great advantage for RNA containing tumor viruses (46, 47). After infection and integration into the cellular DNA, viral information may not be needed for the replication of viral DNA or RNA or for transfer of viral information to progeny cells. Also, since the viral replication has an RNA phase, activation of viral replication could occur without the need for excision of the integrated viral DNA. Oncornaviruses therefore could avoid the need for many complicated regulatory mechanisms to control DNA replication, transcription, and excision such as those required by temperate phages (13, 18).

All of the viral DNA sequences that were vertically inherited in normal chicken cells or that were present 3 weeks after infection in virus-producing leukemic cells appeared to be covalently linked to host DNA. Less than one free viral genome equivalent per cell could have been detected. By contrast, most of the newly synthesized viral DNA was not integrated in CEF infected 2 to 3 days earlier with AMV or RSV (Ali and Baluda, unpublished data). Since the average number of viral DNA copies per cell genome shows a relatively narrow distribution in different normal embryos or leukemic chicks (5, 6), there may be only a limited number of integration sites.

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