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Isolation of Defective Mutant of Avian Sarcoma Virus

(recombination/RNA-dependent DNA polymerase/glycoprotein/endogenous virus)

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ABSTRACT **A colony of transformed cells was isolated from chick-embryo cells infected with a stock of nondefective Schmidt-Ruppin strain of Rous sarcoma virus. The virus recovered from this colony was a stable defective mutant very similar to the Bryan strain of Rous sarcoma virus in the following characteristics: (*i*) noninfectiousness of virus particles released from transformed cells that lack helper factor; (*ii*) formation of infectious pseudotypes by coinfection with avian leukosis virus or by interaction with endogenous-helper factor in chicken cells; (*iii*) ability of the noninfectious form of virus to transform chick-embryo cells in the presence of ultraviolet light-inactivated Sendai virus; (*iv*) absence of glycoprotein in the noninfectious form; (*v*) failure to produce nondefective virus by recombination with avian leukosis virus; and (*vi*) segregation of polymerase-negative virus.**

The morphology of transformed cells is characteristic of those infected by the Schmidt-Ruppin strain. The demonstration of segregation of such a defective virus from nondefective sarcoma virus and failure to detect revertants of this mutant suggest that the deletion of some genes may be involved in this mutation.

Some strains of Rous sarcoma virus (RSV), represented by the Bryan strain (B-RSV), are known to be helper-dependent for the formation of infectious progeny (1, 2). B-RSV appears to be defective in the ability to synthesize or assemble the glycoprotein that determines phenotypic expression of virus relating to the subgroup specificity (3).

Other strains of RSV, such as the Schmidt–Ruppin strain (SR-RSV), have been considered to be nondefective, since they do not require intervention of the helper virus for reproduction (4–7). While this notion has been essentially confirmed, a previous study showed that a significant proportion (10–20%) of the particles in a stock of SR-RSV may contain defective genomes that give rise to transformed cells producing no infectious virus (8). These "nonproducer" transformed cells appeared to be formed by various kinds of mutants or defective forms of viruses. One clone of these cells, which produces virus deficient in viral coat antigen, was examined in great detail in this study. The results show that this particular mutant of SR-RSV is strikingly similar to the defective B-RSV in many characteristics.

Abbreviations: C/E, chicken-embryo cells resistant to subgroup E virus; Q/B, quail-embryo cells resistant to subgroup B virus; gs^-h^-, chicken cells negative for group-specific antigen and for helper activity; gs^+h_H, chicken cells positive for group-specific antigen and having high helper activity. ALV, avian leukosis virus; RAV, Rous associated virus; SR-RSV, Schmidt–Ruppin strain of Rous sarcoma virus; B-RSV, Bryan strain of RSV; SR-RSV-A, SR-RSV of subgroup A; NT-SR-RSV-A, nontransforming variant of SR-RSV-A, further abbreviated as NT-SR-A; chf, chicken-helper factor; RSV(ALV), RSV made by the assistance of ALV; RSV(chf), RSV produced in gs^+h_H cells, therefore, the virus has envelope antigen provided by chf; RSV(−), noninfectious RSV produced in the absence of ALV and chf; UV-Sendai virus, ultraviolet light-inactivated Sendai virus; FFU, focus-forming units.

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MATERIALS AND METHODS

Cell Cultures and Viruses: Chick-embryo cells used in all experiments were negative for the endogenous viral expression (gs^-h^-, C/E type) (9) unless otherwise stated. Chick-embryo cells designated as gs^+h_H are positive for gs antigen in cells and their level of helper activity is high (9). SR-RSV-A used in this work was derived from a virus clone (S-41v) described in the previous paper (8). The virus has been used in our laboratory after recloning in gs^-h^- cells (10–12). The media and conditions for tissue culture, the preparation of ALVs (RAV-1, RAV-2, and RAV-7), B-RSV(RAV-2), and Sendai virus have been described previously (11, 13). NT-SR-RSV-A, a nontransforming variant of SR-RSV-A, isolated from a stock of SR-RSV-A by the method of Vogt (14) will be abbreviated as NT-SR-A.

Infection of Virus in the Presence of UV-Sendai Virus: This was done according to the procedures described by Hanafusa *et al.* (13).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis: Fully transformed cell cultures were incubated with medium containing 16.6 μCi/ml of D-[6-^{3}H]glucosamine hydrochloride (3.6 Ci/mmol) and 2.7 μCi/ml of L[^{14}C]aminoacid mixture (1.5 mCi/mg) (New England Nuclear Corp., Mass.) for successive 12-hr intervals. The double-labeled virus was purified and subjected to protein analysis by sodium dodecyl sulfate–polyacrylamide-gel electrophoresis as described by Scheele and Hanafusa (3).

Isolation of Transformed Cell Clones: The method used for isolation of transformed cell clones was essentially the same as described previously (8). Secondary cultures of chick-embryo cells were exposed to RSV at a multiplicity of infection of 5×10^{-5}. After 4-hr adsorption, the infected cells were trypsinized, diluted properly to form less than 30 colonies per plate (100 mm), and cultured in a soft agar medium. Antiserum against SR-RSV-A, RAV-1, or RAV-2 was usually incorporated into soft agar medium to prevent spread of virus. After 14 days, transformed colonies were separately isolated into test tubes containing 1 ml of medium and grown for 2–4 days. After reaching confluence, cultures were transferred to either 35- or 60-mm plates. Medium was changed every day for confluently transformed cultures.

Polymerase Assay: RNA-dependent DNA polymerase activity was determined by the method of Dr. J. H. Chen (unpublished). Virus was concentrated into a pellet by centrif-

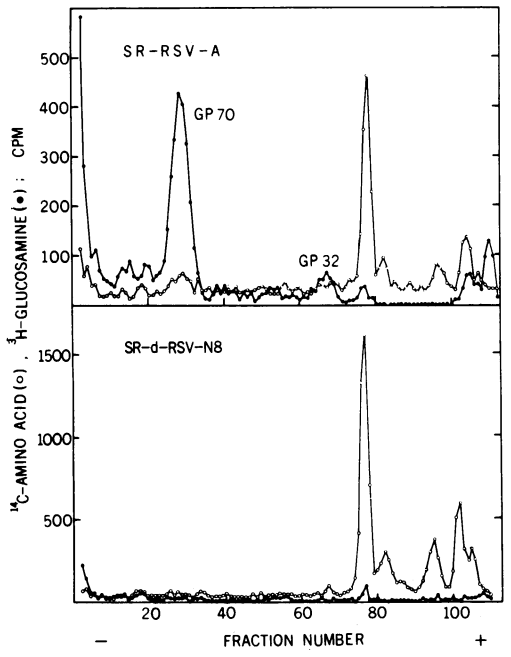

FIG. 1. Sodium dodecyl sulfate–polyacrylamide-gel electrophoresis of the proteins of SR-RSV-A and SR-d-RSV-N8. Virus double-labeled with [^{14}C]amino acid (○) and [^{3}H]glucosamine (●) was purified, dissociated, and subjected to sodium dodecyl sulfate–polyacrylamide-gel electrophoresis. The polypeptides migrated from *left* to *right*. *GP70* and *GP32* represent glycoproteins having molecular weights of 70,000 and 32,000, respectively.

ugation and assayed for polymerase activity in the presence of 7.5 μg of $(rC)_n \cdot (dG)_{12-18}$ (7:3). The reaction mixture in 0.1 ml contained: 0.1% Nonidet-p40; 5 μmol of Tris·HCl (pH 8.3); 0.6 μmol of magnesium acetate; 2 μmol of dithiothreitol; 6 μmol of NaCl, and 580 pmol of [^{3}H]dGTP (480 cpm/pmol). The mixture was incubated at 37° for 1 hr and then the acid-insoluble radioactivity was determined.

RESULTS

A Clone of Transformed Cells Producing Noninfectious Virus. Chick-embryo fibroblasts infected with wild-type SR-RSV-A at a low multiplicity were plated in soft agar medium to obtain colonies of transformed cells. Transformed cells in individual colonies were isolated and grown in cultures. Most of these cultures produced infectious virus but eight out of 46 isolates produced no detectable infectious virus. After a few transfers two of eight converted to producers. One of the clones (no. 8) that remained nonproductive was subjected to extensive characterization.

Clone no. 8 produced no infectious virus but formation of physical virus particles was demonstrated as [^{3}H]uridine-labeled virus in culture fluid after incubation of the cells with this precursor of RNA. The radioactive virus banded at a density of 1.16 g/ml in sucrose gradient centrifugation, and was similar in quantity to the wild-type virus that was labeled under the same condition.

When clone no. 8 cultures were exposed to helper viruses, RAV-1, RAV-2, RAV-7, or NT-SR-A, infectious forms of RSV were produced, as shown in Table 1. This indicates that the virus genome in the cloned transformed cells is defective, and the defective function can be complemented by these helper viruses. The results also show that there was no interference in these cells with infection by helper viruses, including those belonging to the same subgroup, A, as parental SR-RSV-A. Further experiments on the antigenicity, the host range specificity, and the interference relation with other viruses clearly demonstrated that these phenotypes of the activated infectious form of sarcoma virus are determined by the helper virus used for superinfection of the colony culture.

The foci formed by the activated virus derived from clone no. 8 were diffuse and consisted of a mixture of round and spindle-form cells that were often scattered over normal cells. These morphological characteristics of the foci are identical to those induced by the parental SR-RSV-A, but clearly distinct from those formed by the Bryan strain of RSV. Presumably due to the secondary infection, the number of transformed cells in a single focus is sometimes greater with the wild-type SR-RSV-A than with the virus derived from clone no. 8.

Defective Function of the Virus. The analysis of proteins of the noninfectious virus particles released from clone no. 8 was made by gel electrophoresis. As seen in Fig. 1, the patterns of proteins of molecular weight less than 27,000 were alike for the noninfectious virus and for the standard SR-RSV-A. However, two major glycoproteins represented by glucosamine-labeled peaks (molecular weight about 70,000 and 32,000, respectively) are missing in the pattern of the noninfectious virus. The absence of glycoprotein in the virus derived from clone no. 8 was also obvious from the extremely low rate of incorporation of glucosamine into virus particles.

Due to its defectiveness in glycoprotein synthesis, Bryan RSV is released as a noninfectious virus from $gs^{-}h^{-}$ cells, which lack chicken-helper factor (chf). These particles are designated as B-RSV(−) to indicate the lack of a determinant of subgroup specificity on the viral envelope. However B-RSV(−) particles are capable of transforming cells when inoculated together with UV-Sendai virus (15). This was also the case for the noninfectious virus obtained from clone no. 8. About 1000 foci of transformed cells can be seen in cultures exposed to the undiluted culture fluid from clone no. 8 together with 5000 hemagglutinin units of UV-Sendai virus,

TABLE 1. *Formation of pseudotypes*

Superinfecting virus (subgroup)	Titer (10^6 FFU/ml)	Plating efficiency on RAV-7-preinfected cells	Surviving fraction after incubation with	
			Anti-RAV-1	Anti-RAV-2
NT-SR-A(A)	5	1.1	1.0	1.1
RAV-1(A)	9	1.1	<0.001	1.1
RAV-2(B)	15	1.0	1.1	<0.001
RAV-7(C)	12	0.0084	1.1	1.1

Cultures of cell clone no. 8 (containing about 4×10^6 transformed cells) were infected with 1×10^5 infectious units of NT-SR-A, RAV-1, or RAV-7, or 1×10^6 PFU of RAV-2. The cultures were fed with fresh medium every day and culture fluids were harvested on the seventh day after infection and assayed on normal and RAV-7-preinfected chick-embryo cells. Neutralization was performed by incubating about 1×10^4 focus-forming units (FFU) of RSV in 1 ml of 10-fold dilutions of anti-RAV-1 or anti-RAV-2 serum at 37° for 45 min.

while the same virus preparation cannot produce any foci in the absence of UV-Sendai virus. The result suggests that the noninfectious virus is capable of transforming cells if they are incorporated into cells, presumably by modification of the cell surface by Sendai virus. Taken together with the absence of glycoprotein, which seems to be essential in an early virus penetration step, this result suggests that the virus derived from clone no. 8 is defective in the synthesis or assembly of envelope glycoprotein, but contains a genome fully capable of cell transformation. Therefore, we will designate this virus as a defective mutant SR-d-RSV-N8 or SR-d-N8, and its noninfectious phenotype as SR-d-N8(−).

Stability of the Defectiveness of the Virus. The stability of the defective nature of SR-d-N8 was tested in the following experiment. Chicken cells were transformed by infection with SR-d-N8(−) virus in the presence of UV-Sendai virus. Cultures containing transformed cells were kept subcultured until the majority of cells in the cultures became transformed. Then, the culture fluid of the transformed cells was used for the further passage of virus, repeating the same procedure of infection with UV-Sendai virus as before. At the end of each passage, which took about 20 days, culture fluid of confluently transformed-cell cultures was tested for the presence of wild-type SR-RSV-A. As shown in Table 2, no wild-type virus was detected for eight consecutive passages.

At each passage, one plate of cultures was also exposed to RAV-2. The yield of transforming virus was assayed on both chick (C/E) and quail (Q/B) cells. [The quail cells are susceptible only to SR-RSV-A, but C/E cells are susceptible to both SR-RSV-A and SR-RSV(RAV-2)]. The absence of formation of virus infectious for quail cells indicates that the addition of another complete leukosis virus does not elicit the formation of the revertant, SR-RSV-A.

The fact that the virus was passaged over a period of 5 months without formation of a single revertant strongly suggests that the defectiveness of SR-d-N8 is due to a deletion rather than a point mutation in the gene related to the envelope glycoprotein.

Interaction with Endogenous Virus Factor chf. In the foregoing experiments, SR-d-N8 was grown in chick-embryo cells that were negative for the expression of the endogenous virus genome, chf. It is known that chf provides a helper function (a specific envelope glycoprotein) for defective B-RSV (16), and RSV(chf) thus produced is infectious for several avian cells, notably for quail cells. The chf-positive cells (gs^+h_H) (9) also provide a helper function for SR-d-N8. SR-d-N8 grown in gs^+h_H cells produced virus which was infectious for quail cells but not infectious for chick (C/E) cells unless UV-Sendai virus was added, this is the host range expected for SR-d-N8-(chf).

The endogenous chf can be rescued into a leukosis virus, RAV-60, by infecting chf-positive cells with other avian leukosis virus (17). In later experiments, we found that B-RSV grown in chf-positive cells contained also RAV-60. RAV-60 was enriched by repeated transfers of quail cells infected with a stock of B-RSV(chf) before isolation of RAV-60 from end-point dilution of this passaged-virus stock.

Such a complete experiment was not performed with SR-d-N8(chf) grown in chf-positive cells. However, quail cells infected with undiluted or 10-fold diluted SR-d-N8(chf) stock produced SR-RSV with subgroup E type specificity. These results imply that RAV-60 derived from the chf cells was present in the stock of SR-d-N8(chf). The RAV-60 would act as a helper, permitting the defective virus SR-d-N8 to replicate in quail cells, which lack any helper factor. Alternatively, the results may be explained if one assumes the formation of a recombinant between SR-d-N8 and the chf genome. Such a recombinant may have acquired helper-independency as wild-type virus and carry the subgroup E specificity of chf. The interaction of the nondefective Prague strain of RSV with chf has recently been reported (18). However, we have shown that interaction with ALV does not lead to the formation of helper-independent B-RSV (12) or SR-d-N8 as shown in the experiments described below. Thus, the second possibility seems unlikely.

Failure to Produce Nondefective Virus by Recombination with ALV. Vogt (19) and ourselves (12) described the relatively high recombination frequency between nondefective RSV and ALV. In these experiments, the cell transforming capacity and the glycoprotein-determining subgroup specificity were used as two genetic markers. Since SR-d-N8 lacks the glycoprotein, recombination with ALV might convert SR-d-N8 to a nondefective virus. Colonies induced by SR-d-N8-

TABLE 2. *Stability of the defectiveness of the virus*

Virus passage	Superinfecting virus	FFU on C/E	FFU on Q/B
1st	−	0	0
	RAV-2	1.0×10^7	0
4th	−	0	0
	RAV-2	3.0×10^6	0
8th	−	0	0
	RAV-2	1.5×10^7	0

gs^-h^- cells were inoculated with SR-d-N8(−) virus in the presence of UV-Sendai virus. When cultures became confluently transformed, one of the subcultures was inoculated with 1×10^6 PFU of RAV-2. Culture fluids were collected 6 days later and assayed on C/E and Q/B cells. The virus from uninfected cultures was used for the next passage.

TABLE 3. *Lack of reversion to nondefective virus by recombination with ALV*

Virus preparation	Number of colonies isolated	Colonies producing infectious virus	% of colonies producing infectious virus
SR-d-N8(NT-SR-A)*	30	2	6.7
SR-d-N8(RAV-2)*	121	7	5.8
SR-d-N8(RAV-7)*	44	3	6.8
B-RSV(RAV-2)	28	2	7.1

Chick-embryo cells infected with each virus at a multiplicity of 5×10^{-5} were cultured in soft agar medium for 14 days. Transformed colonies were isolated and grown in liquid medium. Culture fluids obtained from subconfluent or confluent cultures of isolated colonies were assayed on chick-embryo cells to test virus production.

* Virus preparations were identical to those used in Table 1.

TABLE 4. *Conversion of α-type virus to β-type virus*

Cells	Virus	Number of colonies isolated	Colonies producing infectious virus: No. of colonies	Colonies producing infectious virus: %
gs $^{+}h_H$	SR-d-N8$_\alpha$(NT-SR-A)	47	2	4.2
	SR-d-N8$_\alpha$(RAV-1)	92	8	8.7
gs^{-}h^{-}	SR-d-N8$_\alpha$(NT-SR-A)	36	2	5.5
	SR-d-N8$_\alpha$(RAV-1)	31	2	6.5

Either chf-positive (gs $^{+}h_H$) or chf-negative (gs^{-}h^{-}) cells (about 1×10^6 cells) were infected with 50 FFU of the viruses that had been obtained by superinfecting transformed cell cultures producing polymerase-negative (α) virus with either NT-SR-A or RAV-1 and cultured in soft agar medium for 14 days. Transformed colonies were isolated and grown in liquid medium. Culture fluids of transformed cells were assayed for the presence of β-type virus. Virus derived from gs $^{+}h_H$ cells were detected by infection of quail cells, and those obtained from gs^{-}h^{-} cells were detected by transformation of C/E cells after inoculation with UV-Sendai virus.

(NT-SR-A), SR-d-N8(RAV-2), or SR-d-N8(RAV-7) were isolated and tested for the production of infectious virus. As shown in Table 3, more than 90% of the isolates failed to produce infectious virus. About 7% of the isolates produced infectious virus, which could be either recombinants with ALV or pseudotypes of SR-RSV coated with ALV due to double infection. Since almost the same fraction of isolated colonies of B-RSV(RAV-2)-infected cells produced infectious virus in a separate experiment, it seemed likely that most of the colonies producing RSV were contaminated with ALV during the isolation process. Indeed, infectious center assay with and without x-irradiation of cells infected with virus derived from these colonies indicated that all of them were helper-dependent, therefore, not recombinant. From these experiments, we concluded that the formation of helper-independent virus through recombination is a rare event; if any recombination occurred, the frequency was less than 1%.

Polymerase-Negative Double Mutant. A small fraction of the virus population of stocks of B-RSV is α-type virus, which is noninfectious in the absence of interaction with ALV, because of a deficiency in virion-associated RNA-dependent DNA polymerase (20). This spontaneous mutation in polymerase gene, presumably due to deletion, might also occur with SR-d-N8. 17 Colonies of chicken cells (gs^{+}h$_H$) transformed by SR-d-N8(RAV-2) were isolated. Because of the presence of chf in these cells the majority of colonies (13 out of 17) produced infectious SR-d-N8(chf), while four produced no detectable infectious virus. Labeling with [^{3}H]-uridine, however, showed that all four colonies were producing physical virus particles. Virions obtained from two cultures were positive for the RNA-dependent DNA polymerase, but two were negative. The properties of the two virus preparations that were polymerase positive and noninfectious have not been studied further.

The characteristics of the polymerase-negative virus, which can be considered as double mutant in genes related to envelope antigen and to polymerase, were similar to those of the α-type B-RSV in many respects. An infectious form of α-type virus can be obtained by superinfecting the α-type SR-d-N8-transformed cells with ALV, which complements the two deficient functions of the double mutants as postulated for B-RSV (20). The majority of colonies formed by a low dose of infectious form of α-type SR-d-N8(ALV) produced the noninfectious, polymerase-negative α-type virus. However, a fraction of the colonies produced polymerase-positive virus (β-type). The rate of conversion was equal whether the colonies were made in gs^{+}h$_H$ or gs^{-}h^{-} cells, indicating that the endogenous viral agent is not the major factor in this conversion (Table 4). It should be pointed out here that (*a*) no spontaneous production of polymerase-positive virus was observed in cultures of α-type transformed cells, and (*b*) the conversion of α- to β-type requires the intervention of ALV. This strongly suggests that the conversion of α-type to polymerase-positive-type requires genetic recombination with ALV, as suggested previously with B-RSV (21).

DISCUSSION

The Bryan strain of RSV is unique among avain leukosis-sarcoma viruses in its defectiveness. Most other avian viruses, including several strains of sarcoma virus, appear to have complete genetic information for their own reproduction. However, the relationship between these two classes of viruses has not been understood. No helper-independent virus has ever been obtained from stocks of the defective Bryan strain of RSV (12). The work presented in this paper demonstrates, however, that a defective virus, similar to the Bryan strain of RSV, can be derived from the nondefective Schmidt–Ruppin strain by spontaneous mutation.

The frequency of this spontaneous mutation has not been determined. In addition to the mutant described here, various other types of mutants have been found in stocks of cloned wild-type Schmidt–Ruppin RSV, in total constituting about 10–20% of the virus population (8). As shown in this paper, some of these mutants induced nonproducing transformed cells that later, during cultivation, became producers, indicating the reversion of point mutants. Thus, a variety of point mutations and deletions at different loci seem to have taken place during replication of this particular Schmidt–Ruppin strain. The generality of these phenomena among other avian sarcoma viruses remains to be studied. In studies of genetic recombination, Vogt (19) described some defective progeny of Prague stain of RSV.

The lack of glycoproteins in the virions could be explained by a deficiency in assembly of the glycoproteins into virions

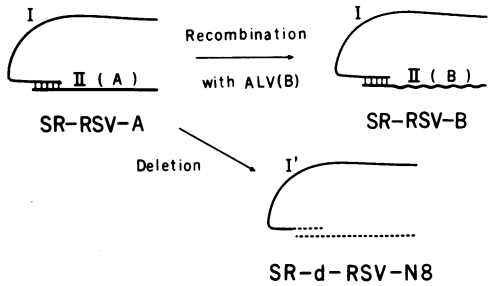

FIG. 2. A possible model for the formation of the defective RSV. Genome *I* contains all information necessary for cell transformation and genome *II* contains genes related to subgroup specificity.

rather than in their intracellular synthesis. However, experiments described in Table 2 show that co-infection of the mutant-infected cells with RAV-2 does not form parental SR-RSV-A, indicating that no subgroup A glycoproteins are formed in the mutant-infected cells. With avian leukosis-sarcoma viruses, it has not been determined which component of glycoprotein, carbohydrate or protein moiety, determines the subgroup specificity. Therefore, we do not know which portion of the glycoproteins is not synthesized in the mutant-infected cells or if both proteins are not synthesized. The absence of glycoprotein on the outer surface of the virions is exactly the same deficiency seen with the Bryan strain of RSV (3). Indeed, detailed characterization showed a remarkable similarity between the two defective viruses. The only critical differences between the two defective strains that could be detected are in the morphology and other biological properties of the cells whose transformation they induce.

The failure to obtain helper-independent revertants after co-infection with avain leukosis virus strongly suggests that the mutant is obtained by the deletion of a certain segment of genes including those related to the synthesis of glycoproteins. If this is the case, the RNA from defective SR-d-N8 should be smaller than that of complete wild-type SR-RSV. Although a hypothesis, based on physical properties of the RNA, proposing the existence of two subunit classes of RNA has been presented (22, 23), a clear picture of the organization of RNA subunits in the virions of RNA tumor virus does not yet exist. On the other hand, on a purely genetic basis, the high frequency of recombination between sarcoma and leukosis viruses with two markers, cell-transforming capacity and subgroup specificity, suggests the existence of at least two subunits containing different genes (12, 19). The high recombination frequency can be explained without difficulty by a model in which two different subunits of two viruses are exchanged. The defective mutant, then, could have a deletion of the terminal sequence that is essential for the linkage of the two RNA subunits. This model is shown in Fig. 2, in which the segment I, containing information for cell transformation, has a deletion at the site which links it to another segment, II, containing genes for specific glycoproteins. Because of the mutation in this particular site, the segment I of the mutant cannot associate with segment II. As already mentioned, we cannot determine whether segment II contains the structural gene for the protein moiety of the glycoprotein, or genes coding for enzymes involved in glycosylation of the protein, or both. Further experiments will be required to test the validity of this model.

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1. Hanafusa, H., Hanafusa, T. & Rubin, H. (1963) "The defectiveness of Rous sarcoma virus," *Proc. Nat. Acad. Sci. USA* **49**, 572–580.
2. Reamer, R. H. & Okazaki, W. (1971) "Evidence for the defectiveness of the Harris strain of Rous sarcoma virus," *J. Nat. Cancer Inst.* **44**, 763–767.
3. Scheele, C. M. & Hanafusa, H. (1971) "Proteins of helper-dependent RSV," *Virology* **45**, 401–410.
4. Hanafusa, H. (1964) "Nature of the defectiveness of Rous sarcoma virus," *Nat. Cancer Inst. Monogr.* **17**, 543–556.
5. Dougherty, R. M. & Rasmussen, R. (1964) "Properties of a strain of Rous sarcoma virus that infects mammals," *Nat. Cancer Inst. Monogr.* **17**, 337–350.
6. Goldé, A. (1966) "Non-défectivité de la souche de virus de Rous de Schmidt–Ruppin," *C. R. H. Acad. Sci.* **262**, 329–331.
7. Duff, R. G. & Vogt, P. K. (1969) "Characteristics of two new avian tumor virus subgroups," *Virology* **39**, 13–30.
8. Kawai, S. & Yamamoto, T. (1970) "Isolation of different kinds of non-virus producing chick cells transformed by Schmidt–Ruppin strain (subgroup A) of Rous sarcoma virus," *Jap. J. Exp. Med.* **40**, 243–256.
9. Hanafusa, T., Hanafusa, H., Miyamoto, T. & Fleissner, E. (1972) "Existence and expression of tumor virus genes in chick embryo cells," *Virology* **47**, 475–482.
10. Kawai, S. & Hanafusa, H. (1971) "The effect of reciprocal changes in temperature on the transformed state of cells infected with a Rous sarcoma mutant," *Virology* **46**, 470–479.
11. Kawai, S. & Hanafusa, H. (1972) "Plaque assay for some strains of avian leukosis virus," *Virology* **48**, 126–135.
12. Kawai, S. & Hanafusa, H. (1972) "Genetic recombination with avian tumor virus," *Virology* **49**, 37–44.
13. Hanafusa, T., Miyamoto, T. & Hanafusa, H. (1970) "A type of chick embryo cells that fails to support formation of infectious RSV," *Virology* **40**, 55–64.
14. Vogt, P. K. (1971) "Spontaneous segregation of nontransforming viruses from cloned sarcoma viruses," *Virology* **46**, 939–946.
15. Robinson, H. L. (1967) "Isolation of non-infectious particles containing Rous sarcoma virus RNA from the medium of Rous sarcoma virus-transformed nonproducer cells," *Proc. Nat. Acad. Sci. USA* **57**, 1655–1662.
16. Hanafusa, H., Miyamoto, T. & Hanafusa, T. (1970) "A cell-associated factor essential for formation of an infectious form of Rous sarcoma virus," *Proc. Nat. Acad. Sci. USA* **66**, 314–321.
17. Hanafusa, T., Hanafusa, H. & Miyamoto, T. (1970) "Recovery of a new virus from apparently normal chick cells by infection with avian tumor viruses," *Proc. Nat. Acad. Sci. USA* **67**, 1797–1803.
18. Weiss, R. A. Mason, W. S. & Vogt, P. K. (1973) "Genetic recombinants and heterozygotes derived from endogenous and exogenous avian RNA tumor viruses," *Virology* **52**, 535–552.
19. Vogt, P. K. (1971) "Genetically stable reassortment of markers during mixed infection with avian tumor viruses," *Virology* **46**, 947–952.
20. Hanafusa, H. & Hanafusa, T. (1971) "Noninfectious RSV deficient in DNA polymerase," *Virology* **43**, 313–316.
21. Hanafusa, H. & Hanafusa, T. (1968) "Further studies on RSV production from transformed cells," *Virology* **34**, 630–636.
22. Duesberg, P. H. & Vogt, P. K. (1970) "Differences between the ribonucleic acids of transforming and nontransforming avian tumor viruses," *Proc. Nat. Acad. Sci. USA* **67**, 1673–1680.
23. Martin, G. S. & Duesberg, P. H. (1972) "The α subunit in the RNA of transforming avian tumor viruses: I. Occurrence in different virus strains. II. Spontaneous loss resulting in non-transforming variants," *Virology* **47**, 494–497.