THE EFFECT OF TRANSFORMATION-DEFECTIVE AVIAN ONCORNAVIRUS MUTANTS ON TUMOR ANTIGEN EXPRESSION

R. Kurth and J. A. Wyke

Department of Tumor Virology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2A 3PX, England

R. R. Friis, L. Rohrschneider, and H. Bauer

Institut für Virologie, Fachbereich Humanmedizin, Justus Liebig Universität, D63 Giessen, West Germany

The recent isolation of conditional (temperature sensitive) and nonconditional transformation-defective mutants of avian sarcoma virus strains has facilitated the investigation of the effect of virus transformation on the cell's phenotype, e.g., with respect to morphology, growth pattern, or cell surface antigenicity. Special emphasis was laid on elucidating the correlation between transformed phenotype and tumor antigen expression.

All of the tested nontransforming deletion mutants and the majority of the temperature-sensitive mutants were unable to induce tumor antigens in phenotypically untransformed cells. However, 3 temperature-sensitive mutants were found which were able to support the expression of tumor specific surface antigens even at restrictive temperature, when cells otherwise exhibited a normal phenotype. The theoretical and practical implications of this association between normal phenotype and tumor antigen expression are discussed.

INTRODUCTION

Malignant cell transformation by oncogenic viruses leads to profound changes in the immunology and biochemistry of the cell surface membrane (CSM). These alterations may be either the cause or a secondary consequence of the cell's transformed phenotype; the most significant manifestation of which is the release of tumor cells from homeostatic growth control. The characterization of cell surface components which are strictly tumor associated may allow the recognition of those cell surface receptors which mediate contact inhibition of growth and subsequently lead to the elucidation of this mechanism (discussed in reference 1).

In the past few years we have studied malignant cell transformation caused by avian leukosis-sarcoma viruses (ALSV), concentrating in particular on the effects on the cell's phenotype and on the CSM. ALSV are single-stranded oncornaviruses, and ASV can easily be distinguished from ALV on the basis of their in vitro transforming abilities (Table 1). Even though ALV are normally unable to transform in vitro fibroblasts of the natural host, the chicken, they are fully oncogenic in vivo, causing mainly neoplasias of the hematopoietic system. Both ALV and ASV can be divided into 7 subgroups according to the serological specificity of their viral envelope antigens (VEA). These antigens are also responsible for the induction of subgroup-specific neutralizing antibodies, which are in general specific for all viruses of a given subgroup, as well as for the interference pattern observed in vitro in superinfection experiments. The ALSV system has recently been reviewed especially with respect to cell transformation and its consequences for the CSM (1–3).

In vitro studies on the phenotype of ALSV-transformed avian and mammalian cells have led to the detection, immunological characterization, and biochemical isolation of at

TABLE I. The Avian Leukosis Sarcoma Virus (ALSV) System

Avian sarcoma viruses (ASV)	Avian leukosis viruses (ALV)
in vitro	
Able to transform chicken embryo fibroblasts	Normally unable to transform chicken embryo fibroblasts
in vivo	
Cause fibrosarcomas	Can cause lymphoproliferative diseases, erythroblastosis, and occasionally other carcinomas

Examples of ASV and ALV strains of different subgroups

Subgroup	ASV	ALV
A	Schmidt-Ruppin strain 1 (SRV-1)	Nontransforming (NT) SRV-1*
	Prague strain A (Prague-A)	RAV-1†
B	Prague strain B (Prague-B)	RAV-2
		Avian myeloblastosis virus-B (AMV-B)
C	Bratislava-77 strain (B-77)	RAV-49
D	Schmidt-Ruppin strain D (SRV-D)	RAV-50
		Nontransforming (NT) SRV-D
E		RAV-O; RAV-60
Endogenous chicken virus		Induced leukosis virus (ILV)
F		RAV-61
Endogenous virus of Ring-neck pheasant		Ring-neck pheasant virus (RPV)
G		Golden pheasant virus (GPV)
Endogenous virus of Golden pheasant		

*Nontransforming mutants were obtained after hydroxylamine treatment of SRV-1 or SRV-D (13).
†RAV, Rous associated virus.

least one tumor specific surface antigen (TSSA) which does not seem to be a component
of the virus particle (4–9). This conclusion is based on the following three observations:
(i) neither intact nor disrupted ALSV particles are able to absorb antibody or lympho-
cytes directed against TSSA (4–7, 9); (ii) virus particles do not contain a 100,000 daltons
Mol wt major structural polypeptide, the size of TSSA (reviewed in references 1, 2);
(iii) direct radioimmunoprecipitation of disrupted virus particles with anti-TSSA anti-
body does not yield TSSA (unpublished observations). Because of its unique character-
istics (Table II) this molecule warrants further detailed studies with the aim of de-
fining its function in the transformation process.

     The recent isolation of transformation-defective ASV mutants (reviewed in
reference 10) enabled us to study their effect on the phenotype and CSM of infected avian
and mammalian cells. We were particularly interested in the mode of expression of TSSA
in mutant-infected but phenotypically normal cells.

TABLE II.   Characteristics of Avian Leukosis Sarcoma Virus Induced TSSA

	Reference no.
1. Expressed on the surface membrane of all ALSV-transformed cells tested.	4–7, 18
2. Absent on productively infected, nontransformed cells.	4, 5
3. Induces humoral and cellular cytotoxic immunity.	5, 6, 18
4. Cross-antigenic on tumors arising in different species.	6, 7, 17, 24–26
5. Group specific for all ALSV strains tested.	4–7, 18
6. Antigenically different from the three embryonic cell surface antigens identified on mouse and chicken fibroblasts.	8
7. Not identical with virus envelope antigens.	4, 5, 17, 18, 22, 23, 27
8. Peripheral 100,000 daltons Mol wt glycoprotein on transformed chicken cells.	9, 19

METHODS

     The details of the methods have been described elsewhere (11, 12).

Cells

     Chicken embryo fibroblasts (CEF) were derived from embryos of a flock of
randomly inbred L-15 chickens kept now at the Institut für Virologie, Universität
Giessen, West Germany. Normal rat kidney (NRK) cells are derived from an Osborn-
Mendel rat and represent a permanent cell line which can be infected by ASV of subgroups
C and D.

Viruses

     Nontransforming (NT) presumed deletion mutants of the ASV strains Schmidt-
Ruppin A and D (NT-SRV-A and NT-SRV-D, respectively) have been described by Graf
et al. (13). The biology of transformation-defective temperature-sensitive (ts) mutants
of Prague strain of Rous sarcoma virus (subgroup A) and the B77 strain of ASV (sub-
group C) has been described by Wyke (10, 14). The NT-deletion mutants (13) as well as
the transformation-defective ts mutants (10) were originally selected for their inability
to transform CEF in vitro or to form colonies in semisolid agar, respectively. Whereas
the NT mutants are nonconditional, the ts mutants transform at 35°C (permissive
temperature). The ts mutants can be classified into T and C classes depending on whether

their ts defect at nonpermissive temperature involves transformation only (T-class) or transformation and replication coordinately (C-class).

Sera

Anti-TSSA sera were prepared by repeatedly injecting subtumorigenic doses of ASV into the wing web of adult chickens (5). It was found originally that only the sera of those birds with the highest virus-neutralizing titer also possessed a suitable level of anti-TSSA antibody (4). Normal chicken sera from birds of the same flock served as negative control.

Assay for TSSA

We now know that the number of TSSA molecules expressed on the transformed cell surface is very low when compared with other viral, xeno- or allogenic antigens (12). Therefore the most sensitive immunological techniques which are available should be applied for its detection. In our hands these techniques involve the use of radiolabeled antibody (11, 12) and a combination of detergent solubilization followed by radio-immunoprecipitation (9).

For the detection of TSSA on mutant infected cells we modified the paired radio-actively labeled antibody technique (PRILAT; 11) in which the same quantities of normal and anti-TSSA chicken IgG are labeled with either ^{125}I or ^{131}I, and their absorption is measured to normal and mutant infected target cells growing in 16 mm microculture dishes.

The inclusion of normal IgG in the incubation mixture quantitates nonspecific absorption. To obtain the required specificity in the reaction for TSSA, all experiments were performed with anti-TSSA serum produced by immunizing chickens with the Schmidt-Ruppin virus strain of subgroup D. Target cells were infected by virus mutants derived either from subgroups A or C, so that no subgroup-specific virus envelope antigens could influence the absorption of antibody. Furthermore, normal and antisera were absorbed with uninfected cells derived from the same embryo which was used to yield the mutant-infected target cells. From the specific radioactivity of the individual IgG preparation, the specific absorption was calculated as the difference between absorption of anti–TSSA IgG minus normal IgG and was expressed as number of anti-TSSA antibodies absorbed per cell.

Isolation and iodination of IgG inevitably results in some loss of antibody titer. Because good anti-TSSA sera are somewhat difficult to obtain, we recently also used an indirect antiglobulin technique in which parallel 16 mm cultures of normal or mutant infected cells were first incubated with normal or antisera, and absorption was subsequently quantitated by the addition of iodinated rabbit anti-chicken IgG antibodies (12).

RESULTS

NT-deletion mutants of ASV (13) are similar to ALV in that they no longer are able to transform CEF in vitro, whereas there is no apparent change in successful virus replication. Like ALV, they have also lost the ability to induce TSSA in CEF (5). No NT-ASV or ALV have yet been found to cause TSSA expression in CEF, indicating that this antigen is closely associated with the process of malignant transformation. NT-ASV have not yet been introduced into mammalian cells.

Ts-mutants of ASV, many selected for their inability to induce colony formation in semisolid agar, have also lost the capacity to induce focus formation at 41°C (in cases

of infected CEF; 10, 11) or 40°C (in cases of infected NRK cells; 12, 15). Similarly, mutant-infected cells growing under restrictive conditions (41°C) have lost a variety of parameters characteristic of tumor cells. Growth control is restored, and cell saturation density normalized (11, 12). Lectin agglutinability (11, 15, 16) and uptake of 2-deoxyglucose (11) likewise return to values shown by uninfected cells. In summary, until the TSSA studies had been performed, mutant-infected cells showed the loss of all tested tumor-specific characteristics at restrictive temperature.

Under permissive conditions (35°C), all mutant-infected cells were virtually indistinguishable from wild-type infected cells. They showed a transformed morphology, had lost contact inhibition of growth, grew to high saturation densities, and formed foci if grown under agar. Lectin agglutinability was facilitated, and sugar uptake enhanced. Likewise, no difference could be observed in the specific absorption of anti-TSSA antibodies (Table III).

This pattern of absorption of anti-TSSA antibodies became considerably more complex when the ts-mutant infected cells were grown under restrictive conditions. As could have been expected, wild-type (Prague-A or B77-C strains) and revertant (ts LA 399-r3/2) infected chicken and rat cells showed an unaltered TSSA expression, quite in agreement with the persistence of their transformed phenotype (11, 12). However, at least three virus mutants (ts LA23, ts LA24 and ts LA31) induce TSSA at both temperatures in both cell types, despite the apparently normal phenotype of the infected cells at high temperature. At first sight this seems to abolish the thus far stringent association between TSSA expression and phenotypic transformation, but two hypothesis will be discussed below which might explain this discrepancy.

TABLE III.   TSSA Expression in ASV Mutant-Infected Chicken Embryo Fibroblasts and Normal Rat Kidney Cells

Mutant number	Temperature-sensitive mutant class	TSSA Expression	
		35°C permissive	40°C/41°C nonpermissive
Uninfected	—	—	—
Wild type	—	++	++
tsLA23	T	++	+
tsLA24	T	++	±
tsLA25	T	++	++
tsLA29	T	++	++
tsLA31*	T	++	++
tsLA33*	T	++	±
tsLA334*	C	++	—
tsLA336*	C	++	—
tsLA339	C	++	—
tsLA339-r3/2†**	—	++	++

T: T-class ts mutants, transformation-defective, replication unaltered.
C:C-class ts mutants, transformation- and replication-defective.
*Not tested in NRK
†Not tested in CEF
**Revertant to wild type
++:absorption of 3–4 × 10^4 anti-TSSA antibodies/cell
 +:absorption of 1–2 × 10^4 anti-TSSA antibodies/cell
 ±:absorption of <1 × 10^4 anti-TSSA antibodies/cell
 —:absorption of <0.5 × 10^4 anti-TSSA antibodies/cell
Some of the data summarized in this Table are taken from references 11 and 12.

The mutant ts LA25 is truly leaky, and infected cells express some residual trans-
formed characteristics at restrictive temperature (11, 12), which is also reflected by the
lowered but still clearly positive level of TSSA expression. Cells infected by all other
tested ts mutants show either a very low (ts LA 29, ts LA33) or no detectable level
(C-class mutants ts LA334, ts LA336, ts LA339) of TSSA under restrictive conditions,
which corresponds fully with their normalized phenotype.

DISCUSSION

In the ALSV system, TSSA seems to represent a unique CSM molecule (Table II)
since it cannot be detected in virus particles yet seems to protect animals against tumor
challenge (17, 18). The recent isolation and purification of TSSA (9, 19) opens new
possibilities of obtaining stronger antisera and looking for the function of this molecule.

It should be stressed, however, that TSSA is a comparatively weak immunogen, which
makes its detection and characterization difficult because specific high-titer antisera are
not easy to obtain. This, however, is not too surprising in light of the fact that the number
of antigenic sites of TSSA is probably below 5×10^4 per cell (11, 12), which is consider-
ably lower than the number of virus envelope antigenic sites inserted into the CSM upon
infection ($>3 \times 10^5$/cell; unpublished observations).

As mentioned above, malignant transformation of cells from different avian and
mammalian species by a wide variety of ALSV strains always leads to the expression of
TSSA on the cell surface (1–3). Conversely, fibroblasts infected but not transformed
by ALV strains do not seem to synthesize TSSA. All NT-deletion mutants and the
majority of the transformation-defective ts mutants of ASV are likewise unable to in-
duce TSSA in phenotypically normal cells. However, three T-class ts mutants (ts LA23,
24, 31) with genetic defects which place them exclusively into two distinct categories (20)
have been found which seem to be able to support TSSA expression in otherwise pheno-
typically normal cells. This apparent contradiction might be explained in two ways.

The three mutants possess a late defect and virus replication at 41°C remains un-
altered. It could be expected that most (e.g., TSSA) but not all transforming proteins
are synthesized and that the block in the transformation process occurs at a
later metabolic stage than TSSA expression. In this case TSSA could still be a prerequisite
for malignant transformation, but its synthesis alone is not sufficient to establish and
maintain the transformed phenotype.

Alternatively, TSSA itself may be the ts-gene product, and under restrictive condi-
tions may still be synthesized, albeit in a functionally inactive form, and inserted into the
membrane. Such a situation would still allow its immunological detection. This hypoth-
esis is particularly attractive and likely if TSSA turns out to represent a tumor cell
surface associated enzyme, e.g., a protease or glycosyltransferase (reviewed in references
1, 21).

The significance of the temperature-independent expression of TSSA by the three
mutants lies in the possible theoretical and practical consequences. First, TSSA expression
is the first CSM marker which allows a further classification of ts mutants and may thus
be useful in future genetic studies. Second, the three TSSA-positive virus mutants to some
extent possess properties which should be a characteristic of potential vaccine viruses
against tumors; they induce TSSA without transforming the host cell (discussed in
references 11, 12). Ts mutants are obviously not suitable candidates for real vaccine
strains, since their reversion rate is too high, but TSSA-positive NT-deletion mutants,

if they exist, are more likely candidates. All NT mutants of ASV tested so far were, however, also TSSA negative, but not enough mutant isolates have been tested to allow a final conclusion (11).

The observation that the three TSSA-positive ts mutants show the same pattern of antigen induction in both CEF and NRK cells does not necessarily mean that TSSA is virus coded. First, the probable size of the virus genome argues against this possibility, and second, one could just as well assume that a virus gene product induces cellular TSSA synthesis. Malignant transformation could lead to a very specific derepression of a cellular gene whose function is otherwise only needed for a limited period of time, for instance, during embryogenesis. The close similarity of all ALSV strains in many biological aspects could account for a common specific derepression of a cellular gene which in turn could explain the cross-reactivity of TSSA on all ALSV-transformed cells from different species. This hypothesis draws further support from the observation that transformation by ASV indeed leads to the reappearance of at least one embryonic antigen (8), but again it is too early for a final conclusion, and in vitro protein synthesis using viral RNA as messenger may be helpful in elucidating the origin of TSSA.

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