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High Spontaneous Mutation Rate of an Avian Sarcoma Virus

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Three genetically distinct types of chicken sarcoma virus Bratislava 77 (B77 virus) differing in their ability to infect duck cells were identified. B77 virus type I does not infect duck cells; B77 virus type II has a low efficiency of infection of duck cells; and B77 virus type III has a high efficiency of infection of duck cells. B77 viruses type I and III are produced by spontaneous mutation during the growth of B77 virus type II in chicken cells. The spontaneous mutation of B77 virus type II to B77 virus type III occurs with a high rate (approximately 1 mutation per 50 infected cell generations), requires cell replication, and neither occurs during the synthesis of viral DNA on an RNA template nor during the transcription of progeny viral RNA from the provirus. The rate of spontaneous mutation of B77 virus type II to B77 virus type I is greater than the rate of spontaneous mutation of B77 virus type II to B77 virus type III.

There is extensive genetic diversity among the avian and murine leukemia and sarcoma viruses. Genetic diversity occurs in virion envelope glycoproteins, internal proteins, and DNA polymerase. Genetic diversity has also been observed in the ability of these viruses to infect heterologous cells, to replicate in permissive cells, to establish and maintain transformation in fibroblast cells or stem cells of the reticuloendothelial system, to form neoplastic tumors, and in the types of tumors formed (30).

The frequency of appearance of this genetic diversity has been quantitatively studied in only a few cases. Clones of Rous sarcoma virus (RSV) which caused round transformed cells frequently spontaneously mutated to RSV which produced fusiform transformed cells (26). Spontaneous temperature-sensitive mutations were observed in several Schmidt-Ruppin RSV (SR-RSV) clones, and the mutants were thermolabile in at least three different characteristics (unpublished data; 29). These spontaneous temperature-sensitive SR-RSV mutants were very unstable and had a high frequency of reversion (unpublished data). Cloned stocks of helper-independent sarcoma viruses spontaneously gave rise to nontransforming viruses with a very high frequency (16, 18, 36, 38). Spontaneous mutations in the virion RNA-directed DNA polymerase and the virion envelope glycoprotein of SR-RSV were found with a high frequency (17). These genetic studies indicated that there is a high frequency of spontaneous variation in avian and murine leukemia and sarcoma viruses.

In the present study, the rate of spontaneous variation in the host range of chicken sarcoma virus of the Bratislava 77 strain (B77 virus) and the phase of the replicative cycle of B77 virus in which these host range variants arose were determined.

MATERIALS AND METHODS

Cell cultures. Cells were propagated in Temin-modified Eagle minimal essential medium (Schwarz/Mann, Orangeburg, N.J.) containing 20% tryptose phosphate broth and supplemented with 2% fetal bovine and 2% calf sera (referred to as complete medium). Fertile chicken eggs were obtained from SPAFAS, Storrs, Conn., and primary cultures of fibroblasts were prepared from 12-day-old embryos by standard techniques (27, 28). The chicken embryos were C/E and were negative for avian leukosis virus, chick helper factor (13, 39), and group-specific antigen of avian leukosis virus (22).

Fertile Muscovy duck (*Cairina moschata*) eggs were obtained from W. Thrun, Madison, Wis., and fertile Peking duck (*Anas platyrhynchos*) eggs were obtained from Abendrath Duck Hatchery, Waterloo, Wis. Fertile eggs of Chinese ring-neck pheasants (*Phasianus colchicus*) were obtained from the Poynette Game Farm, Wisconsin Department of Natural Resources, Poynette, Wis. Fertile eggs of Japanese quail (*Coturnix coturnix* var. *japonica*) were obtained from the Department of Poultry Science, University of Wisconsin, Madison, Wis. Fertile eggs of the Orlopp turkey (*Meleagris galloparvo*) were obtained from Wilmar Poultry Co., Wilmar, Minn. Primary cultures of fibroblasts were prepared from 14- to 15-day-old duck embryos, 10-day-old pheasant embryos, 7- to 9-day-old quail embryos, and 12- to 13-day-old turkey embryos. All duck, quail, turkey, and pheasant cells used were negative for both infec-

tious and noninfectious avian leukosis virus (when assayed for sedimentable DNA polymerase activity as described by Temin and Kassner [32]) and for avian leukosis virus group-specific antigens.

After 1 week of incubation the primary cells were either frozen in liquid nitrogen in complete medium containing 10% dimethyl sulfoxide (7) or transferred once. Secondary or subsequent cultures containing 6 $\times 10^5$ cells were prepared in 60-mm plastic petri dishes.

The Osborne-Mendel rat kidney cell line NRK was obtained from K. Somers, Baylor College of Medicine, Houston, Tex., who had obtained these cells from Duc-Nguyen et al. (8).

Viruses, cloning, and focus assays. B77 virus was previously described (1). The virus had originally been obtained from J. Smida, Cancer Research Institute, Bratislava, Czechoslovakia (24). It was isolated from a fibrosarcoma which spontaneously appeared in the liver of a white Leghorn chicken (34). B77 virus has been propagated in our laboratory only at low multiplicities of infection (<0.01 focus-forming units [FFU] per cell) in chicken cells, and a single clone of B77 virus was used in all the experiments in this paper (1).

B77 virus was recloned in chicken cells under agar by the following technique. Cultures of chicken embryo fibroblasts were inoculated with serial dilutions of virus, and after absorption the cells were overlaid with 5 ml of complete medium containing 0.4% agar. The cultures were fed 3 days after infection with 2 ml of complete medium containing 0.4% agar. Foci of morphologically transformed chicken cells were picked from cultures which had 10 or fewer foci with smallbore Pasteur pipettes, transferred to 0.5 ml of complete medium supplemented with 2 μg of polybrene per ml (35), and frozen (−70 C) and thawed (room temperature) five times in succession to lyse the cells and solubilize the agar plugs.

Virus stocks were prepared from these clones by inoculating cultures of chicken cells with dilutions of the virus in complete medium containing 2 μg of polybrene per ml. The medium from the infected cultures was replaced 4 to 5 days after infection, and the progeny B77 virus was harvested the next day. These clonal virus stocks were then centrifuged, stored frozen, and titrated (1). Titrations performed in all cultures of fowl cells were linear with respect to virus dilution. The standard error of an individual titer was 10% or less in replicate cultures of all the species of cells used.

The efficiency of transformation (EOT) of B77 virus was calculated from the ratio of the virus titer (FFU/ml) in cells of one species (for example, duck) divided by the virus titer (FFU/ml) in chicken cells.

Antiserum. Chicken antisera to purified B77 virus has been described previously (1). For neutralization studies the anti-B77 virus serum or normal chicken serum were incubated for 30 min at 55 C before use. Neutralizations were performed at room temperature for 1 h and terminated by diluting the reaction mixtures into complete medium containing 2 μg of polybrene per ml. The surviving virus was then titrated in chicken cells.

Interference assays. Chicken cells infected with Rous-associated virus (RAV-49), a subgroup C avian leukosis virus, were used to determine the subgroup of different clones of B77 virus (9, 37). The RAV-49 stock (1) was passaged two times at low multiplicities of infection in C/A chicken cells prior to use. Cultures of chicken cells were mock-infected or infected with RAV-49, passaged two times, and used to titrate B77 virus. (The chicken cells infected with RAV-49 were resistant only to focus formation by subgroup C chicken sarcoma viruses.)

RESULTS

Three types of B77 virus. (i) Transforming efficiency of clones of B77 virus grown in chicken and duck cells. B77 virus was recloned under agar in chicken cells as described above. Stocks titrating over 10^6 FFU/ml were prepared in chicken cells from 16 clones, and each stock was titrated in chicken and duck cells. All clones had at least a 400-fold lower efficiency of focus formation (referred to as EOT) in duck cells than in chicken cells (see Table 1).

The virus produced by the infected duck cells was examined to determine whether it had an efficiency of transformation different from the original virus grown in chicken cells. Virus was

TABLE 1. *Transforming efficiency of B77 virus clones grown in chicken and duck cells*[a]

Clone	Titer (FFU/ml) of virus grown in chicken cells assayed in:		Titer (FFU/ml) of virus grown in duck cells assayed in:	
	Chicken cells	Duck cells	Chicken cells	Duck cells
A	7.5×10^6	9.5×10^2	1.5×10^4	<5
B	1.0×10^7	6.0×10^2	6.4×10^4	<5
C	5.0×10^6	1.7×10^2	5.6×10^4	<5
D	2.4×10^6	5.0×10^0	5.0×10^0	<5
E	1.3×10^7	1.3×10^3	7.7×10^4	<5
F	4.5×10^6	1.4×10^2	5.5×10^3	<5
G	8.5×10^5	8.5×10^2	8.5×10^3	<5
H	2.3×10^6	5.0×10^2	3.2×10^4	<5
I	2.5×10^6	1.5×10^3	3.0×10^5	<5
J	7.5×10^6	8.8×10^2	7.2×10^4	<5
K	5.0×10^7	6.9×10^2	2.2×10^4	<5
L	2.6×10^6	3.8×10^3	6.4×10^4	1.9×10^4
M	4.2×10^6	9.5×10^2	7.5×10^5	1.5×10^5
N	1.0×10^7	2.2×10^4	5.5×10^5	1.3×10^5
O	1.0×10^7	5.0×10^3	1.0×10^6	8.7×10^4
P	1.0×10^7	3.9×10^3	2.1×10^6	1.4×10^5

[a] A stock of B77 virus was recloned under agar in chicken cells as described. Virus from 16 clones were grown in chicken cells to prepare stocks of high-titer virus, and the stocks were titrated in chicken and duck cells. Virus was harvested 7 days postinfection from cultures of duck cells inoculated with 0.2 ml of undiluted virus and was titrated in chicken and duck cells.

harvested from cultures of duck cells and was titrated in chicken and duck cells. Eleven out of sixteen clones of B77 virus listed in Table 1 (clones A through K) produced virus that formed foci in chicken cells but did not form any foci in duck cells; such viruses are referred to hereafter as type I B77 virus (B77 virus-I).

In two other reclonings of the original B77 virus stock at a 10-fold higher dilution, 10 additional B77 virus-I clones were obtained. The progeny of 6 of these 10 B77 virus clones grown in chicken cells initially did not form any foci (0 foci formed when 0.2 ml of an undiluted sample was titrated) in duck cells, but had high (up to 6.8×10^6 FFU/ml) focus-forming titers in chicken cells. The other 4 of the 10 clones were similar to B77 virus-I clones A to K (Table 1). Thus, B77 virus-I appeared to be present in the original stock at a high concentration.

In contrast to the B77 virus-I clones, the virus produced by infected duck cells from five clones of B77 virus tested in Table 1 (clones L through P) transformed duck cells with a high efficiency. After a single passage of these viruses in duck cells, the average EOT in duck cells increased from 10^{-3} to 2×10^{-1}. The virus with a high EOT in duck cells is referred to as type III B77 virus (B77 virus-III).

The viral clones (L through P, Table 1) which had a low initial transformation efficiency in duck cells and which gave rise to type III B77 virus after passage through duck cells are called type II B77 virus (B77 virus-II).

The genetic stability of these three types of B77 virus was then examined.

(ii) Stable difference between B77 virus-I and B77 virus-II in their ability to give rise to B77 virus-III. Four clones of B77 virus-II (clones L, M, N, and P from the experiment shown in Table 1) and four clones of B77 virus-I (clones A and E from the experiment shown in Table 1 and two clones of B77 virus-I not shown in Table 1) were passaged in chicken cells four successive times at weekly intervals to determine whether the difference in the ability to give rise to B77 virus-III between B77 virus-I and B77 virus-II was stable. (Table 2 shows representative titers in chicken and duck cells of two of these clones after passage.) None of the fourth chicken passage B77 virus-I clones gave rise to B77 virus-III. In contrast, every B77 virus-II clone gave rise to B77 virus-III. Therefore, B77 virus-I and B77 virus-II are stable genetic variants of B77 virus.

(iii) Stability of B77 virus-III to serial passage in chicken cells. To determine if B77 virus-III was a stable genetic variant of B77 virus, the EOT in duck cells of all five B77 virus-III clones shown in Table 1 (clones L through P after passage in duck cells) were tested during serial passage in chicken cells. As a control, each B77 virus-III clone was also passaged in duck cells. All of the B77 virus-III clones maintained their high EOT in duck cells during five successive serial passages in chicken cells (Fig. 1 and data not shown). There was only a 6- to 11-fold higher EOT of the B77 virus-III clones after serial passage in duck cells. Therefore, B77 virus-III is a stable genetic variant of B77 virus. The higher EOT of the B77 virus-III clones after passage in duck cells could be the result of adaptation by mutation and selection or recombination.

TABLE 2. *Stable genetic difference between B77 virus-I and B77 virus-II*[a]

Clone	Type	4th chicken passage titer (FFU/ml) in:		1st duck passage after the 4th chicken passage titer (FFU/ml) in:	
		Chicken cells	Duck cells	Chicken cells	Duck cells
E	I	3.5×10^7	3.7×10^2	2.8×10^4	<5
L	II	1.0×10^7	4.5×10^4	2.0×10^4	1.5×10^3

[a] B77 virus-I and B77 virus-II stocks from clones of virus grown in chicken cells and described in Table 1 were passaged four times in chicken cells at 5- to 6-day intervals with a 1,000-fold dilution of the virus at each passage and were titrated in chicken and duck cells. Virus was then harvested 7 days postinfection from duck cell cultures inoculated with 0.2 ml of undiluted fourth passage B77 virus and was titrated in chicken and duck cells.

(iv) Subgroup of B77 virus-I, B77 virus-II, and B77 virus-III. Clones of B77 virus-I, B77 virus-II, and B77 virus-III were tested by interference with RAV-49 and by antibody neutralization to determine whether they belonged to avian leukosis-sarcoma virus subgroup C, the subgroup of standard B77 virus and the B77 virus clonal stock used in these experiments (1). All three viruses were neutralized more than 95% by antiserum to B77 virus and were interfered more than 99% by RAV-49 (Table 3). Therefore, the three types of B77 virus belonged to subgroup C. The differences in the degree of neutralization of the clones of B77 virus-I, B77 virus-II, and B77 virus-III may reflect type-specific antigenic determinants (for review see 30). These results also confirm that there is no simple correlation between sarcoma virus envelope subgroup and efficiency of transformation of heterologous cells (1, 9).

(v) Characteristics of the infection of duck cells with B77 virus-I. Previous experiments (Table 1 and data not shown) have shown that

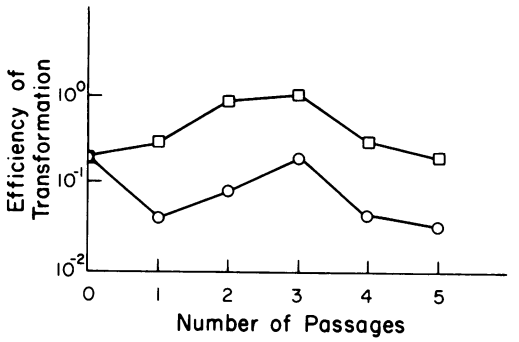

FIG. 1. *Stability of B77 virus-III to serial passage in chicken and duck cells. Cultures of chicken and duck cells were inoculated with dilutions of B77 virus-III (clone M after duck passage from the experiment described in Table 1) and overlaid with complete medium. Seven days after infection, virus was harvested from chicken and duck cultures containing approximately 100 to 200 foci. These viruses were passaged from chicken to chicken cells or from duck to duck cells, and the efficiency of transformation of duck cells was determined at each passage. Symbols: □, duck EOT of B77 virus-III passaged in duck cells; ○, duck EOT of B77 virus-III passaged in chicken cells.*

TABLE 3. *Subgroup of B77 virus-I, B77 virus-II, and B77 virus-III*

Index	B77 virus-I	B77 virus-II	B77 virus-III
Neutralization[a]	1×10^{-4}	2×10^{-2}	4×10^{-2}
Interference[b]	7×10^{-3}	6×10^{-3}	2×10^{-3}

[a] Clones of B77 virus-I, B77 virus-II, and B77 virus-III were grown in chicken cells, and the viruses were incubated with chicken antiserum to B77 virus or control chicken serum (0.3 ml of virus plus 0.2 ml of serum) and were diluted and titrated in chicken cells as described. The neutralization indexes represent the ratios of the titers obtained after the viruses were treated with antiserum to B77 virus to the titers after incubation with control serum. All control B77 virus titers were approximately 10^6 FFU/ml.

[b] Cultures of RAV-49-infected or mock-infected chicken cells were used for titration of B77 virus-I, B77 virus-II, and B77 virus-III (the same virus stocks used in *a*, above) as described. The interference indexes were calculated from the ratios of the virus titers in chicken cells infected with RAV-49 to those in mock-infected chicken cells.

B77 virus-I formed foci at very low efficiency or not at all in duck cells. To test the possibility that B77 virus-I was able to infect duck cells without causing transformation and production of progeny capable of forming foci, cultures of chicken and Muscovy duck cells were inoculated with a high-titer stock of a clone of B77 virus-I which did not form foci in duck cells. These cultures were tested for the production of nontransforming B77 virus (36) and for the production of viral particles containing either endogenous or exogenous DNA polymerase activity or viral RNA (14). Control cultures of chicken cells infected with B77 virus-I produced large amounts of progeny viral particles. In contrast, no evidence was obtained indicating that infectious nontransforming virus or noninfectious viral particles were released from duck cells exposed to B77 virus-I (data not shown). Furthermore, cultures of duck cells inoculated with a high-titer stock of B77 virus-I contained no detectable (<0.3 U/30 μg of protein) avian leukosis virus complement-fixing antigens. Parallel B77 virus-I-infected chicken cells contained over 100-fold more avian leukosis virus complement-fixing antigens (data not shown). Therefore, except for the phenotypic leakiness of some clones of B77 virus-I, we did not detect any expression of B77 virus-I in Muscovy duck cells.

High-titer clonal stocks of B77 virus-I also did not form foci (or only produced a few foci through phenotypic leakiness) in cultures of Peking duck cells. In contrast, B77 virus-I had a relatively high efficiency of plating in cultures of turkey cells (EOT = 10^0), ring-neck pheasant cells (EOT = 10^{-1}), and Japanese quail cells (EOT = 10^{-1}) (data not shown).

Origin of B77 virus-III from B77 virus-II. (i) Fluctuation tests. The change from B77 virus-II to B77 virus-III could occur (i) in chicken cells by mutation or (ii) in duck cells by adaptation. To distinguish between these two possibilities, fluctuation tests were performed (19). If mutation occurred randomly during the growth of B77 virus-II in chicken cells (hypothesis i), then some clones would contain many B77 virus-III, whereas others would contain few or no B77 virus-III; that is, the number of B77 virus-III in each clone of B77 virus-II would exhibit large fluctuations. In contrast, if the change in B77 virus-II occurred in duck cells (hypothesis ii), all of the B77 virus-II would be alike at the time they infected the duck cells, and each B77 virus-II would have an equal probability of changing to B77 virus-III.

Several cultures of chicken cells were infected at low multiplicities with serial dilutions of a high-titer stock of a clone of B77 virus-II (clone N from the experiment described in Table 1). Twenty-two foci were picked, and each virus clone was titrated. Table 4 shows the number of viruses in each clone that formed foci in chicken and duck cells. To determine whether the virus causing foci in duck cells was B77 virus-III, the efficiency of transformation of duck cells by the

TABLE 4. *Fluctuation test*[a]

No. of viruses forming foci		
Observed in chicken cells	Observed in duck cells	Expected in duck cells
40,000	640	1,076[b]
22,600	0	608
21,400	1,540	576
19,300	110	519
17,600	0	473
17,100	1	460
16,000	17	430
13,900	0	374
10,500	137	282
10,100	400	272
10,000	0	269
8,300	0	223
4,000	31	108
3,100	0	83
2,300	100	62
1,900	2	51
1,700	2	46
1,370	0	37
1,000	400	27
800	0	22

[a] Cultures of chicken cells were inoculated with 0.2 ml of serial 10-fold dilutions of B77 virus-II clone N (virus from the experiment described in Table 1), and, after absorption, the cells were overlaid with complete medium. On the following day, the medium was removed, medium containing 0.4% agar was added, and the cultures were fed 5 days later. Eight days after infection, foci were picked and stored as described. Each virus clone (0.5 ml in complete medium containing 2 μg of polybrene per ml) was serially diluted and titrated in chicken and duck cells undiluted or diluted 10- or 100-fold. All foci in duck cells were shown to be the result of primary infection in other experiments where the virus was titrated by two-fold serial dilutions going beyond the end point. Two clones which formed no foci in duck cells were found to be B77 virus-I and are not shown in this table.

[b] The expected values were calculated for each clone by multiplying the number of foci observed in chicken cells by the average of the EOT for each clone, 2.69 B77 virus-III per 100 B77 virus-II. (The EOT of clones which had no foci in duck cells were calculated on the basis of 1 duck-plating virus.)

progeny from those cultures of duck cells with foci was determined. In all cases, the progeny virus had an efficiency of transformation of approximately 10^{-1} in duck cells (data not shown).

There was no correlation between the number of viruses in a clone capable of plating in chicken and duck cells. The last column of Table 4 shows the number of viruses expected to transform duck cells assuming a uniform EOT. To determine if the distribution observed was statistically different from a Poisson distribution, the deviation of the observed number of foci from the expected number of foci was checked by a χ^2 test. The probability was less than 0.001 that the observed data fit a Poisson distribution. Therefore, the results are inconsistent with hypothesis (ii) of adaptation in which each B77 virus-II had an equal probability of changing to B77 virus-III in duck cells. This experiment was repeated four times with similar results (data not shown).

Experiments were performed to determine whether those clones which did not produce foci in duck cells (Table 4) were B77 virus-I. Aliquots (0.05 ml) of the eight viral clones shown in Table 4 which did not form foci in duck cells were diluted 10-fold and inoculated in cultures of chicken cells to prepare high-titer viral stocks. These stocks were titrated in chicken and duck cells. All of these eight clones had a duck EOT of approximately 10^{-3}, and the virus produced by the transformed duck cells had a duck EOT of approximately 10^{-1} (data not shown). Therefore, the clones of B77 virus-II which originally did not form foci in duck cells (Table 4) were not B77 virus-I.

However, two other viral clones present in the experiment described in Table 4 were B77 virus-I. These clones have been removed from the data shown in Table 4. (Data for these two clones are shown in Table 6 with other mutant subclones of B77 virus-II clone N.)

These findings permit an estimation of the rate of mutation of B77 virus-II to either B77 virus-I or to B77 virus-III (see Discussion).

(ii) Isolation of B77 virus-III from clones of B77 virus-II by indirect selection. If the genetic change in B77 virus-II could occur by a process of mutation in chicken cells, it should be possible to isolate B77 virus-III by indirect selection (5) of the sibs of B77 virus-III spontaneously arising in a clone of B77 virus-II.

From the fluctuation test shown in Table 4, B77 virus-II clones which had high percentages of B77 virus-III were selected (clones 15 and 19, Table 4). These clones were inoculated in cultures of chicken cells, and the cultures were overlaid with complete media containing 0.4% agar. The cultures were fed, 54 foci were picked, and 0.2-ml aliquots were titrated (diluted 10-fold or more) in chicken and duck cells. Eight clones which produced large numbers of foci in duck cells were used for selection. The viruses produced by the infected chicken cells were harvested to prepare high-titer stocks. These stocks were then titrated in chicken and duck cells. Table 5 shows the results of examining

TABLE 5. *Isolation of B77 virus-III from clones of B77 virus-II by indirect selection*[a]

Clone	Titer (FFU/ml) of virus grown in chicken cells when assayed in:		Clone genotype[b]
	Chicken cells	Duck cells	
N17-1	2.6×10^6	9.4×10^5	III
N17-2	1.8×10^6	7.1×10^5	III
N17-3	2.4×10^6	8.2×10^5	III
N17-4	6.7×10^5	5.3×10^5	III
N17-5	3.5×10^5	3.3×10^5	III
N17-6	4.0×10^5	3.4×10^5	III
N17-7	1.0×10^6	3.2×10^5	III
N17-8	5.0×10^5	2.1×10^5	III

[a] B77 virus from clone 15 in Table 4 which produced 2,300 foci in chicken cells and 100 foci in duck cells was inoculated in cultures of chicken cells, and the cultures were overlaid with complete media containing 0.4% agar. Fifty-four foci were picked, and aliquots were titrated in chicken and duck cells. Eight clones which produced large numbers of foci in duck cells (70 to 530 foci from a 0.2-ml undiluted aliquot of a 0.5-ml clone) were selected, and the virus was harvested from the parallel infected cultures of chicken cells to prepare high-titer stocks. These viral stocks, which had not been passaged in duck cells, were titrated in chicken and duck cells.

[b] Viral harvests were prepared 7 days after the infection of duck cells with 0.2 ml of a 100-fold dilution of the high-titer virus stocks and were titrated in chicken and duck cells. All viruses had a duck EOT of approximately 10^{-1} (data not shown) and, therefore, were B77 virus-III.

these eight viral subclones (virus from clone 15, Table 4) selected to contain large numbers of duck-plating viruses. Each clone (which had been grown in chicken cells only) had an efficiency of transformation of duck cells of approximately 10^{-1}, and the virus produced by the infected duck cells also had a duck EOT of approximately 10^{-1} (data not shown). B77 virus-III was also indirectly selected in chicken cells from subclones of clone 19 (Table 4). Therefore, B77 virus-III arose by spontaneous mutation during the growth of B77 virus-II in chicken cells.

Origin of B77 virus-I from B77V-II. B77 virus-III originates from B77 virus-II (Tables 1, 2, 4, and 5). It was observed in fluctuation tests (Table 4) that B77 virus-I could also originate from B77 virus-II. As a further test, B77 virus-II clone N (from the experiment described in Table 1) was passaged four times in chicken cells with 100-fold dilutions of the progeny virus at each passage. After the fourth serial passage, the virus was recloned in chicken cells under agar. A total of 116 clones were screened for their ability to form foci in duck cells. Several (11) viral clones that formed few or no foci in duck cells were selected, the original viral clones were propagated in chicken cells to prepare high-titer stocks, and these stocks were titrated in chicken and duck cells (Table 6). Six of these clones (N-2, N-4, N-5, N-6, N-7, N-9) did not form foci when initially assayed in duck cells but had high focus-forming titers in chicken cells; that is, they were B77 virus-I. Four other clones (N-1, N-3, N-8, N-10) initially formed a few foci in duck cells, but the progeny virus from the infected duck cells did not form foci in duck

TABLE 6. *Origin of B77 virus-I from B77 virus-II*[a]

Clone	Titer (FFU/ml) of virus grown in chicken cells when assayed in:		Clone genotype[b]
	Chicken cells	Duck cells	
N-1	2.0×10^6	2.9×10^2	I
N-2	1.5×10^6	<5	I
N-3	3.0×10^6	7.5×10^1	I
N-4	2.9×10^5	<5	I
N-5	9.5×10^6	<5	I
N-6	3.3×10^5	<5	I
N-7	2.0×10^6	<5	I
N-8	5.5×10^6	5	I
N-9	3.5×10^6	<5	I
N-10	2.5×10^6	5	I
N-11	3.6×10^7	1.6×10^4	II
N-12	4.2×10^6	6.5×10^1	I
N-13	1.0×10^7	<5	I

[a] Cultures of chicken cells were infected with B77 virus-II clone N (from the experiment described in Table 1), and the progeny virus was harvested 5 days later. This virus was passaged four times in chicken cells and then recloned in chicken cells under agar as described. A total of 116 viral clones were picked, and 0.2-ml portions were titrated in duck cells. Eleven clones (N-1 to N-11) which formed few or no foci in duck cells were selected, and 0.2 ml of fivefold dilutions of the original clones were inoculated in cultures of chicken cells to prepare high-titer stocks as described. These viral stocks were titrated in chicken and duck cells. Clones N-12 and N-13 originated from subclones of B77 virus-II clone N from the fluctuation test shown in Table 4. These viruses were not multiply passaged, and high-titer stocks of these clones were prepared in chicken cells and titrated in chicken and duck cells.

[b] Viral harvests were prepared 7 days after the infection of the duck cells with the high-titer stock viruses and were titrated in duck cells. Harvests from clones of B77 virus-I did not form foci in duck cells. Clones of B77 virus-II gave rise to B77 virus-III after passage in duck cells.

cells and, therefore, these clones were also B77 virus-I. Clones of B77 virus-II were also present (e.g., clone N-11) and gave rise to B77 virus-III (duck EOT = 10^{-1}) after passage in duck cells. Clones N-12 and N-13 are subclones of B77 virus-II clone N from the fluctuation test shown in Table 4 and are also B77 virus-I. Therefore, B77 virus-I spontaneously arose from B77 virus-II propagated in chicken cells.

Provirus mutation (i) Lag in the appearance of B77 virus-III. B77 virus replicates via a DNA intermediate (provirus) integrated into the host cell genome (for review see 30). To get a clonal distribution of mutants, a mutant provirus must be formed in chicken cells. For example, a mutant provirus could be formed when the parental B77 virus RNA is copied into DNA (RNA to DNA information transfer), when it becomes integrated into the host cell DNA, or when the integrated provirus is replicated with the host genome (DNA to DNA information transfer). Mutations could also occur during transcription of progeny viral RNA (DNA to RNA information transfer) with a mutant provirus formed in a second cycle of infection by this mutant viral RNA.

The time of appearance of B77 virus-III during the growth of B77 virus-II in chicken cells was studied to distinguish among these possibilities. A clone of B77 virus-II selected because it did not contain any B77 virus-III (virus from the experiment described in Table 4) was inoculated in chicken cells, and the progeny virus produced each day was titrated in chicken and duck cells (Fig. 2). There was a 2-day lag in the time of appearance of B77 virus-III compared with the time of appearance of progeny virus capable of forming foci in chicken cells. After this time, there was an exponential increase in the amount of B77 virus-III produced. If a mutant provirus had been formed soon after infection of chicken cells (for example, when the information in the parental B77 virus-II RNA was transferred into DNA), then B77 virus-III would have appeared in the first progeny. (The broken curve in Fig. 2 is a theoretical curve of B77 virus titer in duck cells calculated by multiplying the duck EOT observed in the day 5 viral harvest by the virus titer in chicken cells.) These results and similar results with four other clones of B77 virus-II (viruses also from the experiment described in Table 4) indicate that a mutant provirus is not formed early after infection.

(ii) Distribution of B77 virus-III in clones of chicken cells. Experiments were performed to distinguish between the possibilities that

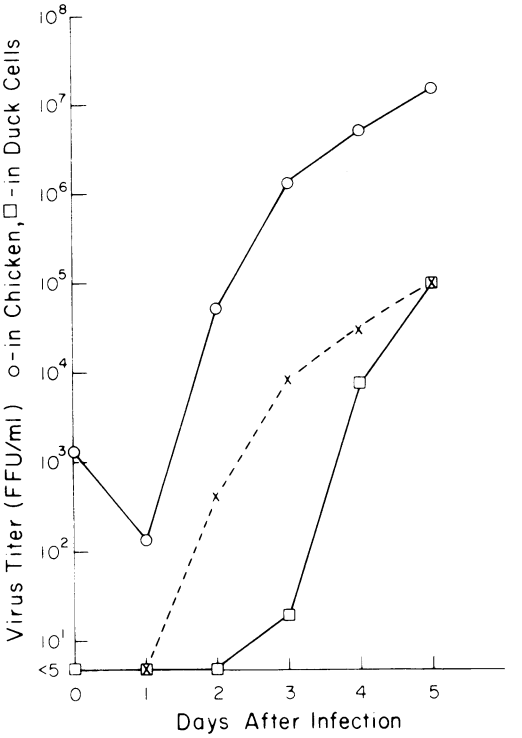

FIG. 2. *Lag in the time of appearance of B77 virus-III. Duplicate cultures of chicken cells were inoculated with a clone of B77 virus-II which did not contain any B77 virus-III (virus from the experiment described in Table 4) at a multiplicity of approximately 0.001 FFU per cell. After absorption, 2.0 ml of complete medium was added to each plate, and the virus was harvested every day by removing the medium and adding 2.0 ml of fresh complete medium. Each virus harvest (total pool volume of 4.0 ml) was titrated in chicken and duck cells. Symbols: ○, B77 virus titer (FFU/ml) in chicken cells; □, B77 virus titer (FFU/ml) in duck cells; ×, calculated B77 virus titer (FFU/ml) in duck cells. These titers were calculated by multiplying the B77 virus titer in chicken cells by the duck EOT of 6.0 × 10^{-3} observed with the day 5 viral harvest.*

mutant proviruses arose during provirus replication or during transcription of proviral DNA followed by secondary infection of other cells. Fluctuation tests were performed under conditions allowing no secondary infection, so that all foci were cell clones as well as virus clones. Cultures of chicken cells were infected with a clone of B77 virus-II, and the cells were seeded sparsely on rat cell feeder layers and overlaid with media containing agar. Viral clones were picked from foci of transformed cells growing under agar, and the viruses were titrated in chicken and duck cells (Table 7). To determine whether the virus which formed foci in duck

TABLE 7. *Fluctuation test in clones of chicken cells*[a]

No. of cells per focus	No. of viruses forming foci	
	Observed in chicken cells	Observed in duck cells
75	5,100	270
20	4,100	190
35	3,800	74
35	1,380	260
75	1,020	22
45	1,000	350
40	900	0
30	700	9
31	690	0
33	660	43
42	600	37
16	500	92
23	52	6

[a] Cultures of chicken cells were infected with B77 virus-II at a multiplicity of approximately 0.03 FFU per cell. After absorption, the cells were overlaid with complete medium, and, 4 h later, the cells were washed, removed from the plate with trypsin, counted, and seeded at either 10^5 or 2×10^4 cells/culture in cultures containing 2×10^5 attached NRK cells. After allowing the infected chicken cells to attach overnight in medium without serum, the medium was removed, and complete medium containing 0.4% agar was added to each culture. An additional 2.0 ml of medium containing 0.4% agar was added on days 4 and 8 after infection. Ten days after infection, the number of cells in each focus was counted, the clones were picked, and the virus was titrated in chicken and duck cells. All viral clones which produced no foci in duck cells were tested to determine whether they were B77 virus-I or B77 virus-II. A single clone of B77 virus-I (duck EOT $< 10^{-6}$) was found and removed from the data shown.

cells was B77 virus-III, the efficiency of transformation of duck cells by the progeny of each culture containing transformed duck cells was tested and found to be approximately 10^{-1} (data not shown).

No correlation was observed between the amount of B77 virus-III in a clone and either the total number of viruses capable of forming foci in chicken cells or the total number of cells in the focus. From a χ^2 test, the probability was less than 0.001 that the results fit a Poisson distribution. Therefore, when secondary RNA to DNA information transfers were blocked neither the frequency of appearance of B77 virus-III mutants nor the clonal distribution of mutants was affected. The results of this, two similar experiments (data not shown), and the previous experiments indicate that spontaneous mutation in the provirus did not primarily occur during the original synthesis of viral DNA (RNA to DNA information transfer) or during provirus transcription (DNA to RNA information transfer). (A high rate of mutation during DNA to RNA transcription is not consistent with the observed clonal distribution of mutants unless there is new provirus formation by the mutant RNA.)

(iii) Dependence on cell replication of mutation of B77 virus-II to B77 virus-III. The mutation of B77 virus-II to B77 virus-III could (i) be a function of the amount of time the B77 virus-II DNA is present in infected cells or (ii) occur by a mechanism which required cell replication. To distinguish between these two possibilities, the effect of preventing cell replication on the mutation of B77 virus-II to B77 virus-III was determined.

Cultures of stationary chicken cells were infected at a low multiplicity with stocks of B77 virus-II selected to contain no B77 virus-III (virus from the experiment described in Table 4). The infected cells were kept stationary for an additional 5 or 10 days, serum was added, and the cells divided (14). The virus produced each day was titrated in chicken and duck cells (Fig. 3). After the addition of serum there was a 5-day lag in the appearance of B77 virus-III from cells held stationary for 5 days and a 6-day lag from cells held stationary for 10 days. In contrast, the amounts of B77 virus capable of forming foci in chicken cells increased exponentially after the addition of serum. Control cultures which received no serum during the periods of release from stationary phase produced background levels of approximately 10^4 FFU/ml in chicken cells (14) and no foci (<5 FFU/ml) in duck cells.

The results of these and similar experiments with two other B77 virus-II clones (data not shown) show that mutation of B77 virus-II to B77 virus-III depends on cell replication and not on the length of time spent by the viral genome in the infected chicken cells.

DISCUSSION

Avian and mammalian leukosis-sarcoma viruses after passage through heterologous cells often possess an expanded host range with high EOT for the cells of the new host (for review see 30). For example, following the in vivo inoculation of RSV into ducklings, Duran-Reynals (10) obtained early and late tumors. Virus recovered from rapidly growing early duck tumors was not infectious for ducks. This type of RSV apparently had a host range like that of B77 virus-I. However, the RSV recovered from late duck tumors caused markedly different tumor cell

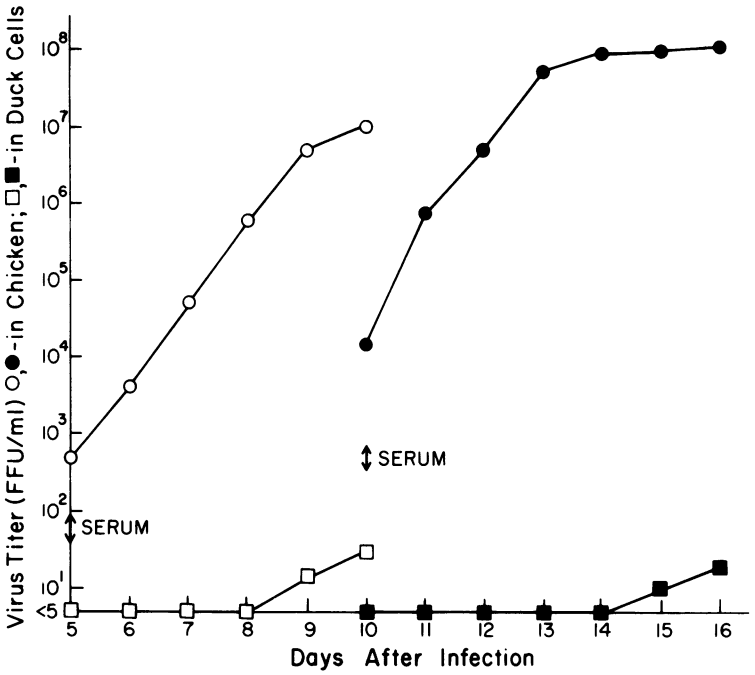

FIG. 3. *Dependence on cell replication of mutation of B77 virus-II to B77 virus-III. Duplicate cultures of stationary chicken cells in serum-depleted medium (14) were infected with a clone of B77 virus-II containing no B77 virus-III (virus from the experiment shown in Table 5). After absorption, the cells were washed and overlaid with 5.0 ml of the original serum-depleted medium. The infected cells were incubated for an additional 5 or 10 days. At 5 or 10 days after infection, the medium was removed from one-half of the cultures, and 2.0 ml of fresh medium containing 1% calf serum and 1% fetal bovine serum was added. Virus was harvested every day by removing the medium and adding fresh medium containing serum. Each viral harvest (total pool volume of 4.0 ml) was titrated in chicken and duck cells. Symbols:* ○, *B77 virus titer (FFU/ml) in chicken cells, serum added at 5 days;* □, *B77 virus titer (FFU/ml) in duck cells, serum added at 5 days;* ●, *B77 virus titer (FFU/ml) in chicken cells, serum added at 10 days;* ■, *B77 virus titer (FFU/ml) in duck cells, serum added at 10 days.*

morphologies, had affinities for different types of tissues, and, like B77 virus-III, had high infectivity for ducks. Clonal lines of B77 virus recovered from B77 virus-transformed rat cells also possessed significantly higher efficiencies of transformation of rat cells than the parental virus (1).

These sarcoma virus host range variants have been considered to originate either (i) as viral mutants which spontaneously occur during replication of the virus in cells of their natural hosts with selection upon infection of the heterologous host cells or (ii) to originate by adaptation in the heterologous cells (1).

The results of the studies presented here are incompatible with the hypothesis of adaptation as an explanation for the origin of B77 virus-III. Statistical analysis of the fluctuations in the numbers of B77 virus-III arising in chicken cells infected with B77 virus-II indicated that each B77 virus-II did not have an equal probability of plating in duck cells. The fluctuations in the distribution of mutants reflected spontaneous mutations occurring during the growth of the clones of B77 virus-II in chicken cells. This statistical evidence (Tables 4 and 7) coupled with the results of indirect selection experiments (Table 5 and data not shown) demonstrate that B77 virus-III host range mutants originate by spontaneous mutations which occur in chicken cells and that these mutants are selected in duck cells. It is possible that additional variation in B77 virus (Fig. 1) occurred in duck cells through host-induced modifications including viral acquisition of normal duck cell genetic information (23) or further mutation and selection. (Possible back mutation from B77 virus-III to B77 virus-II would not have been seen in the experiments presented here.)

B77 virus-I also originates from B77 virus-II by spontaneous mutation in chicken cells (Table 6). However, B77 virus-I was not observed to give rise to either B77 virus-II or B77

virus-III after serial viral or infected cell passages, including passage of infected duck cells (unpublished observations). A high rate of back mutation from B77 virus-I to B77 virus-II was not expected because there would be little or no experimental distinction between B77 virus-II and a B77 virus-I that back mutated to B77 virus-II.

Mutations in the B77 provirus occurred after the original synthesis of viral DNA and not during transcription of the viral RNA (Fig. 2 and Tables 4 and 7). B77 virus mutation also required cell replication (Fig. 3). The molecular mechanism of these mutations might involve viral DNA replication directly and/or provirus repair or replacement. However, these experiments do not directly exclude the possibility of mutation during a process of progeny viral RNA to DNA transcription followed by replacement of the resident B77 virus provirus by a mutant one.

The fluctuation data (Tables 4 and 7) permits an estimation of the rate of virus mutation. In the fluctuation test shown in Table 4, 12 out of 20 clones contained B77 virus-III mutants, and, in the fluctuation test shown in Table 7, 11 out of 13 clones contained B77 virus-III mutants. These clones contained an average of about 40 to 50 transformed cells per clone. Therefore, there was a probability of approximately 1 of a mutational event occurring in about 50 cumulative cell replications.

The relative rate of spontaneous mutation of B77 virus-II to B77 virus-I can also be estimated from the fluctuation experiments. In Tables 4 and 7, respectively, 2 out of 22 and 1 out of 14 parental virus were B77 virus-I. However, in Tables 4 and 7 none of the parental virus was B77 virus-III. Therefore, the mutation rate of B77 virus-II to B77 virus-I was greater than the mutation rate of B77 virus-II to B77 virus-III.

These spontaneous mutation rates for the B77 virus host range gene(s) are the highest known for any animal virus character (11). Preliminary experiments indicate that the mutation affecting the viral host range occurs in the B77 viral envelope gene (unpublished observations).

The high rate of spontaneous mutation in the B77 virus host range gene(s) is probably not a unique property limited to this particular genetic marker. As mentioned previously, there also appears to be a high frequency of spontaneously occurring nonconditional and conditional-lethal mutations in several other viral genes including the sarcoma virus gene controlling the transformed cell state (26, 29). The low frequencies (10^{-6}) of back mutation reported for some mutagen-induced temperature-sensitive avian and murine leukemia and sarcoma virus mutants are possibly the result of selection of the least leaky mutants, including double or multiple mutants in these studies (12, 21, 25).

The endogenous ribodeoxyvirus-related genes detected by nucleic acid hybridization in normal avian (15, 20, 33) and mammalian (2, 3, 4, 6) cells also appear to be hypermutable (31). It is possible that mechanisms similar to those responsible for producing a high rate of spontaneous mutation in the DNA of an exogenously infecting strain of avian sarcoma virus, such as B77 virus, also produce mutations in some of the endogenous ribodeoxyvirus-related genes present in normal avian and mammalian cells.

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