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Synthesis of Avian Oncornavirus DNA in Infected Chicken Cells

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The intracellular synthesis and integration of viral DNA (vDNA) into the host cell genome was studied in cultured chicken embryo fibroblasts infected with avian sarcoma or leukemia viruses. The newly synthesized vDNA was detected by hybridization with 70*S* viral RNA. Extraction of infected cell DNA by the selective procedure of Hirt resulted in the enrichment of newly synthesized vDNA in the low molecular weight supernatant fraction while leaving the bulk of cellular DNA containing integrated vDNA in the high molecular weight pellet fraction. This approach led to detection of intracellular vDNA synthesis within 1 h after infection and to vDNA integration into cellular DNA within 24 h. There was a several-fold increase in the vDNA content of infected cells during the initial phase of virus infection. But only a part of this newly synthesized vDNA appeared to become covalently linked with high molecular weight cellular DNA. Most of the remaining unintegrated vDNA gradually disappeared. The sedimentation profiles of minimally sheared cellular DNA in alkaline sucrose velocity gradients suggest that vDNA is synthesized as free linear molecules of approximately 3×10^6 daltons which subsequently are covalently linked to host cell DNA.

The replication of RNA tumor viruses (oncornaviruses) via a DNA intermediate is well documented (19). The intracellular presence of the proviral DNA (vDNA) has been confirmed by genetic (10, 11) and biochemical evidence (5-15, 20). Recent evidence from this laboratory and from other workers has indicated the covalent linkage of oncornavirus vDNA to host cell DNA (2-8, 13, 21). Since the components necessary for this process (RNA template and primers, reverse transcriptase) are enclosed within the virion, the synthesis of vDNA should take place in the early phase of virus infection (19). Some support for this expectation had been provided by studies indicating that iododeoxyuridine (IUdR) may be incorporated into vDNA within 1 h after virus infection (1), and by autoradiographic detection in the cell nucleus of ^{3}H-labeled parental viral RNA also within 1 h after infection (7).

The present study was undertaken to obtain some information about the intracellular synthesis of vDNA and its integration with cellular DNA. The synthesis of vDNA after infection of cultured chicken embryonic fibroblasts (CEF) with avian myeloblastosis virus (AMV) or sarcoma viruses (RSV) was monitored by hybridization of purified AMV 70*S* RNA with cellular DNA extracted at various time intervals after infection. The fractionation of DNA from infected cells into high molecular weight and low molecular weight components by the selective procedure of Hirt (12) made possible the separation of nonintegrated and integrated vDNA. This procedure had been successfully used to obtain similar information about SV-40 DNA integration (6, 17). The integration of vDNA into cellular DNA was verified by alkaline sucrose velocity sedimentation of minimally sheared cellular DNA as described earlier (13). The integrated vDNA sedimented with high molecular weight cell DNA, whereas the unintegrated free vDNA sedimented with low molecular weight DNA. These results are in agreement with a recent publication of Varmus et al. (21) in which the authors demonstrated unintegrated vDNA early after infection of mouse or duck cells by RSV and integrated vDNA in transformed cells.

MATERIALS AND METHODS

Cells. CEF were cultured from individual 10-day-old embryonated eggs of White Leghorn, cross K-137, or its subline SPF-K-137, purchased from Kimber Farms of California (3). The growth medium consisted of modified Eagle medium, 10% tryptose phosphate broth, and 5% each of fetal calf and chicken sera. Because of possible congenital infection or

spontaneous production of avian leukosis viruses (ALV) in apparently normal chicken embryonic cells (10, 23, 24), all cell cultures were tested for ALV production (3) and only non-virus-producing cells were used. In addition, in order to have a uniform background of endogenous viral DNA, cells derived from a single embryo were used for each experiment.

Viruses. Prague strain (subgroup A) of Rous sarcoma virus (PR-RSV-A) was grown in CEF tested for absence of ALV production. Most of these cells were transformed 2 to 3 days after PR-RSV-A infection and produced virus for at least 2 weeks. The culture supernatant fluid was harvested every 12 h and was spun at 8.5 K for 30 min at 2 to 4 C in a Sorvall centrifuge. The supernate, containing at least 10^6 infectious virus particles per ml, was either used directly or after concentration and purification by density gradient centrifugation (9).

AMV BAI strain-A (subgroup B) was obtained from the plasma of acutely leukemic chicks. The leukemic plasma was frozen, thawed, and centrifuged at 8.5 K for 30 min to remove blood cells. The isolation and purification of ^{3}H- or ^{32}P-labeled 70*S* RNA from RSV or AMV have been described (13). Tritiated SV-40 DNA (forms I and II) was a gift from John Jordan (UCLA) and circular ϕX-174 DNA was a gift from Daniel Ray (UCLA).

Cell infection. Secondary or tertiary cultures of CEF in confluent monolayers were trypsinized, and then replated without dilution in fresh growth medium. Four to 6 h later, the medium containing unattached cells was aspirated and the virus inoculum was added. After allowing 30 to 60 min for virus adsorption, fresh medium was added and the cultures were reincubated for the desired length of time. This method achieves partial synchronization of the processes of cell division and virus infection (1, 9). The input multiplicity of infection (MOI) of PR-RSV(A) determined from the focus forming titer (22) and from the cell number per plate was either 2 or 4 focus forming units (FFU) per cell in an inoculum volume of 0.2 ml of growth medium without serum.

Isolation and fractionation of cellular DNA. The extraction and purification of cellular DNA for DNA-RNA hybridization have been described (3, 5). The Hirt fractionation of large and small molecular weight cellular DNA with 0.6% sodium dodecyl sulfate (SDS) and 1 M NaCl at 0 to 4 C, followed by centrifugation, has also been described (12, 17). Alkaline sucrose sedimentation of cellular DNA was carried out by the method of Sambrook et al. (17) as modified by Markham and Baluda (13). DNA-RNA hybridization was carried out by the filter method in ^{3}H- or ^{32}P-labeled 70*S* AMV RNA excess (10^6 to 2×10^6 counts per min per ml; specific activity 0.7×10^6 to 10^6 counts per min per μg) (5, 15). After hybridization the amount of DNA attached to each filter was determined by the Burton diphenylamine reaction (5).

RESULTS

Intracellular synthesis of vDNA. The vDNA content of CEF infected with AMV or PR-RSV was measured at various time intervals after infection by hybridization with ^{3}H-labeled 70*S* AMV RNA (3, 5, 15). The absence of an accidental oncornavirus contamination was checked at the beginning and at the end of each experiment in uninfected CEF from the same embryo cultured under the same conditions as the experimentally infected cells. These uninfected cells also served as controls for endogenous vDNA content (3, 15).

After infection of CEF with PR-RSV(A) at an input MOI of 2 FFU per cell, the synthesis of oncornavirus-specific DNA sequences can be detected within 12 h, appears to continue up to 36 h, and is followed by a slight but repeatedly observed drop in vDNA content per infected cell 72 h after infection (Table 1). The continuous synthesis of vDNA for 36 h reflects either (i) asynchronous initiation of replication, or (ii) several cycles of vDNA synthesis.

vDNA synthesis early after infection. If vDNA is synthesized as relatively small molecules, it should be found in the Hirt supernatant fraction before its integration into cellular DNA. Extraction of DNA from infected cells by the Hirt procedure (12) which separates small and large molecular weight DNA molecules should also facilitate the detection of vDNA by virtue of the several-fold concentration of small molecular weight DNA in the supernatant fraction. CEF were infected with PR-RSV at an MOI of 2 FFU per cell and were subjected to Hirt fractionation at various time intervals between 1 and 24 h after infection. A twofold increase in the vDNA content of the Hirt supernate was noted at 9 h postinfection (Table 2). Further increase in vDNA content of the small DNA fraction continued to occur up to 24 h, when the experiment was terminated. The vDNA content of the Hirt pellet, presumably representing integrated vDNA, did not show an appreciable increase up to 24 h.

To ensure the simultaneous infection of all the cells and facilitate the earlier detection of newly synthesized vDNA, cells were infected at a higher multiplicity of input virus with leukemic chick plasma which contains high infectious titers of AMV (10^8 to 10^9 infectious units of virus per ml of plasma) (4). AMV is capable of productive infection but not of morphological transformation in CEF (4). Table 3 shows the appearance of AMV specific DNA in the Hirt supernatant fraction of CEF infected with leukemic plasma diluted two times. A good separation of the small and large DNA species resulting in a much greater enrichment (nearly 15-fold) of small DNA in the supernatant fraction and perhaps the high MOI enabled us to detect

TABLE 1. *Appearance of vDNA after infection of CEF with PR-RSV(A)*

Time after infection[a]	Counts per min of RNA hybridized	
	Per filter	Per 100 μg of DNA
Uninfected cells	472 ± 26[b]	1,600 ± 55[b]
12 h	438 ± 72	2,380 ± 96
24 h	814 ± 41	2,627 ± 171
36 h	654 ± 52	3,121 ± 125
48 h	670 ± 38	3,044 ± 192
72 h	931 ± 87	2,411 ± 60
Reference DNA[c]	1,388 ± 68	4,550 ± 100

[a] Tertiary cultures of CEF were infected with RSV at an input multiplicity of 2 and harvested at different time intervals after infection. DNA from these cells was extracted, purified, immobilized on nitrocellulose filters, and hybridized with ^{3}H-labeled 70*S* AMV RNA in 4× SSC (1× SSC = 0.15 M NaCl and 0.015 M Na citrate) containing 3 mg of unfractionated mouse RNA per ml and 0.05% SDS at 70 C for 10 h.

[b] Mean of five filters ± standard deviation.

[c] Leukemic chick DNA containing 31 DNA equivalents of the 35*S* AMV RNA subunit used as a reference.

newly synthesized vDNA as early as 1 h postinfection.

Intracellular concentration of vDNA late after infection. The intracellular level of PR-RSV specific DNA was determined for several days after infection with an input MOI of 4 FFU per cell (Table 4). The amount of vDNA recovered in the Hirt supernate increased for about 48 h and then decreased. There was a rise in the concentration of vDNA in the Hirt pellet for about 72 h, after which time it remained relatively constant. The comparable concentrations of vDNA in unfractionated cell DNA and in pellet DNA might result from the loss of newly synthesized unintegrated vDNA if DNA extraction and purification is carried out with unfractionated cells.

A similar pattern of vDNA synthesis and distribution in the pellet and the supernatant fractions also appears to occur in CEF infected with AMV (Table 5). With both AMV and PR-RSV the de novo synthesis of vDNA appears to take place for only a few days after infection. The data presented in Tables 2 to 5 also show that the pattern of vDNA synthesis and integration appears to be similar for RSV and AMV irrespective of the fact that RSV can transform CEF whereas AMV cannot.

Integration of vDNA into the host cell genome. The presence of newly synthesized vDNA in the Hirt pellet does not seem to result from entrapment. This is demonstrated by the absence of newly synthesized vDNA in the pellet at the early stages of infection, and by the constant amount of vDNA in the Hirt pellet even at later stages in infection when the vDNA content of the Hirt supernate is decreasing.

The size distribution of DNA in the Hirt pellet and of that in the Hirt supernate was determined by alkaline sucrose velocity sedimentation (Fig. 1). The single-stranded pellet DNA had a broad size distribution with an average sedimentation coefficient of about 34*S*, whereas the supernate DNA formed a sharper peak at approximately 13*S*. The separation point between the two fractions was approximately 20*S* and from Studier's relationship for estimating the molecular weight of DNA in alkaline sucrose (18), this corresponds to denatured DNA molecules of approximately 2.8 × 10^6 daltons.

The nature of the association of vDNA with host cell DNA in the Hirt pellet was investigated by alkaline sucrose velocity sedimentation of minimally sheared DNA from infected

TABLE 2. *Detection of vDNA in the HIRT fractions of CEF infected with PR-RSV(A)*

Time after infection[a] (h)	Counts per min of RNA hybridized per 100 μg of DNA	
	Pellet	Supernate
Uninfected cells	1,032 ± 61[b]	925 ± 91
	(322 ± 52)	(168 ± 13)
1	1,192 ± 36	989 ± 20
	(180 ± 16)	(193 ± 10)
3	1,046 ± 74	1,002 ± 29
	(315 ± 35)	(217 ± 9)
6	1,084 ± 82	1,020 ± 70
	(270 ± 38)	(152 ± 6)
9	1,191 ± 88	2,045 ± 45
	(341 ± 36)	(379 ± 21)
12	1,163 ± 35	2,575 ± 169
	(341 ± 18)	(576 ± 32)
24	1,239 ± 61	2,702 ± 228
	(310 ± 22)	(758 ± 93)

[a] Tertiary cultures of K-137 CEF were infected with RSV at an input MOI of 2 and lysed after various time intervals at room temperature in 0.6% SDS, 0.01 M Tris, pH 7.4, and 0.01 M EDTA. The lysate was adjusted to a final concentration of 1 M with NaCl, was allowed to precipitate at 0 C for 16 h, and was centrifuged at 35,000 × *g* for 1 h. DNA from the pellet or the supernate was extracted, purified, immobilized on filters, and hybridized with ^{3}H-labeled 70*S* AMV-RNA. The ratio of DNA recovered from the pellet to that recovered from the supernate was approximately 2:1 in each case.

[b] Mean of 3 to 5 filters ± standard deviation; mean counts per minute hybridized per filter ± standard deviation are shown in parenthesis below each figure.

TABLE 3. *Detection of vDNA in the HIRT fractions of CEF infected with AMV*

Time after infection[a]	Pellet DNA:[b] supernate DNA	Counts per min of RNA hybridized per 100 μg of DNA		
		Pellet	Supernate	Corrected supernate increase[c]
Uninfected cells	3.5	551 ± 55[d] (213 ± 24)	687 ± 15 (169 ± 22)	0
1 h	14.6	609 ± 20 (214 ± 14)	1,673 ± 35 (153 ± 35)	236
3 h	14.9	615 ± 105 (283 ± 60)	3,480 ± 436 (325 ± 33)	656
6 h	4.0	656 ± 46 (190 ± 8)	1,588 ± 28 (186 ± 26)	1,030
Reference DNA[e]		1,475 ± 30 (570 ± 27)		

[a] Tertiary cultures of SPF-K-137 CEF were infected with AMV in leukemic chick plasma diluted 1 to 2 with growth medium and were subjected to Hirt fractionation at various times after infection. After extraction, purification and processing the DNA from each fraction was hybridized with ^{3}H-labeled 70*S* AMV-RNA.

[b] Ratio of DNA recovered in the Hirt pellet to that recovered in the supernate calculated from the respective absorbancy at 260 nm.

[c] Corrected to the same enrichment factor (pellet DNA:supernate DNA), i.e., (column 4-687) × (3.5/column 2).

[d] Mean of 3 to 5 filters ± standard deviation. Average counts per minute hybridized per filter ± standard deviation are shown in parenthesis below each figure.

[e] Leukemic chick DNA containing 21 DNA equivalents of the 35*S* AMV RNA subunit.

TABLE 4. *Distribution of vDNA in HIRT fractions of CEF late after PR-RSV(A) infection*

Time after infection[a]	Pellet DNA: supernate DNA	Counts per min of RNA hybridized per 100 g of DNA			
		Unfractionated cells	Pellet	Supernate	Corrected supernate increase[b]
Uninfected cells	7.7	851 ± 90[c] (114 ± 28)	998 ± 71 (220 ± 15)	1,163 ± 116 (100 ± 3)	0
24 h	2.7	1,412 ± 161 (160 ± 17)	1,567 ± 45 (391 ± 18)	4,140 ± 454 (315 ± 31)	8,490
48 h	3.1	1,811 ± 62 (334 ± 24)	1,857 ± 44 (420 ± 78)	6,666 ± 1,036 (688 ± 55)	13,669
72 h	8.7	2,074 ± 123 (365 ± 43)	2,103 ± 118 (486 ± 54)	14,901 ± 631 (788 ± 25)	12,159
96 h	5.4	1,960 ± 107 (332 ± 34)	1,802 ± 75 (541 ± 25)	5,098 ± 310 (543 ± 174)	5,611
120 h	3.2	1,887 ± 138 (265 ± 39)	1,700 ± 50 (450 ± 14)	2,230 ± 102 (204 ± 18)	2,567
Reference DNA[d]		1,475 ± 30 (570 ± 27)			

[a] Secondary cultures of SPF-K-137 CEF were infected with PR-RSV at an input MOI of 4 and were subjected to Hirt fractionation at various time intervals after infection. DNA was then extracted, purified, and hybridized with ^{3}H-labeled AMV RNA. At each time interval the DNA from one-fifth of the culture plates was directly extracted from whole cells without prior Hirt fractionation (unfractionated cells).

[b] (Column 5-1163) × (7.7/column 2).

[c] Mean of 3 to 5 filters ± standard deviation. Average of counts per minute hybridized per filter ± standard deviation are shown in parenthesis below each figure.

[d] Leukemic DNA containing 21 DNA equivalents of the AMV 35*S* RNA subunit.

cells. Cells infected 60 h earlier with PR-RSV at an input multiplicity of at least 2 FFU per cell were lysed directly on top of alkaline sucrose gradients (13, 17) and were centrifuged for 6 h at 82,500 × *g* (Fig. 2). The gradients were collected from the top, and divided into five fractions according to their sedimentation coefficient values. As found in a previous study (13), most of

the ^3H counts per minute at the top of the gradient did not appear to be in DNA since they were acid soluble and did not bind to filters (Table 6). The newly synthesized vDNA was located by hybridization of DNA in each fraction with ^{32}P-labeled 70S AMV RNA (Table 6).

Approximately half of the newly synthesized vDNA 60 h postinfection is smaller than 40S and represents an unintegrated form or possibly fragmented cellular DNA containing integrated vDNA. Approximately 37% of the free vDNA has a sedimentation coefficient smaller than 28S, and from Studier's relationship (18) this corresponds to DNA with an approximately molecular weight of less than 6.5×10^6, assuming that it is linear. The vDNA in fraction III (28 to 40S) could represent either (i) several covalently linked linear vDNA subunits, or (ii) a circularized form of a single 35S subunit transcript. Fractions I and II appear to correspond to DNA recovered in the Hirt supernate fraction, whereas fractions III, IV, and V would correspond to the Hirt pellet. At 60 h after infection with PR-RSV, approximately 50% of the vDNA is associated with the high molecular weight cellular DNA in fractions IV and V of the alkaline sucrose velocity gradient. This result is in good agreement with the amount of vDNA recovered in the Hirt pellet at similar time intervals after infection (Table 4) and substantiates the assumption that vDNA recovered in the pellet represents integrated viral DNA.

DISCUSSION

The synthesis of provirus DNA has been detected within 1 h after virus infection. Because of its relatively smaller size, the newly synthesized vDNA can be partially separated from the bulk of cellular DNA by the Hirt fractionation procedure (12). Extraction of DNA by this method results in a several-fold enrichment of vDNA in the supernatant fraction, thereby facilitating the early detection of small increases in the viral DNA content of infected cells. For example, the 15-fold increase of vDNA in the Hirt supernate, seen 1 h postinfection in Table 3, would have amounted to only an insignificant increase of about 67 counts per min per 100 μg of DNA if DNA from unfractionated cells had been tested. The appearance of newly synthesized vDNA within 1 h after infection is in agreement with the report of Bader (1). These findings are also consistent with the report by Salzberg et al. (16) indicating that, in the murine system, the transcription of virus specific RNA from vDNA can be detected within 6 to 7 h after infection.

In these experiments it cannot be determined whether several copies of proviral DNA can be made directly from one RNA subunit or by

TABLE 5. *Distribution of vDNA in HIRT fractions of CEF late after AMV infection*

Time after infection[a]	Pellet DNA: supernate DNA	Counts per min of RNA hybridized per 100 μg of DNA			
		Unfractionated cells	Pellet	Supernate	Corrected supernate increase[b]
Uninfected cells	5.2	334 ± 10[c] (121 ± 7)	405 ± 35 (129 ± 27)	594 ± 51 (72 ± 18)	0
Expt. 1					
5 days[d]	5.6	3,972 ± 228 (632 ± 137)	1,622 ± 93 (580 ± 66)	16,145 ± 2822 (1,543 ± 302)	14,440
Expt. 2					
10 days[d]	2.9	1,080 ± 178 (350 ± 39)	838 ± 22 (340 ± 39)	1,343 ± 105 (325 ± 41)	1,343
15 days[d]	3.2	950 ± 76 (270 ± 24)	768 ± 185 (295 ± 24)	1,415 ± 182 (126 ± 9)	1,334
Reference DNA[e]		1,630 ± 52 (529 ± 48)			

[a] Secondary cultures of SPF-K-137 CEF were infected with AMV in leukemic chick plasma diluted 1 to 3 with growth medium and subjected to Hirt fractionation at various time intervals after infection. DNA was purified, processed and hybridized with ^3H-labeled 70S AMV-RNA.

[b] (Column 5-594) × (5.2/column 2).

[c] Mean of 3 to 5 filters ± standard deviation. Average counts per minute hybridized per filter ± standard deviation are shown in parenthesis below each figure.

[d] Two different stocks of virus were used for infection: one for the 5-day infection and another for the 10- and 15-day infection. Cells from the same embryo were used in both experiments.

[e] Leukemic chick DNA containing 21 DNA equivalents of the AMV 35S RNA subunit.

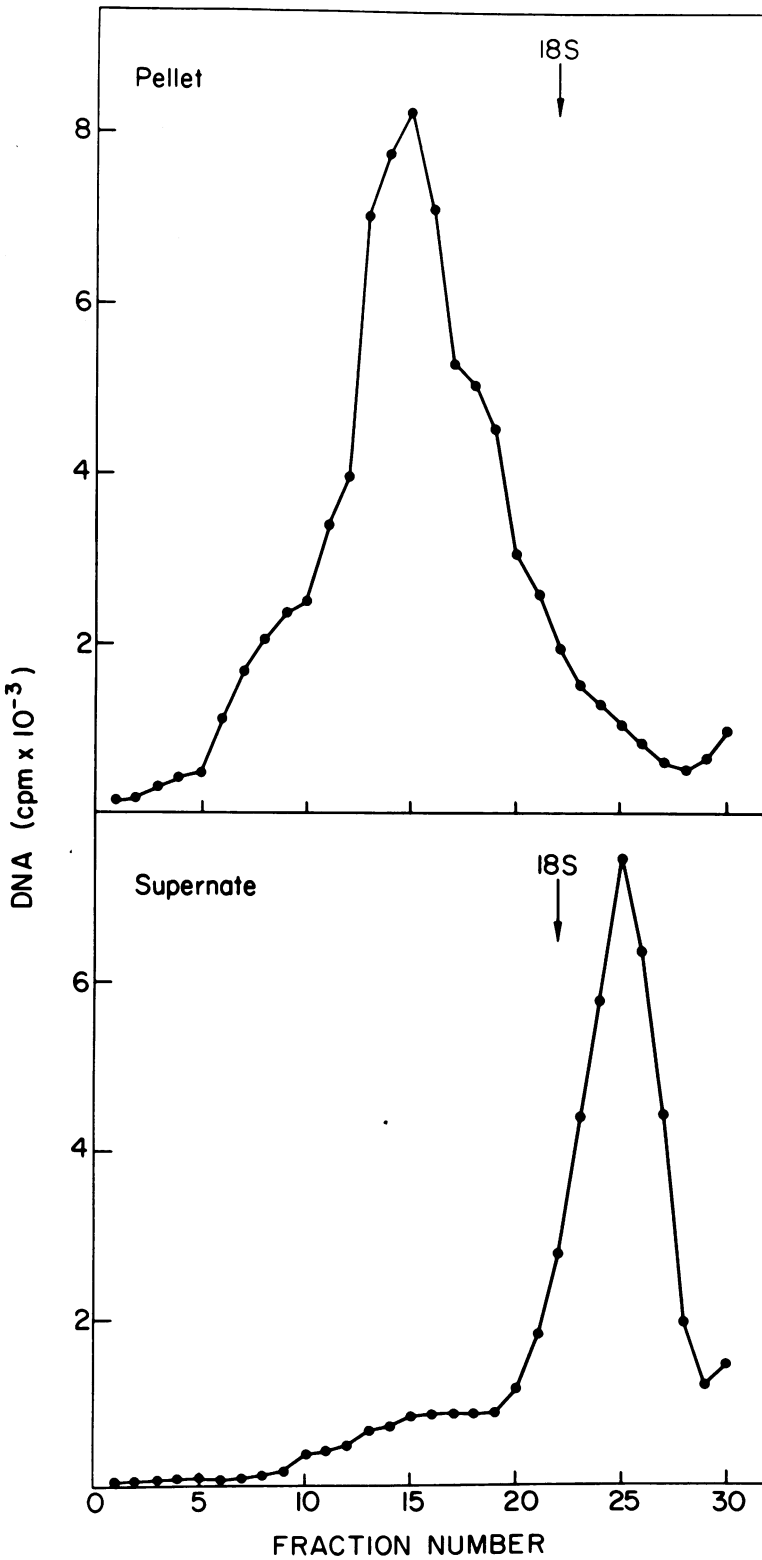

FIG. 1. *Alkaline sucrose velocity sedimentation of DNA isolated from RSV-infected CEF by Hirt fractionation. CEF were labeled with* [^{3}H]*thymidine (2 μCi/ml) for 8 h, the label was chased for 10 h in cold medium, and the cells were subjected to Hirt fractionation as described under Table 2. The DNA from the pellet or the supernatant fraction was gently extracted and treated with RNase A (50 μg/ml) and RNase* T_1 *(50 units/ml) in 1× SSC for 30 min. After re-extraction, the DNA was dialyzed in 0.1× SSC and run on a 5 to 40% alkaline sucrose gradient (0.5 M NaCl and 0.3 N NaOH) at 82,500 × g for 16 h. φX-174 DNA (18 S) was used as a marker.*

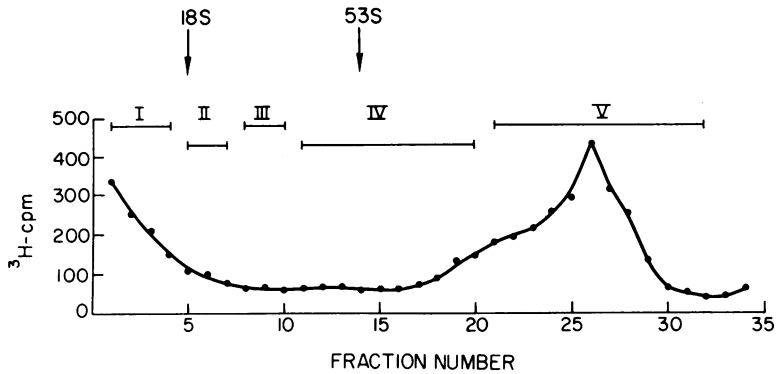

FIG. 2. *Alkaline velocity sedimentation of DNA from RSV-infected CEF lysed directly on top of gradient. Tertiary cultures of CEF were infected with PR-RSV(A) and 40 h later were labeled with [^{3}H]thymidine (2 μCi/ml) for 8 h. The label was chased for 12 h in cold medium, and the cells were harvested 60 h postinfection. After washing three times with PBS, 2 to 3 × 10^6 cells were allowed to lyse for 12 h at 4 C in 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.5 NaOH, and 0.8 M EDTA on top of a 32-ml 10 to 30% alkaline linear sucrose gradient (0.3 N NaOH, 0.5 M NaCl, 0.01 M EDTA, and 0.05% sodium deoxycholate). The gradients were then centrifuged at 25,000 rpm for 6 h in a Beckman SW-27 rotor at 4 C, and DNA was collected (30 to 35 fractions) from the top of the gradient by pumping 70% sucrose into the bottom of each tube. Each fraction was neutralized with 1 N HCl and diluted 1:1 with water. Each gradient was divided into five fractions according to the sedimentation velocity of cell DNA by using SV-40 DNA (forms I and II) as a marker. Similar fractions from 90 such gradients were pooled and ethanol precipitated after adding 5 μg of yeast RNA per ml as a carrier. The approximate range of sedimentation coefficients for each fraction are: fraction I, <18S; fraction II, 18 to 28S; fraction III, 28 to 40S; fraction IV, 40 to 80S; fraction V, 80 to 122S.*

TABLE 6. *Alkaline sucrose velocity sedimentation of virus-specific DNA in CEF 60 h after infection with PR-RSV(A)*[a]

Fraction	Counts per min of [^{3}H]DNA × 10^{-3}			Counts per min of [^{32}P]RNA hybridized		Newly synthesized vDNA	
	Per fraction[b]	Per filter[c]	Per μg of DNA	Per filter	Per 100 μg of DNA	Counts per min per fraction[d]	%
Uninfected whole cells	ND	ND	ND	508 ± 44[e]	2,133 ± 353		
RSV-infected whole cells	2,190	236.6 ± 4.1	10.5	1,022 ± 102	4,221 ± 376		
I (<18S)	303	35.8 ± 2.5	12.2	102 ± 7	3,273 ± 35	283	9.0
II (18-28S)	178	27.3 ± 9.5	10.6	190 ± 74	7,324 ± 621	872	27.6
III (28-40S)	179	28.6 ± 1.3	12.4	130 ± 40	5,331 ± 608	462	14.6
IV (40-80S)	410	47.1 ± 6.5	12.4	106 ± 20	2,780 ± 380	214	6.8
V (80-122S)	2,383	154.5 ± 7.1	14.2	339 ± 14	2,925 ± 232	1,329	42.1

[a] CEF were infected with PR-RSV(A), labeled with [^{3}H]thymidine, and fractionated by alkaline sucrose velocity sedimentation as described under Fig. 2. The DNA from various fractions was extracted, processed, and hybridized with ^{32}P-labeled 70S AMV RNA. After hybridization the amount of DNA attached to each filter was determined by the Burton diphenylamine reaction.

[b] The total ^{3}H counts per minute per fraction were determined before hybridization.

[c] Mean of two to five filters ± standard deviation. The ^{3}H counts per minute per filter were counted after hybridization.

[d] (Column 6-2133) × (column 2/column 4) × (1/100).

[e] Mean of two to five filters ± standard deviation. An average background of 131 counts/min of [^{32}P]RNA hybridized to mouse DNA filters has been subtracted from counts per minute of [^{32}P]RNA hybridized per filter.

vDNA directed vDNA synthesis. It appears, however, that de novo proviral DNA synthesis only occurs during a limited time (48 h) period after infection. The newly synthesized DNA is then either integrated or diluted by cellular multiplication and perhaps degraded.

Newly synthesized vDNA appeared in the Hirt pellet with the bulk of cellular DNA within

24 h after infection. It is conceivable, however, that the earlier integration of small amounts of vDNA may have escaped detection. Our results support the recent report of Markham and Baluda (13) demonstrating the integration of vDNA in uninfected cells and in AMV induced leukemic cells analyzed several weeks after infection with AMV. These authors also noted the absence of free vDNA in uninfected cells and in leukemic cells transformed for a relatively long time. Additional support for the integration of oncornavirus DNA into host cell DNA comes from the studies of Balduzzi (2), Gelb et al. (8), and Varmus et al. (21), who arrived at the same conclusion using different experimental approaches.

Approximately 50% of the total vDNA synthesized after infection seems to be integrated. Also, different embryos from the same chicken species contain a similar average cellular amount of vertically transmitted vDNA sequences and after AMV infection most leukemic cells contain approximately the same average number of vDNA copies. These facts suggest that there may be a limited number of integration sites. Another explanation for limited integration could be that only part of the total vDNA synthesized is suitable for, or capable of, integration. This includes the possibility of uneven and disproportionate intracellular transcription of viral RNA into DNA resulting in the accumulation of excessive amounts of some part(s) of the viral genome.

Although it is difficult to estimate the size of the vDNA transcripts from the data presented, partly because of the low resolution in the alkaline gradients, the present findings are compatible with the hypothesis that vDNA is synthesized as individual transcripts of the 35*S* viral RNA subunit. Covalent linkage of several molecules or circularization of a single molecule preceding integration, however, is possible and may be the reason for the sedimentation of some vDNA at higher *S* values.

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