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# DNA Complementary to Viral RNA in Leukemic Cells Induced by Avian Myeloblastosis Virus*

**M. A. Baluda† and D. P. Nayak‡**

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE, LOS ANGELES

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**Abstract.** Nucleic acid hybridization studies were made between 71S-AMV-RNA and DNA from leukemic myeloblasts and from normal chicken cells. There was homology between the viral RNA and chicken cell DNA and to a greater extent between viral RNA and leukemic cell DNA. Leukemic cell DNA hybridized approximately twice as much viral RNA as did normal chicken DNA. Thermal melting studies showed that the viral RNA bound to normal and leukemic cell DNA consists of long polynucleotides ($T_m$ = 87° and 92°C, respectively, in 2× saline citrate). This suggests that the leukemic cells contain a DNA template of the viral RNA.

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**Introduction.** Avian myeloblastosis virus (AMV), an avian tumor virus, possesses an RNA genome. Two single-stranded RNA components with approximate molecular weights of $12 \times 10^6$ and $3 \times 10^4$ daltons are isolated from preparations of purified radioactively labeled virions.[1–5] The small component consists of cellular tRNA and may be a cellular contaminant either inside or outside the virions.[6–8] Indirect evidence obtained with inhibitors of nucleic acid synthesis and function suggests that avian myeloblastosis virus and other RNA tumor viruses replicate their RNA genome via a DNA intermediate.[9–16] This DNA template would be synthesized within 12 to 14 hr after infection and would require continuous transcription for synthesis of viral RNA.[4, 9, 15, 17, 18] The inability to detect complementary viral RNA in cells producing avian myeloblastosis virus or other avian leukosis viruses also indirectly supports this hypothesis.[19, 20] The present nucleic acid hybridization studies were undertaken to test whether the presence of DNA complementary to AMV-RNA could be demonstrated in virus producing leukemic myeloblasts transformed by avian myeloblastosis virus. Similar studies by other workers have been inconclusive.[3, 12, 21–24]

**Materials and Methods. DNA:** Leukemic myelob'asts, chick embryonic (CE) cells, or mouse embryonic cells were resuspended in extraction buffer (NaCl $10^{-1}$ *M*; Tris $2 \times 10^{-2}$ *M*, pH 8.5; EDTA $10^{-3}$ *M*) at no more than 0.4 ml of packed cells/10 ml of buffer. After addition of 1% mercaptoethanol and 1% SDS, the cells were incubated at 60°C for 10 min. Chick or mouse embryonic cells were obtained by homogenization of decapitated embryos in a Waring Blendor for 1 min. The DNA was extracted twice with 10 ml of chloroform-isoamyl alcohol (24:1 v/v) and then it was extracted three times with 10 ml of cold phenol[25] and spooled out of the aqueous phase with 2 vol of ethanol. The DNA was then dissolved in 0.3 *N* KOH and incubated at 30°C for 16 hr,

after which time it was neutralized with 1 *M* HCl in 0.2 *M* Tris base and dialyzed for three days (six changes) against 200 parts of 0.1 × saline citrate (1 × saline citrate consists of 0.15 *M* NaCl and 0.015 *M* sodium citrate). *E. coli* $K_{12}$ (λ) DNA was purchased from General Biochemicals (Chagrin Falls, Ohio) and re-extracted three times with phenol prior to treatment with KOH. The DNA was stored at 4°C in 0.1 × saline citrate with a few drops of chloroform.

The DNA was boiled for 10 min immediately prior to fixation which was performed by gravity filtration at a DNA concentration of 4 μg/ml in 4× saline citrate, on nitrocellulose filters (Millipore HA 0.45 μm). The filters had been soaked in 4× saline citrate and washed with 20 ml of 4× saline citrate prior to use. After DNA fixation the filters were washed with 20 ml of 4× saline citrate, dried, and stored at room temperature. The DNA filters were incubated at 70° for 4 hr just prior to hybridization.

**AMV-RNA:** All four $^3$H-labeled ribonucleosides were used and the intracellular viral RNA pool was prelabeled in the preparation of $^3$H-labeled AMV-RNA, as previously described.[20]

**Chick embryonic RNA:** Cold RNA was obtained from decapitated 9- or 10-day-old chick embryos homogenized in a Waring Blendor, either by direct cold phenol extraction or after removing DNA from the chick embryo DNA preparation. After alcohol precipitation, the RNA was treated with DNase (20 μg at 37°C for 15 min) in Tris-magnesium buffer (Tris-HCl $10^{-2}$ *M*, pH 7.4; $MgCl_2$ $10^{-3}$ *M*), re-extracted with phenol, precipitated with 2 vol of ethanol, and stored in NTE-ethanol (1:2 v/v) at −10°C.

**DNA-RNA hybridization:** The procedure used was similar to that of Gillespie and Spiegelman[26] and that of Westphal and Dulbecco.[27] The hybridization mixture contained $^3$H-labeled AMV-RNA and unlabeled CE-RNA (1.5 mg/ml or 3 mg/ml) in 4× saline citrate containing 0.05% SDS. Usually five filters containing DNA and two blank filters were placed in one vial containing 2.5 or 3 ml of hybridization mixture. In experiments involving hybridization of $^3$H-labeled cellular RNA with myeloblast or chick embryonic DNA, yeast RNA (1.5 mg/ml) was used instead of unlabeled chick embryonic RNA. Hybridization was performed at 70°C for 10 hr. After hybridization, the vials were chilled in ice water, each filter was washed twice in 250 ml of 4× saline citrate, placed on Millipore filter holders, and each side washed with 75 ml of 4× saline citrate. The filters were then RNase treated (25 μg/ml RNase A, 10 units/ml RNase $T_1$ in 2× saline citrate for 1 hr at 25°), washed twice in 250 ml of 4× saline citrate, and each side rewashed with 75 ml of 4× saline citrate on filter holders. The center portion of each filter was then cut, dried, and counted for radioactivity in toluene scintillation fluid. Each filter was washed in chloroform and the amount of DNA trapped on each filter was determined by the modified diphenylamine reaction.[28] In order to ascertain that the hybridized radioactivity was in viral RNA, and not in DNA, some filters were treated with 0.3 *N* KOH for 16 hr at 30°C and the trichloroacetic acid-precipitable and -soluble radioactivities were determined.

**Results. Hybridization of 71S-AMV-RNA with DNA from leukemic myeloblasts or from normal chick embryonic cells:** Only the 71S RNA component from purified AMV was used. In order to have a large variety of DNA and RNA species from the widest possible spectrum of multiplying and differentiating cell types, decapitated 9- or 10-day-old chick embryos were chosen as a source of control DNA and of unlabeled RNA. Table 1 shows that leukemic cell DNA immobilized 1.7 times as many counts as normal cell DNA. The standard deviation calculated for each set of filters establishes that the higher degree of hybridization shown by leukemic cell DNA is well above the significance level. Similar results were obtained if the control DNA was extracted from CE-fibroblast cultures instead of from whole embryos. All the hybridized counts were rendered trichloroacetic acid-soluble if the filters were treated with 0.3 *N* KOH.

TABLE 1. *Hybridization between 71S-AMV-RNA and DNA from leukemic and normal cells.**

| | Input RNA† ($\mu$g/ml) | cpm‡ Hybridized per 100 $\mu$g DNA from: Myeloblasts | Chick embryos | Excess cpm hybridized by myeloblasts |
|---|---|---|---|---|
| Expt. 1 | 0.57 | 334 ± 34 (5) | 190 ± 40 (5) | 144 |
| | 2.8 | 1156 ± 119 (5) | 710 ± 139 (5) | 446 |
| | 5.7 | 2095 ± 127 (5) | ... | ... |
| | 8.5 | 2663 ± 150 (5) | ... | ... |
| Expt. 2 | 6.3 | 2869 ± 325 (5) | 1544 ± 323 (5) | 1325 |

* Each vial contained 2 ml of 4× saline citrate with 0.05% SDS, 1.5 mg CE-RNA/ml plus $^3$H-71S-RNA, and seven filters (five experimental and two blanks). The amount of DNA remaining on the filters after hybridization was determined and the hybridized cpm of each filter were normalized to an average of 100 $\mu$g DNA (only the center portion of each filter was utilized to minimize unwashable noise). The same $^3$H-AMV-RNA preparation was used but different DNA's were used in both experiments. Average amount of DNA recovered per filter: 23 $\mu$g.

† Specific activity of input $^3$H-71S-AMV-RNA: $4.01 \times 10^5$ cpm/$\mu$g.

‡ Counts per minute ± standard deviation and in parentheses, the number of filters (cpm in filters containing no DNA have been subtracted). All samples were counted at least 40 min.

Even in the presence of 1500 $\mu$g of unlabeled CE-RNA, CE-DNA immobilized relatively many counts of $^3$H-labeled viral RNA. These counts represent in part noise and in part specific homology, as will be shown subsequently. Under similar conditions, *B. subtilis* DNA and *E. coli* DNA immobilized 10–12½% of the counts and mouse DNA about 50%. The noise results from an insufficient amount of complementary cold cellular RNA in the hybridization mixture to saturate all the DNA sites containing short sequences fortuitously complementary to the viral RNA. This is because the saturation of 100 $\mu$g of chicken DNA would require 600 mg of complementary RNA/ml of the hybridization mixture according to the data obtained by Westphal and Dulbecco with polyoma DNA and its complementary RNA.[27] Only 3 mg were used and the majority of this was ribosomal and tRNA.

Table 1 also shows that there are more than four copies of the viral genome DNA equivalent per leukemic cell. This was calculated from the data of experiment 2, taking into account that one 71S RNA molecule corresponds to $8 \times 10^{-6}$ cpm, and that 100 $\mu$g of chicken DNA contains $4.2 \times 10^7$ cell genomes.[29] Since a plateau was not reached in experiment 1, the actual number of copies remains undetermined.

**Hybridization of normal cellular RNA's to DNA from normal and leukemic cells:** In spite of the fact that we took great care in the extraction and purification of DNA, and used only DNA preparations with an $OD_{260}$:$OD_{280}$ ratio greater than 1.8, the increased immobilization of $^3$H-AMV-RNA by leukemic cell DNA might be nonspecific. It might result from the fortuitous selection during extraction from myeloblasts of a DNA species with a higher binding capacity for any type of RNA or from the contamination of leukemic cell DNA by some unknown factor with a high nonspecific binding capacity for RNA. To test this possibility, leukemic DNA was compared with CE-DNA for its ability to hybridize with different species of $^3$H-labeled RNA from normal CE fibroblasts. Table 2 shows that DNA isolated from either leukemic or normal cells hybridizes to the same extent with large molecular weight nuclear RNA, 28S or

TABLE 2. *Hybridization of normal cellular RNA to DNA from normal and leukemic cells.*

| Input RNA* | cpm Hybridized per 100 μg DNA† from: Myeloblasts | Chick embryos |
|---|---|---|
| Nuclear RNA (heavier than 45S) 4.45 × $10^4$ cpm/ml | 181 ± 8 (2) | 198 ± 2 (2) |
| Nuclear RNA (heavier than 45S) 4 × $10^6$ cpm/ml | 18,873 ± 714 (5) | 20,257 ± 1,988 (5) |
| 28S-RNA 1.23 × $10^6$ cpm/ml | 1,172 ± 100 (4) | 1,120 ± 94 (4) |
| 18S-RNA 3.5 × $10^5$ cpm/ml | 2,376 ± 64 (3) | 2,240 ± 171 (3) |
| 4S-RNA 7.5 × $10^4$ cpm/ml | 418 ± 26 (4) | 440 ± 67 (4) |

* The input RNA is measured in cpm per ml because the specific activities of these RNA preparations were unknown.

† Different DNA preparations were used in each experiment. The amount of DNA recovered per filter varied from 20 to 24 μg.

18S ribosomal RNA, or 4S RNA. Therefore, leukemic cell DNA does not possess a higher nonspecific binding capacity for RNA.

**Competition hybridization of unlabeled and $^3$H-labeled viral RNA:** If the difference in immobilized counts between leukemic cell DNA and normal DNA represents the specific hybridization of viral RNA with homologous DNA, competition with unlabeled AMV-RNA should eliminate this difference, provided the concentration of the unlabeled RNA is high enough to saturate its homologous DNA. Competition hybridization experiments were therefore performed with 3.98 μg/ml of $^3$H-71S-AMV-RNA and 62.7 μg/ml of unlabeled 71S-AMV-RNA in the presence of 1500 μg/ml of unlabeled CE-RNA. The purification of AMV, extraction of RNA, and isolation of unlabeled 71S-AMV-RNA were performed as in the preparation of $^3$H-labeled 71S-AMV-RNA.

As shown in Table 3, a 16-fold excess of unlabeled viral 71S-RNA eliminated the difference (1318 cpm) between the amount of $^3$H-labeled 71S-RNA immobilized by leukemic cell DNA and by normal cell DNA. The results also indicate the existence of homology between AMV-RNA and normal chicken DNA, because there is a 46% reduction in cpm immobilized by normal DNA (from 2565 to 1386 cpm) in the presence of excess cold viral RNA. The cold cellular RNA (1500 μg/ml) in the hybridization mixture did not contain a sufficient amount, if any, of cold complementary RNA to compete with that part

TABLE 3. *Competition between unlabeled and $^3$H-labeled 71S-AMV-RNA.**

| Input RNA† | cpm Hybridized per 100 μg DNA‡ from: Myeloblasts | Chick embryos |
|---|---|---|
| $^3$H-71S RNA (3.98 μg/ml) plus cold CE-RNA (1.5 mg/ml) | 3883 ± 286 (5) | 2565 ± 263(5) |
| $^3$H-71S-RNA (3.99 μg/ml) plus cold 71S-RNA (62.7 μg/ml) plus cold CE-RNA (1.5 mg/ml) | 1479 ± 110 (5) | 1386 ± 240 (4) |
| $^3$H-CE-28S-rRNA (10.8 μg/ml) plus cold yeast soluble RNA (1.5 mg/ml) | 3103 ± 291 (4) | 3476 ± 391 (4) |

* Each vial contained 2.5 ml and seven filters (five experimental plus two blanks).

† Specific activities:
$^3$H-71S-AMV-RNA: 4.7 × $10^5$ cpm/μg
$^3$H-28S-CE-RNA: 2.3 × $10^5$ cpm/μg

‡ Average amount of DNA recovered per filter: 21 μg.

of the $^3$H-labeled AMV-RNA which is homologous to chicken DNA. Even if as much as $10^{-3}$ of the normal chicken RNA were the product of the DNA which is complementary to AMV-RNA, there would be only 1.5 $\mu$g of this complementary RNA/ml of the hybridization mixture.

The remaining counts bound by chick embryonic DNA probably represent noise. Under similar hybridization conditions in the absence of cold viral RNA, mouse DNA consistently immobilized 50% less $^3$H-labeled 71S-AMV-RNA than did CE-DNA, and as will be shown in the next section, only short nucleotide sequences are involved.

Table 3 also shows that in a control test the leukemic DNA and the normal DNA hybridized to the same extent with chicken 28S ribosomal RNA.

**Thermal chromatography of DNA-RNA complexes:** To determine the extent of the homology involved in the previous hybridization experiments, the heat stability of the DNA-RNA hybrids was investigated. To reduce background noise, the concentration of unlabeled CE-RNA was increased to 3 mg/ml and immediately prior to RNase treatment the filters were heated at 50°C for 10 min. Table 4 reveals that the cpm immobilized by mouse DNA represent noise since 79% of them are eluted by heating at 60°C. The same number of noise cpm are also removed at 60°C from the total cpm hybridized by leukemic or normal DNA. The stability to heat of the DNA-RNA complexes formed between viral RNA and chicken DNA demonstrates that long RNA molecules are involved.[30-34] That part of the AMV-RNA which is homologous to chicken DNA elutes from its complementary DNA with a sharp melting profile and a $T_m$ of 87°C (Fig. 1*A*). The part of the viral RNA which is complementary to DNA present only in leukemic cells also has a sharp melting curve but a higher $T_m$ of 92°C (Fig. 1*C*).

TABLE 4. *Melting of ($^3$H)-AMV-RNA-DNA hybrids with increasing temperatures.*

| Temperature (°C) | cpm Hybridized per 100 $\mu$g DNA from: Myeloblasts | Chick embryos | Mouse embryos |
|---|---|---|---|
| ... | 483 ± 21 | 322 ± 16 | 128 ± 13 |
| 60 | 387 ± 8 | 193 ± 13 | 27 ± 6 |
| 65 | 357 ± 22 | 192 ± 10 | 27 ± 9 |
| 70 | 352 ± 3 | 194 ± 10 | 20 ± 0 |
| 75 | 328 ± 19 | 165 ± 14 | 18 ± 6 |
| 80 | 327 ± 5 | 141 ± 4 | 18 ± 4 |
| 85 | 274 ± 34 | 121 ± 3 | 19 ± 10 |
| 90 | 202 ± 8 | 91 ± 3 | 22 ± 7 |
| 95 | 83 ± 4 | 33 ± 10 | 17 ± 6 |

Hybridization was performed in four vials with 30 DNA filters in each. Each vial contained 5 ml of 4 × saline citrate with 0.05% SDS, 15 mg of chick embryo RNA, and 15 $\mu$g of 71S-$^3$H-AMV-RNA (spec. act.: 3.63 × $10^5$ cpm/$\mu$g). After hybridization all the filters were washed and treated as usual except that they were incubated at 50°C in 2 × saline citrate for 10 min prior to the RNase treatment. After the rims were cut off, the filters were treated three at a time for 10 min at the indicated temperature in 300 ml of 2 × saline citrate. They were then immediately treated a second time with RNase at 37° for 30 min in 2 × saline citrate. After two further washings in 300 ml of 4 × saline citrate, the filters were dried and counted. Six filters of each type of DNA were subjected to the same treatment but were not heated and served as controls. After counting, the filters were washed twice in chloroform and their DNA content was determined. The amount of DNA recovered per filter varied between 34 and 45 $\mu$g. Even after heating at 95°, 71 to 73% of the DNA still remained attached to the filter.

Background (34 cpm/filter without DNA) was subtracted. All cpm were normalized to 100 $\mu$g of DNA and the standard deviation was calculated. *E. coli* DNA (six filters) retained 51 ± 17 cpm/100 $\mu$g after heating at 50° for 10 min.

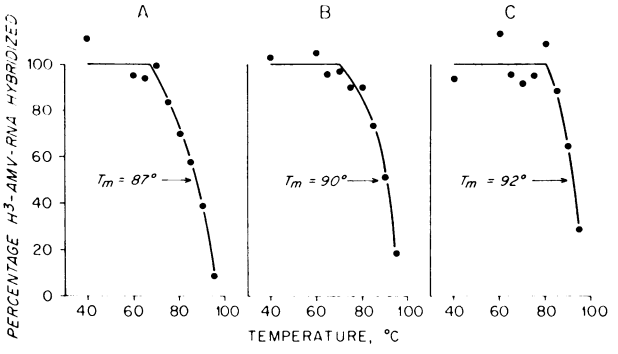


FIG. 1.—Melting curves of DNA-RNA hybrids. The data of Table 4 are plotted as percentages of RNase-resistant $^3$H-AMV-RNA versus heating temperatures. (*A*) Viral RNA homologous to normal chicken DNA (cpm hybridized to CE-DNA minus cpm hybridized to mouse DNA). (*B*) Total viral RNA (cpm hybridized to leukemic DNA minus cpm hybridized to mouse DNA). (*C*) Viral RNA complementary to DNA present only in leukemic cells (cpm hybridized to leukemic DNA minus cpm hybridized to CE-DNA).

The total viral RNA has an intermediate $T_m$ of 90°C (Fig. 1*B*). Therefore, the viral genome appears to be made up of two parts with different G+C content.[34–36] These two viral RNA regions must be of approximately equal lengths since twice as much AMV-RNA hybridizes with leukemic DNA as with normal DNA and since the $T_m$ of the DNA-RNA hybrid formed using leukemic DNA is intermediate between the $T_m$ of the hybrids formed with the two individual parts.

**Hybridization of small (5S) viral RNA component with leukemic and normal DNA:** The small (5S) RNA component isolated from purified AMV hybridizes equally well with both types of DNA and also with mouse embryonic DNA (Table 5). Competition-inhibition experiments with unlabeled CE-RNA and with 4S-CE-RNA indicate that the 5S viral RNA is cellular RNA. These results, coupled with the findings that the 5S viral RNA has amino acid binding capacity,[6] is methylated to the same extent, and has the same base composition as cellular tRNA,[7] show that the small viral RNA consists of cellular tRNA.

TABLE 5. *Competition hybridization between $^3$H-labeled 5S-AMV-RNA and cold CE-RNA.**

| Input RNA (μg/ml) | cpm Hybridized per 100 μg DNA† | | |
|---|---|---|---|
| | Myeloblasts | Chick embryos | Mouse embryos |
| 0.39 μg $^3$H-AMV-RNA (5S) only | 1494 ± 73 (3) | 1292 ± 156 (3) | 1331 ± 33 (3) |
| 0.39 μg $^3$H-AMV-RNA + 187 μg cold CE-RNA (unfractionated) | 258 ± 50 (4) | 187 ± 30 (4) | 162 ± 21 (4) |
| 0.39 μg $^3$H-AMV-RNA + 485 μg cold CE-RNA (unfractionated) | 203 ± 45 (4) | 115 ± 54 (4) | 99 ± 35 (4) |
| 0.39 μg $^3$H-AMV-RNA +168 μg cold CE-RNA (4S only) | 286 ± 22 (4) | 239 ± 41 (4) | 184 ± 21 (4) |

* The specific activity of $^3$H-5S AMV-RNA was assumed to be the same as that of the $^3$H-71S RNA ($4.59 \times 10^5$ cpm/μg) and was used to calculate the amount of $^3$H-5S RNA used. All vials contained 1 mg of yeast soluble RNA/ml of 4× saline citrate plus 0.05% SDS. Each vial contained 14 filters in 3 ml.

† Average amount of DNA recovered per filter: 23 μg.

The small, but consistently higher degree of hybridization with myeloblast DNA is caused by the presence of degraded 71S RNA in the 5S viral RNA preparation.

This experiment also confirms the specificity of the hybridization of 71S viral RNA with myeloblast or CE-DNA which was not inhibited by 3000 $\mu$g of CE-RNA (Table 4); in contrast, the hybridization of the 5S viral RNA was 83% inhibited by only 187 $\mu$g of CE-RNA.

**Discussion.** This study demonstrates that the 71S component of AMV-RNA hybridizes specifically with DNA from leukemic myeloblasts and to a lesser extent with DNA from normal chick embryos. The heat stability and high $T_m$ of the DNA-RNA hybrids in 2× saline citrate indicate that sequences of at least 100 nucleotides are involved[34] and, therefore, that extensive homology not dependent on accidental similarities exists between the two nucleic acids.

The leukemic cell DNA which is complementary to AMV-RNA could represent either a viral DNA intermediate[9] or a virus-induced redundancy of normal chicken DNA sequences homologous to the viral RNA. The $T_m$ of the DNA-RNA complex which forms only between AMV-RNA and leukemic DNA was found to be 5°C higher than the $T_m$ of the hybrid formed between the viral RNA and normal DNA. If this difference can be definitely established, the DNA replicative intermediate hypothesis would be favored, since two regions of the viral genome, differing by about 10% in G+C content, would be involved.

The hybridization of viral RNA with normal DNA is about half that with leukemic DNA. It could result either from a natural homology between AMV-RNA and chicken DNA or from the contamination of the chick embryos by some avian leukosis virus partially homologous to the avian myeloblastosis virus. The latter hypothesis is suggested by the fact that approximately 10% of the chick embryos used in this study (White Leghorn, strain K-137 from Kimber Farms) develop "spontaneous" leukotic neoplasias.[37] If homology exists between viral RNA and normal chicken DNA, it may be a necessary prerequisite for initiation of virus production and for malignant transformation.[38] The question arises whether the presence of the homology is related to the susceptibility of the cells to transformation since in some bird species AMV is able to multiply, but not to transform, and in others it does not even multiply.[39]

There are at least four DNA equivalents of the viral genome per leukemic cell, although it is not known whether they correspond to entire genomes or parts of it. Experiments are in progress to determine their exact number and also whether the viral DNA is chromosomal, mitochondrial, or other. If the leukemic cell DNA is complementary to only part of the viral genome, an RNA intermediate could still be involved in the replication of RNA tumor viruses. Two recent reports suggest this possibility.[40, 41]

Abbreviations: AMV, avian myeloblastosis virus; CE, chick embryonic.

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