RECOVERY OF AVIAN SARCOMA VIRUS FROM TUMORS INDUCED BY TRANSFORMATION-DEFECTIVE MUTANTS*

By HIDESABURO HANAFUSA, CAROL C. HALPERN,† DOROTHY L. BUCHHAGEN,§ AND SADAAKI KAWAI

(From The Rockefeller University, New York 10021, and the Department of Oncology, The Institute of Medical Science, University of Tokyo, Takanawa, Tokyo, Japan)

The nondefective strains of Rous sarcoma virus (RSV)¹ derived from the original Rous chicken tumors (1) are known to contain both transforming and replicating functions (2-4, 4a). With relatively high frequencies, these sarcoma viruses spontaneously convert to transformation defective (td) mutants which retain only replicating functions (5, 6). The genomic RNA of the td mutants is about 15% smaller than the parental RSV RNA, corresponding to the loss of ≈1,500 nucleotides (7-10). The analysis of oligonucleotides obtained after hydrolysis of the RNAs of parental RSV and of td mutants showed that the deletions occur in a specific portion of the RSV genome, located near the 3' terminus (11, 12). Since this particular segment of the genome, deleted in td virus RNA, is also absent in naturally occurring nonsarcomagenic oncoviruses (avian leukosis viruses), it is thought to be responsible for sarcomagenic transformation, and was designated as the src gene (13-15).

The size of the deletions in td mutants has been considered to be relatively uniform (8, 15a). However, recent studies by us and others suggested the existence of some variations (16, 17; M. C. Lai, S. Hu, and P. K. Vogt, personal communication). Certain isolates of td mutants of the Schmidt-Ruppin strain of RSV (SR-RSV-A), which were found to contain RNA slightly larger than that of other td mutants and leukosis viruses, were able to interact with an RSV mutant which is temperature-sensitive in transformation to form a recombinant with a reduced temperature-sensitivity (17). Thus, these td mutants appear to retain a small portion of the src gene which is not sufficient to cause cell transformation in vitro.

As part of the survey of characteristics of these partially deleted td mutants, we examined their potential to produce tumors in chickens. In this paper, we will report that some of the td mutants were capable of producing sarcomas in chickens after an unusually long latent period at sites distant from the original injection, and that fully transforming sarcoma virus was recovered from every one of these tumors. Evidence will be presented that this tumor production is

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¹ Abbreviations used in this paper: chf, chicken helper factor; gs antigen, group-specific antigen; r-ASV, recovered avian sarcoma virus; RAV, Rous-associated virus; RSV, Rous sarcoma virus; src gene, gene responsible for sarcoma transformation; SR-RSV-A, Schmidt-Ruppin strain of RSV, subgroup A; td, transformation defective; TS, temperature-sensitive.
not due to contamination of the \textit{td} virus preparations by parental strain of RSV. The formation of the new sarcoma virus is, therefore, most likely a result of genetic interaction between the \textit{td} virus and some host cell component in chickens.

\section*{Materials and Methods}

\textit{Virus.} Isolation of \textit{td} mutants was described (6, 17). These \textit{td} viruses were present in a stock of SR-RSV-A as spontaneous deletion mutants and were isolated by cloning. The \textit{td} virus stocks contained generally $-10^7$ infectious units per ml. The preparations of Rous-associated virus-1 (RAV-1) and SR-RSV-A have been described before (6, 18). Avian erythroblastosis virus, strain ES4, was kindly provided by Dr. R. Ishizaki, Duke University, Durham, N. C.

\textit{Cells.} Chicken embryo fibroblasts used were prepared from fertile eggs obtained from a flock free of group-specific \textit{(gs)} antigens (SPAFAS, Inc., Norwich, Conn.). These cells were also negative for chicken helper factor \textit{(chf)}. Conditions for cell culture and virus titration have been described (19, 20).

\textit{Chickens.} Two flocks of chickens (SPAFAS, Inc.) were used. One flock is called COFAL-negative and another GS-negative. Both flocks are free of avian leukemia virus but $\sim 30\%$ of the COFAL-negative chickens are positive for the expressions of \textit{gs} antigen and \textit{chf}, whereas chickens of the GS-negative flock are almost entirely free ($>95\%$) of these expressions (21).

\textit{Inoculation of Chickens.} 0.1 ml (containing $-10^6$ infectious units) of cell-free virus in culture medium was inoculated into wing webs for testing sarcoma formation. Injections were usually subcutaneous but due to the wing size of baby chicks some may have been intramuscular or intravenous. Chickens were examined by palpation for the presence of tumors every 3-4 days. When tumors were removed, they were cut into small pieces. A portion was formalin-fixed for pathological examination, a portion was frozen for later homogenization, and the remainder was trypsinized in the same manner as used for preparing primary cultures of chicken embryo cells (20). The trypsinized tumor cells were plated into tissue culture dishes with or without addition of normal chicken embryo cells.

\section*{Results}

As described previously, \textit{td} mutants isolated from SR-RSV-A were shown to be free of detectable transforming virus in repeated tests examining either direct cell transformation or the production of transforming virus (17). In the first attempt of this series (exp. 1, Table I), we inoculated these tested virus stocks of \textit{td}101, 105, 107, and 108 into the wing web of newborn chicks (within 1 day). As expected, no sarcomas were produced at the site of injection. However, solid tumors were recognized in several chickens (exp. 1, Table I) between 61 and 68 days after infection at sites distant from the original injection, such as breast, legs, or back. When tumor cells were co-cultured with normal chicken embryo fibroblast cells, cultures became transformed within a few days and displayed morphological characteristics similar to those of cells transformed by RSV. We observed, however, that the morphology of both foci and individual transformed cells induced by these recovered agents was different from that induced by SR-RSV (see below). High titers of transforming virus ($>10^9$ focus-forming units per ml) were obtained from co-culture-derived transformed cells. Transforming virus was also recovered by direct homogenization of tumors, and 20\% tumor homogenates contained $10^4$-$10^5$ focus-forming units per ml. The virus recovered produced sarcomas within 10 days upon injection into 1-wk-old chickens. These preliminary experiments strongly suggested that some \textit{td} mutants were capable of producing solid tumors after a long latent period, and


**Table I**

| Exp. | Virus | State of purification | Flock of chickens | Route of injection | Total | With solid tumors | Dead without tumors | Remained negative |
|------|-------|-----------------------|-------------------|-------------------|-------|------------------|--------------------|-------------------|
| 1    | td101 | COFAL s.c.            | 5                 | 2                 | 3     |                  |                    |                   |
|      | td105 | COFAL s.c.            | 5                 | 1                 | 4     |                  |                    |                   |
|      | td107 | COFAL s.c.            | 4                 | 2                 | 1     |                  |                    |                   |
|      | td108 | COFAL s.c.            | 4                 | 3                 | 0     |                  |                    |                   |
| 2    | RAV-1 | COFAL s.c.            | 8                 | 0                 | 2     | 6                |                    |                   |
|      | td101 | Purified              | COFAL s.c.        | 8                 | 2     | 6                | 0                  |                   |
|      | td107 | Purified              | COFAL s.c.        | 8                 | 2     | 4                | 2                  |                   |
|      | td108 | Purified              | COFAL s.c.        | 8                 | 0     | 4                | 4                  |                   |
|      | td109 | Purified              | COFAL s.c.        | 8                 | 5     | 3                | 0                  |                   |
| 3    | td101 | Purified              | GS i.v.           | 7                 | 6     | 1                | 0                  |                   |
|      | td107 | Purified              | GS i.m.           | 8                 | 5     | 3                | 0                  |                   |
|      | td107A| Purified              | GS i.v.           | 8                 | 0     | 3                | 5                  |                   |
|      | td108 | Purified              | GS i.m.           | 7                 | 0     | 2                | 5                  |                   |
|      | td109 | Purified              | GS i.m.           | 7                 | 2     | 2                | 3                  |                   |
|      | Medium| Purified              | GS i.m.           | 8                 | 0     | 0                | 8                  |                   |

* Some td mutants were "repurified" by one or more additional terminal dilution passages.
† Two chicken flocks designated as COFAL-negative and GS-negative were used.
§ s.c., subcutaneous; i.m., intramuscular; i.v., intravenous.
† All chickens in these experiments were injected within 1 day after hatching. Observations were terminated at 13 wk in exps. 1 and 2, and at 14 wk in exp. 3.

that the resulting tumors produce transforming virus which is similar to RSV in many respects.

**Further Purification of td Viruses and Their Specificity of Tumor Production.** Although the original td virus preparations were repeatedly tested for the absence of transforming virus and have been shown to contain genomic RNA similar in size to those of nontransforming oncoviruses (17), the possibility still remained that tumors were induced by very minute amounts of SR-RSV existing as contaminants in the td virus preparation. Such contaminants may have escaped detection due to the presence of a large excess of nontransforming virus which could cause viral interference. To eliminate such a possible contaminant, chicken cells were infected with serial 10-fold dilutions of td virus stocks (td101, 107, 108), and virus was recovered after three transfers from terminal dilutions (10⁻⁶ for all three) which gave positive interference against SR-RSV-A. For td101, purification by this terminal dilution was repeated twice. As shown in Table I (exps. 2 and 3), td101 and 108 were active in tumor production after this purification. All tumors except one (12 wk) were found at 8-9 wk after injection. Unpurified td109 was also found to be active in tumor production (exp. 2).

On the other hand, td107, which was recovered from the terminal dilution, lost its tumor-inducing activity. This was anticipated from the composition of viral RNA of td107 analyzed by gel electrophoresis (17) which showed the presence of two RNA components in td107, a minor component of larger-sized RNA and a major component of smaller-sized RNA. If the ratio of these two
RNA components reflected the ratio of virus particles containing these two size classes of RNA, and if the virus containing the larger-sized RNA was responsible for tumor formation, then the tumor-producing component may have been selected against by the terminal dilution. To distinguish between the original td107 preparation and the recloned virus, td107 obtained from the terminal dilution which was negative in tumor formation will be designated td107A. In addition to td107A, td106 was negative in tumor formation in one experiment with a limited number of animals. Control chickens, which received either 0.1 ml of RAV-1 (exp. 2), or unused tissue culture fluid (exp. 3) did not develop solid tumors within the 14-wk-period of observation.

In both the second and third experiments, a significant fraction of chickens died because of either poor development, enlargement of the heart, liver, or kidneys, or without an identifiable symptom. The deaths were apparently due to td virus infection as none of the control chickens (exp 3) died, but we have made no further attempt to examine the cause of death. Lymphoid leukemia is known to appear rarely before 14 wk of age (22).

A comparison was made between chickens of two flocks termed gs antigen-negative and COFAL-negative. No significant difference was found between the two types of chickens in the frequency of tumor formation by td101 and 108, indicating that endogenous viral information responsible for the expression of gs antigen and chf is not directly related to the tumor formation by td viruses.

The route of injection does not seem to be an important factor in the frequency of tumor formation. However, the comparison of various routes (Table I) was not conclusive since both subcutaneous and intravenous injections sometimes resulted in the leakage of inoculum to surrounding muscle tissues due to either the size of the wing webs or the fragility of the blood vessel of the newborn chicks.

Pathological Findings. Tumors appeared in various parts of the body: often in the leg, wing, chest, or back, and organs such as the kidney, liver, and heart. We also found malignant growths in the abdominal cavity and tissues surrounding the eye. Frequently, more than one tumor was found in a single chicken. The size of tumors was variable but was sometimes larger than 3 cm in diameter. Histologically, the tumors consisted predominantly of sarcomas—possibly of muscle origin—and were often associated with hemorrhage or necrotic areas. It seems clear that primary tumors were produced at various sites in the injected chickens. In two cases kidney tumors were associated with areas of nephroblastomas. However, some tumors in internal organs seemed to have been formed secondarily by metastasis.

Transforming virus was recovered from most of these tumors, including kidney tumors. Thus far no difference was found in the morphology of transformed cells induced by these virus isolates.

Some Characteristics of Recovered Transforming Viruses. Transforming virus was recovered from all 18 tumors examined. All isolates thus far tested (12 samples) were capable of producing sarcomas in the wing web of chickens at a rate comparable to Bryan or Schmidt-Ruppin strains of RSV. Therefore we will call the new isolates collectively “recovered avian sarcoma virus” (r-ASV) and individual isolates will be distinguished by the number of the tumor from which the virus was recovered.
The r-ASV isolates were indistinguishable from SR-RSV-A and the td mutants in subgroup specificity, as shown both by the specificity of interference and antigenic determinants. It was somewhat surprising to find that some isolates of r-ASV did not contain td virus in concentrations higher than r-ASV. In preliminary attempts, most foci made by such isolates (r-ASV 50 and 165) were found to produce infectious transforming virus, suggesting that at least these isolates are nondefective.

The focus morphology of the r-ASV-transformed cells is shown in Fig. 1 in comparison with that of SR-RSV-A transformed cells. It is remarkable that there was very little, if any, difference in the morphology of foci produced by the isolates of r-ASV recovered from many different tumors. The foci of r-ASV generally consisted of small, round transformed cells tightly held together (Fig. 1 A-D). Thus, r-ASV-induced transformed cells were readily distinguishable from foci of SR-RSV-transformed cells, which are often spindle-shaped and scattered over the layer of normal cells into a relatively large area (Fig. 1 E, F). Foci induced by r-ASV often have an area of cell displacement, thus making a small hole, the extent of which perhaps exceeds similar changes often seen with the Bryan strains of RSV. Because of this effect, r-ASV-transformed cells had a tendency to detach from the substratum when these transformed cells became the majority of the cell population in a culture.

There seem to exist variations in the virus titer depending on the original td virus samples. High titers of virus (0.2–2 × 10^8 focus-forming virus per ml) were uniformly recovered from tumors induced by td101. On the other hand, relatively low titers of virus (4 × 10^1, 6 × 10^1, 3.3 × 10^2, and 7.8 × 10^3 focus-forming units per ml) were obtained from tumors induced by td109.

Discussion

Genetic interaction between leukosis type virus and host cell genes has recently attracted attention as a possible mechanism for the formation of sarcoma-inducing viruses. First, most strains of murine sarcoma virus were obtained from tumors experimentally developed after serial passage of leukemia virus in rats or mice (23–26). Molecular hybridization analysis revealed that specific sequences, that were gained by these sarcoma viruses, and are thus apparently responsible for sarcomagenic transformation by these viruses, are of cellular origin. The specific sequences of rat-derived sarcoma virus are not homologous to those of mouse-derived virus, and the specific sequences of each virus are homologous to cellular DNA of their respective animal species (27–32). Second, all avian sarcoma viruses have been obtained from tumors found in the field. No example of the derivation of avian sarcoma virus from leukemia virus has been reported. However, the presence of genetic information specific to the src gene of RSV has been demonstrated within DNA and RNA of normal tissues of various avian species by molecular hybridization (15, 33, 34). Third, in addition to these endogenous src genes, chicken cells are known to contain endogenous oncovirus genetic information which is homologous to other portions of oncovirus genome (35–37). Genetic recombination of these endogenous viral genes with exogenously infected leukosis viruses has been shown to yield a new leukosis-type virus, RAV-60, with the envelope glycoprotein gene specific to the endogenous virus (38, 39).
FORMATION OF NEW AVIAN SARCOMA VIRUS
Fig. 1. Cells transformed by SR-B and r-ASV in chicken embryo fibroblasts at 7 days after infection. (A, B) r-ASV 106; (C, D) r-ASV 144; (E, F) SR-B-RSV-A.
In a previous study, we found that the src gene in some of the td mutants of SR-RSV is only partially deleted (17). If recombination with endogenous (cellular) sarcoma-specific sequences is the key to the genesis of sarcoma virus, these partially retained src sequences of td mutants may greatly facilitate an interaction that would increase the frequency of formation of sarcoma virus. This consideration was the rationale behind our first attempts to generate sarcoma viruses by inoculation of td virus into 1-day-old chickens in this study.

The formation of tumors and the recovery of sarcoma-inducing virus, however, might be attributed to other more trivial reasons. First, parental SR-RSV might still be present at very low titer in td virus preparations, and be responsible for these phenomena. This seems, however, highly unlikely for a number of reasons. The original isolates of each td mutant have been extensively tested for focus-forming virus in tissue culture (17). The absence of tumor formation at the site of inoculation in this study can be considered as another line of evidence for the absence of transforming virus, since other experiments by us showed that tumor formation was at least as sensitive as focus formation in tissue culture for detection of infectious SR-RSV-A particles.

Furthermore, we have purified the td viruses by the limiting dilution method in which final virus stocks were isolated from cultures infected with a $10^{-6}$ dilution of the original stock. The presence of $10^7$ infectious units of nontransforming virus (RAV or td virus) per $10^6$ cells is known to cause 70-90% suppression of infection by simultaneously added RSV (18). Thus, the failure to detect focus-forming virus in the td mutant stocks (1 ml) indicates that the titer of RSV, if any, in the original td virus stocks was not $>10$ focus-forming units per ml. The probability of recovering RSV in the cultures infected with a $10^{-6}$ dilution of a td virus stock would then be below $10^{-5}$. td101 was further purified by repeating this procedure. The fact that td107A was no longer active in tumor induction demonstrated that the limiting dilution procedure does effectively eliminate a smaller component from a mixture of viruses.

If the tumors were formed by a contaminant SR-RSV, we should have recovered SR-RSV from the tumors. As shown in Fig. 1, the loci formed by all of the r-ASV isolated had similar morphologies, but were quite different from those of SR-RSV.

One might argue that some of the td mutants are not deleted within the src gene, but contain point mutations; the difference in the size of the RNA of these mutants could be deletions in other portions of the genome. In this case, spontaneous reversion of the point mutations could be responsible for tumor formation. Experiments described in this paper do not exclude this possibility. However, preliminary experiments analyzing the content of src gene information in the RNA of td mutants seems to indicate that the deletions indeed occurred within the src gene. For example, DNA complementary to the src gene of SR-RSV hybridized only 22% with td101 RNA. (W. S. Hayward and H. Hanafusa, unpublished results).

As another explanation, one can consider that infection by td virus might have induced an endogenous sarcoma virus. However, the endogenous src gene and the other viral genes essential for replication are apparently integrated into different chromosomes of normal chicken cells (40). Thus, the formation of transforming virus by simple induction seems highly unlikely. Furthermore,
the differences in the virus titers of r-ASVs recovered from tumors induced by td101 and 109 suggests that some properties of r-ASV are dependent on the original td virus. The fact that r-ASV belongs to the same subgroup A as td viruses also supports the above suggestion, since the endogenous viral sequences in chicken cells appear to contain only subgroup E specific information for envelope glycoproteins (36-38).

Thus, tumor formation by td virus and the recovery of r-ASV can be best explained by an assumption that td mutants which contain partial deletions in the src gene become converted to r-ASV after genetic interaction with some host cell component. Preliminary studies indicate that the RNA of some isolates of r-ASV is in fact restored to the size of the RNA of SR-RSV. Whether or not this is due to recombination with the cellular sarcoma-specific sequences should be clarified by a comparison of the sequences of the src genes of r-ASV, SR-RSV, and chicken cells.

The recovered virus is considered to be a sarcoma virus, based on many characteristics of in vitro and in vivo infection. Avian erythroblastosis virus is known to produce sarcomas by injection into the wing web, and causes cell transformation in fibroblast cultures (41, 42). However, sarcoma production by r-ASV was much faster and more consistent than that by erythroblastosis virus. Infection by the latter virus often results in erythroblastosis (8-10 days after infection) before sarcomas are formed (42). Within the 3-wk observation period, chickens inoculated with r-ASV produced sarcomas but none of them developed erythroblastosis. Finally, r-ASV caused more readily identifiable cell transformation in fibroblast cultures than erythroblastosis virus.

We have previously demonstrated that some td mutants can recombine with a temperature-sensitive mutant of RSV, TS68, and have considered this to be evidence for the presence of a partial src gene in these mutants (17). The fact that all of these mutants, td107, 108, and 109, were capable of producing r-ASV supported this idea. However, some mutants, td101 and 105, were negative for recombination with TS68 and yet positive for r-ASV formation. This may be a result of the requirement of a specific region of the src gene in the td mutant to replace the TS lesion of TS68, whereas recombination with the cellular src gene may not require a specific set of src sequences in the td mutants.

In general, the size of td virus RNA described in the previous paper (17) correlated with its tumor-producing capacity. The td viruses with larger RNA, td107 and 108, were tumorigenic whereas td106 was not. However, td101 and 109, which were shown to be similar to a standard td virus in the size of RNA, were active in the formation of r-ASV. The reexamination of the size of these td virus RNAs and their correlation with the recovery of r-ASV will be described elsewhere together with more detailed properties of some of the r-ASV isolates.

The period required for the tumor formation after injection with td viruses would be shorter than 60 days if chickens were examined by autopsy rather than by palpation. If one assumes that tumor formation was initiated by r-ASV after it had been generated and that the tumor formation required about 15 days, then the time period required for the generation of r-ASV would be about

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45 days. The requirement of this long period could be a reflection of the fact that genetic interactions leading to the formation of a stable recombinant between td virus and cellular genes is a relatively rare event. But, at the same time, it occurs rather consistently as shown by the formation of tumors in a high percentage of td virus-infected chickens.

Transforming virus has not been detected in tissue culture cells infected with td virus. Murine sarcoma viruses have also been obtained only after the passage of leukemia virus in vivo. It remains to be determined whether this is due to a difficulty in performing such long-term experiments in tissue culture, to much greater numbers of cells involved in in vivo infection, to higher susceptibility of neighboring tissues in vivo to immediate transformation by newly formed r-ASV or to some specific physiological conditions in animals which are not available in tissue cultures.

In studies of biological activities of avian leukosis viruses, occasional formation of sarcomas in chickens has been described (20, 41, 43, 44), but no report has been made of the direct recovery of a typical sarcoma virus. In this study, RAV-1, included as one of the controls, did not produce sarcomas. It seems clear that td viruses containing a partial src gene have an advantage over other standard leukosis viruses for the generation of r-ASV. A 26S RNA containing endogenous (cellular) src genetic information was identified in normal chicken cells, and this RNA species does not contain sequences homologous to other portions of the viral genome (45). The presence of the partial src gene in td virus could then be crucial since it would provide homologous sequences which may be required for recombination with the endogenous src gene. Further studies on this system should provide information concerning relationships between the cellular src sequences, present in normal cells, and carcinogenesis.

Summary

Transformation-defective (td) mutants of the Schmidt-Ruppin strain of Rous sarcoma virus (RSV), which contains deletions in the gene responsible for transformation (src gene), are unable to transform chicken embryo fibroblasts in vitro. Injection of some of these td mutants into newborn chickens resulted in the formation of sarcomas from which sarcoma virus was unfailingly recovered. The possibility that transforming RSV was present in the td virus preparations was excluded by further purification of the td viruses. Morphology of the foci induced by the newly recovered sarcoma virus was distinct from that of foci induced by the parental Schmidt-Ruppin strain of RSV. It is suggested that the new sarcoma virus was generated as a result of the genetic interaction between the genomes of td virus and chicken cells.

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