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Host Induced Alteration of Avian Sarcoma Virus B-77 Genome

(RNA·DNA hybridization/viral RNA/recombination/host modification/viral integration)

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ABSTRACT **The genome of an avian oncornavirus was altered after infection of a heterologous host. This was studied with avian sarcoma virus B-77 in duck embryonic fibroblasts (DEF) and chicken embryonic fibroblasts (CEF). To detect alteration of the viral genome, we hybridized 35S B-77 RNA with normal duck DNA by either one of two techniques: when viral RNA was in excess and when DNA was in excess. The RNA of B-77 passaged only in gs$^-$ chf$^-$ CEF does not have homology with duck DNA. However, after several passages of B-77 through DEF the viral genome acquired duck specific RNA sequences. After 4 and 10 passages, B-77 RNA acquired 2.2 and 6.6%, respectively, complementarity to normal duck DNA. The duck specific RNA sequences were found to be covalently linked to the B-77 RNA genome. Also, the host specific sequences acquired by the virus appear to be from a region of the duck DNA which is repeated four to six times per cell. After 5 back passages in CEF some of the duck specific RNA sequences in the viral genome were lost.**

Avian oncornaviruses have the ability to be modified by the host through which they are passaged. Even Rous sarcoma virus (RSV) may have had to be adapted to induce tumors and replicate in chickens (1). After the isolation of chicken tumor no. 1 by Rous in 1911, the tumor cells could cause sarcomas only in chickens of the same strain as that in which the tumor originated and only by inoculation of large numbers of tumor cells. Subsequently, tumors could be induced in other strains of chickens but only after several more passages could sarcomas be induced by a cell-free filtrate. After more than 60 years of laboratory manipulations, RSV can now rapidly induce tumors in a wide host range including primates (2, 3). Duran-Reynals reported that after several passages in ducks, a duck adapted variant of RSV appeared (4). In addition to the ability to cause tumors in ducks, the duck-adapted virus had acquired new tissue affinities causing tumors in bone, skin, and digestive tract, whereas the original RSV did not. Furthermore, the duck-adapted virus had altered surface antigenic properties. A similar phenomenon has also been noted with RSV that has been rescued from transformed rat cells (5–12). These findings led Altaner and Temin (6) to suggest that the alteration of physical and biological properties in the virus may reflect recombination between viral and host genetic material. Host-induced alterations of murine sarcoma viruses have also been reported after passage in rat cells (13). Kirsten and Harvey sarcoma viruses appear to contain murine and rat oncornavirus specific RNA sequences but these sequences have not been shown to be covalently linked. Also, in those studies the DNA probe synthesized *in vitro* with reverse transcriptase may have contained cellular as well as viral sequences since oncornaviruses contain cellular messenger RNA and small-molecular-weight RNA species associated with the 60–70S RNA species (14).

The present study was undertaken to determine whether duck genetic information is acquired by B-77 avian sarcoma virus (ASV) produced in duck cells and whether the duck specific sequences are covalently linked to the viral genome. The helper independent B-77 ASV was isolated from a spontaneous sarcoma in the liver of a chicken and had been maintained by passage in chicken cells (15). Duck embryo fibroblasts (DEF) support the replication of B-77 virus and are efficiently transformed *in vitro*. In addition, noninfected duck cells do not contain DNA sequences which are homologous with any known chicken oncornavirus (ref. 16 and our unpublished findings). To eliminate the possible contamination of the viral RNA probe used in DNA·RNA hybridization by cellular RNA species, we used only purified 35S viral RNA. Our findings indicate that the 35S RNA from B-77 virus replicated in chicken cells contain very little, if any, homology with normal duck DNA, whereas virus passaged several times through duck embryo fibroblasts acquires duck genetic information which is covalently linked to the viral genome. In addition, after 5 back passages through chicken cells, B-77 virus seemed to have lost some of the duck specific sequences acquired through 10 passages in duck cells.

MATERIALS AND METHODS

Cells. White Leghorn cross K-137 fertile chicken eggs were purchased from Kimber Farms, Pomona, Calif. White Leghorn SPF-K-137 fertile chicken eggs free of infection by subgroups A and B of avian leukosis viruses were purchased from Kimber Farms, Niles, Calif. Fertile chicken eggs (C/E) were obtained from SPAFAS, Inc., Storrs, Conn. Individual embryos were identified for the status of group specific (gs) antigen and chicken helper factor (chf) by Prof. H. Hanafusa, the Rockefeller University, according to methods described previously (8), and the embryos used in this study were negative for both gs and chf. Fertile duck eggs were supplied by Ward Duck Farm, LaPuente, Calif. Preparation of primary and secondary cultures from avian embryos and of culture media followed published procedures (17) except that for the culture of DEF, heat-treated chicken serum was replaced by heat-treated fetal calf serum. All cultures were tested for avian oncornavirus production and only nonproducer cells were used (18).

Abbreviations: DEF, duck embryo fibroblasts; CEF, chicken embryo fibroblasts; RSV, Rous sarcoma virus; AMV, avian myeloblastosis virus; C_0t, nucleotide concentration (mol/l) × time (sec); TCA, trichloroacetic acid.

TABLE 1. *Filter hybridization of 35S ^{3}H-labeled B-77 RNA with duck or chicken DNAs*

Exp.	Viral RNA: Host cells[a]	Viral RNA: No. of passages	Viral RNA: Specific activity (cpm/μg)	cpm hybridized/100 μg of DNA from: Leukemic chicken myeloblasts	K-137 chicken embryos[b]	SPF K-137 chicken embryos[c]	Duck embryos
1	CEF	4	1.8×10^6	2414 ± 26[d]	379 ± 33	N.D.	11 ± 4 (6.1 ± 2.2 pg)[e]
	DEF	4	2.0×10^6	2864 ± 161	436 ± 7	N.D.	87 ± 3 (43.8 ± 1.5 pg)
2	CEF	10	1.1×10^6	971 ± 36	N.D.	654 ± 20	0 (0)
	DEF	10	8.3×10^5	836 ± 127	N.D.	309 ± 38	110 ± 11 (132.5 ± 13.3 pg)
	CEF	5 (after 10 passages in DEF)	1.1×10^6	1155 ± 43	N.D.	411 ± 36	48 ± 14 (42.9 ± 12.5 pg)

Hybridization was carried out as described earlier. 4.5×10^5 cpm of ^{3}H-labeled viral 35S RNA per ml was hybridized at 70° for 10 hr. Four nitrocellulose filters were used. Counts hybridized with mouse DNA were subtracted as nonspecifically-bound RNA. After hybridization each filter contained 40–60 μg of DNA. N.D., not determined.

[a] All CEF cultures were from gs^- chf^- embryos. All DEF cultures were tested for absence of oncornavirus production.

[b] K-137 chicken embryos from Kimber Farms, Pomona, Calif. tested for absence of oncornavirus production.

[c] SPF-K-137 embryos from Kimber Farms, Niles, Calif. tested for absence of oncornavirus production.

[d] Mean of four filters ± SD.

[e] Numbers in parentheses are the amounts in picograms of viral RNA hybridized/100 μg of duck DNA.

Virus. B-77 (Bratislava) strain of avian sarcoma virus subgroup C passaged only through chicken cells was used.

Infection and Passage of Virus through CEF and DEF. Secondary cultures were prepared by plating approximately 4×10^6 cells per 100 mm Falcon plastic dish. They were infected within 4 hr after plating with 1 ml of undiluted supernate (unknown virus titer) from transformed cells. When nearly all the cells were morphologically transformed and were virus producers, within 6–7 days after three cell subcultures, the supernate from these cells was used to infect other secondary, or later passage, CEF and DEF.

Nucleic Acids. The isolation of cellular RNA and DNA, the isolation of 35S[^{3}H]RNA from purified virions labeled with [^{3}H]uridine and [^{3}H]cytidine, and the hybridization of viral RNA with cellular DNA either on filters when RNA was in excess or in liquid when DNA was in excess, and the separation of single- and double-stranded nucleic acids by hydroxylapatite chromatography followed published procedures (19–22). Modifications, if any, are described in footnotes and figure legends.

RESULTS

Filter hybridization of 35S [^{3}H]RNA from B-77 produced in CEF or DEF

The 35S [^{3}H]RNA from B-77 which had been passaged 4 or 10 times through CEF or DEF was hybridized to denatured avian DNAs immobilized on cellulose nitrate filters (Table 1). DNA from leukemic chicken myeloblasts transformed by avian myeloblastosis virus (AMV) contained more B-77 specific DNA sequences than DNA from normal chicken cells. This results from the homology that exists between B-77 RNA and RNA from AMV or chicken endogenous virus (19). The virus grown in CEF hybridizes very little or not at all with normal duck DNA. Similar results, obtained with 35S RNA from AMV, RAV-0, RAV-2, RAV-60, and RAV-61 (data not shown), demonstrated the absence of these oncornavirus specific DNA sequences in normal duck DNA. However, virus passaged through DEF acquires some duck specific sequences in its 35S RNA. The duck specific RNA sequences in viral RNA appear to be three times more numerous in passage 10 virus than in passage 4 virus. A 3-fold reduction in these duck RNA sequences is observed when duck passage 10 virus is back-passaged five times through CEF. Also, even virus passaged 10 times in duck cells hybridizes with leukemic or normal chicken DNA as well as chicken-passaged virus does. This indicates that the genome of the duck-passaged virus still retains most of its original nucleotide sequences, as will be better demonstrated in the next section.

Liquid hybridization of 35S viral RNA in DNA excess

The previous filter hybridization studies suggested the acquisition of RNA sequences complementary to duck DNA after passaging B-77 virus in DEF. In order to learn more about the nature of the acquired duck sequences and their proportion in viral RNA, we hybridized 35S B-77 RNA with an excess of chicken or duck DNA (Fig. 1 and Table 2). B-77 duck passaged 4RNA hybridizes about 3% with normal duck DNA, while maximum hybridization obtained with passage 10 viral RNA was found to be about 6.6% at C_0t 15,000 mole·sec·liter^{-1} (C_0t: nucleotide concentration in moles/liter × times in seconds). With virus passaged 10 times through DEF followed by 5 passages through CEF, 3.4% of the viral RNA was made RNase-resistant at C_0t 15,000. As observed with the filter hybridization in viral RNA excess, there is no significant hybridization between normal duck DNA and 35S B-77 RNA passaged in chicken cells. We also failed to detect any hybridization between duck DNA when DNA was in excess and 35S RNA from AMV, RAV-0, RAV-2, RAV-60, or RAV-61 (data not shown). Under similar conditions, 30–40% of the 35S RNA from B-77 passaged four times through CEF or DEF hybridized with DNA from normal chicken cells or DNA from leukemic myeloblasts with almost identical kinetics of hybridization (Fig. 2). However, 70–75% of the 35S B-77 RNA hybridized with DNA from DEF transformed by B-77 virus at C_0t 25,000 (M. Dastoor, M. Shoyab, and M.

TABLE 2. *Liquid hybridization of 35S B-77 RNA to normal duck DNA when the DNA is in excess*

Exp.	Host cells[a]	No. of passages	Maximal percentage of input [^{3}H]RNA hybridized
1	CEF	4	0.74 ± 0.29[b]
	DEF	4	2.16 ± 0.64[b]
	DEF	4 (prehybridized with chicken DNA)[c]	4.57 ± 0.21[d]
2[e]	CEF	10	0.12 ± 0.15
	DEF	10	6.6
	CEF	5 (after 10 passages in DEF)	3.4

The hybridization conditions are described in legend of Fig. 1.

[a] All the CEF were from gs$^-$ chf$^-$ chicken embryos and all the DEF were negative for oncornavirus production.

[b] Mean of five maximal values ± SD between C_0t 10^3 and 2.3×10^4.

[c] 35S RNA from B-77 virus (4 passages in DEF) was enriched for duck RNA sequences by prehybridization in excess of leukemic and normal chicken DNAs which hybridize with approximately 50% of the B-77 viral RNA sequences.

[d] Mean of two determinations at C_0t 10^3 and 10^4.

[e] Data are the same as those shown in Fig. 1.

Baluda, data unpublished). Therefore, it appears that the duck-specific sequences acquired by B-77 35S RNA after 10 passages in DEF make up approximately 7% of the viral RNA. These data also show that B-77 viral RNA has about 50–60% homology with the DNA sequences from chicken endogenous oncornavirus or from AMV (Fig. 2A) and that the extent of this homology is not noticeably altered by passage through duck cells (Fig. 2B). The kinetics of hybridization of 35S RNA from B-77 passaged through DEF gives a $C_0t_{1/2}$ of hybridization of 1400–1800 mole·sec·liter^{-1} for all three types of viral RNA hybridizing with normal duck DNA (Fig. 1). This $C_0t_{1/2}$ corresponds to an amount of DNA represented approximately four to six times per diploid duck genome if compared with the $C_0t_{1/2}$ of *Escherichia coli* cRNA hybridized with *Escherichia coli* DNA as reported previously (22).

Covalent linkage between acquired duck RNA sequences and viral genome

The hybridization data presented in the preceding paragraphs could be explained in several different ways: (*i*) the passage of B-77 virus in duck cells may induce the synthesis of some duck endogenous virus, (*ii*) the infecting B-77 virus inoculum may contain another virus having RNA homologous to duck DNA which has a selective replication advantage in duck cells, or (*iii*) there may be a true recombination phenomenon between duck nucleic acid sequences and the B-77 genome either in DNA or RNA form. To discriminate between these different possibilities we designed the following experiment.

Regardless of size, nucleic acid molecules containing a double-stranded segment of at least 50 nucleotide pairs behave as completely double-stranded molecules during hydroxylapatite column chromatography (23). Therefore, unsonicated 35S [^{3}H]RNA from virus passaged through DEF was hybridized with an excess of normal sonicated duck DNA (6S fragments), and the mixture was subjected to hydroxylapatite fractionation as described earlier (21). If hypotheses *i* and *ii* were correct, only a small fraction of the viral RNA would be eluted in the double-stranded fraction, whereas if hypothesis

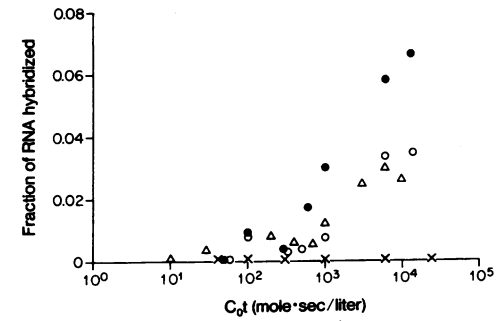

FIG. 1. Kinetics of hybridization in duck DNA excess of 35S [^{3}H]RNA from B-77 virus passaged in CEF or DEF. The hybridization mixture contained 4 mg/ml of sonicated duck DNA (6.4 S), 2000 cpm/ml of sonicated 35S viral RNA (8–10 S) and 0.1% sodium dodecyl sulfate in 0.4 M phosphate buffer (pH 6.8). The hybridization was carried out as previously described (22). Samples of 0.5 ml were taken at different time intervals and diluted with cold water. One-half of each sample was then treated with pancreatic and T_1 ribonucleases to determine the fraction of viral RNA rendered ribonuclease-resistant, as described earlier. The specific activity of the RNA is given in Table 1. ●, RNA from B-77 ASV passaged 10 times through DEF; Δ, RNA from B-77 ASV passaged four times through DEF; O, RNA from B-77 ASV first passaged 10 times through DEF and then passaged five times in CEF; ×, RNA from B-77 ASV passaged through CEF.

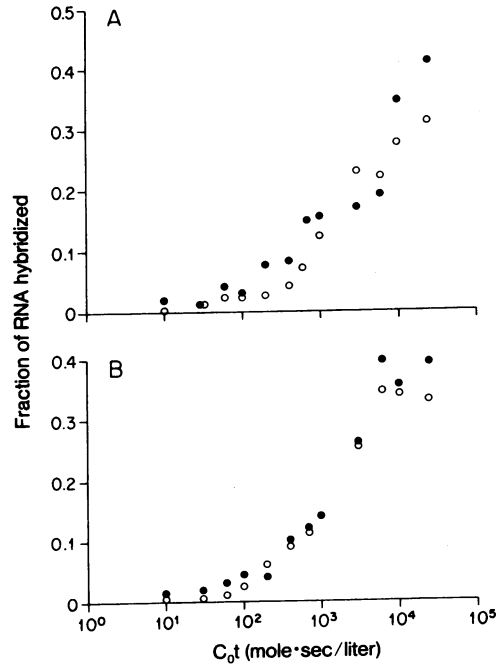

FIG. 2. Kinetics of hybridization in normal or leukemic chicken DNA excess of 35S [^{3}H]RNA from B-77 ASV passaged in CEF or DEF. The conditions of liquid hybridization were the same as those described in Fig. 1 except that DNA from leukemic chicken myeloblasts and from normal gs-negative chick embryos were used and only 0.25 ml aliquots were used at each point. (A) RNA from B-77 ASV replicated only in CEF, (B) RNA from B-77 ASV passaged four times through DEF. ●, Leukemic DNA; O, gs-negative CEF DNA.

TABLE 3. *Hydroxylapatite chromatography of 35S B-77 RNA hybridized with normal duck DNA*

Exp.	Host cells[a]	No. of passages	Sonication[b]	TCA-precipitable [^{3}H]RNA[c] cpm: Single-stranded	In hybrids	% In hybrids
1	CEF	8	+	1580	102	6.1
	CEF	8	−	650	280	30.1
	DEF	8	+	613	78	11.3
	DEF	8	−	76	355	82.4
2	CEF	10	+	495	34	7.0
	CEF	10	−	694	52	6.4
	DEF	10	+	731	67	8.4
	DEF	10	−	370	995	72.9
3[d]	DEF	10	+	877	59	6.3
	DEF	10	−	772	138	15.2

Normal duck DNA (3 mg), 600–1600 cpm of trichloroacetic acid (TCA) precipitable 35S viral RNA in 0.5 ml of 0.5 M phosphate buffer (pH 6.8), containing 0.2% Sarkosyl were denatured for 3 min at 100° and hybridized at 65° for 20 hr. After hybridization, the mixture was diluted with cold distilled water containing 0.1% Sarkosyl to obtain a final phosphate concentration of 0.05 M. The mixture was applied to a column of hydroxylapatite (6 ml bed volume of DNA grade BioGel HTP from BioRad) at 60°, and the column was washed with 25 ml of 0.05 M phosphate buffer (pH 6.8), containing 0.1% Sarkosyl. Single-stranded and double-stranded nucleic acids were eluted with 0.15 and 0.4 M phosphate buffer containing 0.1% Sarkosyl as described (21).

[a] All CEF were gs$^-$ chf$^-$. All CEF and DEF were tested for absence of oncornavirus production.

[b] Five 1 min pulses giving an RNA size of 8–10 S.

[c] Viral RNA specific activity: Exp. 1: 1.1×10^6 cpm/μg for both CEF 8 and DEF 8 grown virus; Exp. 2: 1.1×10^6 cpm/μg for CEF 10 grown virus and 8.3×10^5 cpm/μg for the DEF 10 grown virus.

[d] Normal duck DNA was replaced by sonicated mouse DNA. After denaturation the reaction mixture was quenched and processed on hydroxylapatite as in Exps. 1 and 2.

iii were correct all the viral RNA should be in the double-stranded nucleic acid fraction. Furthermore, sonication of the viral RNA into fragments of 8–10 S would not affect the results if hypotheses *i* and *ii* were correct, but if hypothesis *iii* were correct, sonication of the viral RNA would greatly reduce its binding to hydroxylapatite. The results of two such experiments are presented in Table 3 which also includes control experiments with 35S RNA from B-77 virus passaged only through CEF. The data are consistent with hypothesis *iii* in that after hybridization with normal duck DNA when DNA was in excess, 73–82% of the unsonicated 35S RNA from virus passaged in DEF was present in the double-stranded fraction and sonication reduced this fraction to 8.4–11%. We did not get 100% binding in 0.15 M phosphate buffer probably due to experimental limitations since RNA undergoes thermal scission during long periods of hybridization, and a single break in an RNA molecule would reduce the fraction of RNA which could be bound to hydroxylapatite as part of an RNA·DNA hybrid. In control experiments with 35S RNA from virus replicated in CEF, only 7–30% of the unsonicated viral RNA and 6–7% of the sonicated viral RNA were present in the double-stranded nucleic acid fraction. We do not have an explanation in Exp. 1 for the relatively high percentage (30%) of unsonicated RNA from CEF replicated virus which is retained by hydroxylapatite in the double-stranded fraction. In another control experiment (Exp. 3), mouse DNA replaced duck DNA in the hybridization mixture that had been fractionated by hydroxylapatite chromatography directly after boiling and ice-cooling. Only 15% of the intact 35S RNA from duck-passaged B-77 virus eluted in the double-stranded fraction, whereas 6.3% of the sonicated viral RNA did so. This experiment indicates that the retention on hydroxylapatite of 73–82% of the unsonicated 35S RNA from duck-passaged virus in Exps. 1 and 2 is due to hybridization to duck DNA and not to intramolecular double-strandedness of the large RNA nor to nonspecific binding to duck DNA or to hydroxylapatite. The significant difference in hydroxylapatite binding between RNA from B-77 replicated in CEF and RNA from B-77 replicated in DEF, demonstrates the covalent linkage between the duck specific RNA sequences and viral RNA.

DISCUSSION

B-77 avian sarcoma virus produced in chicken cells can cause transformation and replicates in duck embryonic fibroblasts. After passage through DEF, the virus retains most of its original genome but acquires some genetic information of duck origin which is covalently linked to the viral RNA. The duck specific nucleotide sequences acquired by the viral genome appear to arise from a region of DNA which is reiterated four to six times per diploid duck cell. This suggests that at least one end of the proviral DNA is integrated into that region of the host genome which represents either duck DNA or endogenous viral DNA. These results demonstrate indirectly that avian oncornaviruses integrate their proviral DNA into host cell DNA as shown earlier (16, 24) and are consistent with an integration unit size which corresponds to a viral RNA molecule of 3×10^6 daltons (25, 26). The present findings could result if a DNA-dependent RNA polymerase copies cellular DNA sequences which are adjacent to the integrated B-77 DNA during the transcription of viral RNA. These sequences could remain as an integral part of the viral genome in matured virions. However, there are other possible mechanisms for this recombination: (*i*) it could take place at the DNA level between proviral DNA and a DNA copy of some duck messenger RNA before integration, since reverse transcriptase can efficiently copy mRNA (27–29), or (*ii*) it could even take place at the RNA level since RNA ligases have been detected in mammalian cells (30).

The acquired duck sequences which are either from the host or from an endogenous virus may be responsible for modification of the physical and biological properties of the virion as reported by Duran-Reynals (4). Host-induced alterations in viral biological properties have also been noted in other oncornaviruses replicated in heterologous hosts (6, 13, 31, 34). The duck specific sequences in passage 10 virus could code for a polypeptide of approximately 21,000 daltons since they make up 6.6% of the viral genome and the base sequence complexity of the avian oncornaviruses genome is about 3.3×10^6 daltons (see ref. 32 for references).

The genetic alteration of RNA tumor viruses by acquisition of host cell genetic information or by recombination with an integrated endogenous virus might be involved in tumor virus formation and in information transfer from cell-to-cell (33). This phenomenon may also provide a pathway for the evolu-

tion of oncornaviruses from a common primordial ancestor. Duran-Reynals had demonstrated the possibility of causing experimental tumors not observed under natural conditions by infection of heterologous hosts (4). For instance, it might be possible to generate an oncornavirus containing human specific RNA linked covalently with the genome of exogenous non-human oncornaviruses produced in human cells. Such laboratory-born recombinants might give false-positive tests in the detection of putative human oncornaviruses.

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