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Correlation between Tumor-specific Surface Antigens and *src* Gene Expression in Rous Sarcoma Virus-induced Rat Tumors*

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Abstract—*Immunization with Rous sarcoma virus (RSV)-induced mouse tumors or with SR-3Y1 or NY8-3Y1 rat fibroblasts transformed by a wild type RSV or an* env *gene deletion mutant of RSV induced complete transplantation resistance against an RSV-induced mouse tumor (CSA1M) in syngeneic hosts. On the other hand,* 10 *of* 19 *mice immunized with ts68-3Y1 rat fibroblasts transformed by an* src *gene temperature-sensitive mutant of RSV (permissive temperature:* 33–35°C) *could not reject the CSA1M. SR-3Y1, NY8-3Y1 and RSV-induced mouse tumors expressed a common tumor-specific cell-surface antigen (TSSA) detected by a syngeneic rat antiserum against NY8-3Y1. In ts68-3Y1, expression of the TSSA was temperature-sensitive, TSSA being detected only when ts68-3Y1 was cultivated at the permissive temperature. Immunoprecipitation showed that serum from a rabbit bearing an RSV-induced tumor detected a* 60-kdalton *protein in cell extracts from SR-3Y1 and NY8-3Y1. Antiserum to NY8-3Y1 failed to detect this protein. These results suggest that the tumor-specific surface antigen(s) on RSV-induced mammalian tumors was coded for by an RSV* src *gene that was not identical with the simple form of the* 60-kdalton *protein identified.*

INTRODUCTION

RETROVIRUS infection can induce a variety of growth-related, biochemical and morphological changes in the host cell [1]; a process called 'transformation'. Virus-directed changes in the antigenic constitution of the surface of transformed cells have also been demonstrated [2]. Such antigens, termed tumor-specific surface antigens (TSSA), have been assayed by transplantation experiments *in vivo* and by *in vitro* techniques. It has been reported that transforming proteins encoded by Abelson murine leukemia virus or feline sarcoma virus are localized on the plasma membrane and that these molecules can function as TSSAs [3, 4].

Avian sarcoma virus (ASV)-transformed cells from a variety of avian or mammalian species express TSSA [5–8]. Genetic analysis of ASV has identified and mapped four genes [9]: *env* coding for the glycoproteins of the viral envelope; *gag* coding for the internal structural proteins of the virion; *pol* coding for the RNA-directed DNA polymerase; and *src* coding for a non-structural protein that is necessary for both the initiation and maintenance of neoplastic transformation [1]. The product of the *src* gene has been identified as a phosphorylated polypeptide of mol. wt 60,000 daltons (designated $pp60^{src}$) by the use of antisera obtained from young rabbits bearing tumors induced by the Schmidt–Ruppin strain of Rous sarcoma virus (SR-RSV) [10].

We have investigated possible relationships between expression of TSSA and expression of the *src* gene.

MATERIALS AND METHODS

Cells

The cell lines used are listed in Table 1. A Fischer rat fibroblast cell line 3Y1, an SR-RSV-

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Table 1. Cell lines used in the experiments

Cell lines	Strain	Oncogenic agents	Histology
Rat			
SR-3Y1	Fischer	SR-RSV	Sarcoma
NY8-3Y1	Fischer	rdNY8*	Sarcoma
ts68-3Y1	Fischer	tsNY68†	Sarcoma
B77-3Y1	Fischer	B77-ASV	Sarcoma
Sp6	BDX	Spontaneous	Sarcoma
3Y1	Fischer		Fibroblast
Mouse			
CSA1M	BALB/c	SR-RSV	Sarcoma
CSA9F	BALB/c	SR-RSV	Sarcoma
CBr2F	BALB/c	SR-RSV	Brain tumor
S908D2	B10D2	SR-RSV	Sarcoma
B6SA1M	C57BL/6	SR-RSV	Sarcoma
B6SA3F	C57BL/6	SR-RSV	Sarcoma
C3SA1F	C3H	SR-RSV	Sarcoma
MCSA1F	BALB/c	Methylcholanthene	Sarcoma
MCSA4M	BALB/c	Methylcholanthene	Sarcoma
RL♂1	BALB/c	Radiation	Leukemia
BW5147	AKR	Spontaneous	Leukemia

*An *env* gene deletion mutant of SR-RSV.
†An *src* gene temperature-sensitive mutant of SR-RSV (permissive temperature: 33–35°C; non-permissive temperature: 30–40°C).

transformed 3Y1 (SR-3Y1), an *env* gene deletion mutant (rdNY8-SR)-transformed 3Y1 (NY8-3Y1) and an *src* gene temperature-sensitive mutant (tsNY68-SR)-transformed 3Y1 (ts68-3Y1) were kindly given by Dr. S. Kawai (the Institute of Medical Science, Tokyo University, Tokyo, Japan). Characterization of these mutant viruses and the infected cells has been described previously [11–13]. A B77 ASV-transformed 3Y1 (B77-3Y1) was supplied by Dr. H. Mitsui (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). The SR-RSV-induced mouse tumor cell lines CSA1M, CSA9F, CBr2F of BALB/c origin, B6SA1M, B6SA3F of C57BL/6 origin and C3SA1F of C3H mouse origin were also used [14]. An SR-RSV-induced B10D2 mouse tumor, S908D2, was given by Dr. S. Fujimoto (Kochi Medical University, Kochi, Japan). A spontaneously induced BDX rat sarcoma Sp6, methylcholanthrene-induced BALB/c mouse tumor cell lines MCSA1F and MCSA4M, a radiation-induced tumor RL♂1 (given by Dr. T. Takahashi, Aichi Cancer Institute, Nagoya, Japan) and a spontaneously induced AKR mouse leukemia BW5147 were also used. All cell lines were maintained in Dulbecco-modified Eagles' Medium (dMEM) supplemented with 5–10% newborn calf serum. Embryo cells from 13-day gestation BALB/c mouse embryos and normal spleen cells from a BALB/c mouse were also used.

Transplantation experiments

In tests for transplantation immunity, BALB/c mice (7 weeks old) were immunized with 5×10^6 mitomycin C-treated mouse cells or the same number of viable rat cells. Three weeks after the immunization, all recipients, including untreated mice, received 400 rad whole-body irradiation to avoid non-specific immunization effects. Next day, 1×10^5 viable CSA1M cells were inoculated subcutaneously into the syngeneic untreated and immunized mice. Developing tumors were followed by regular caliper measurements. The mice were kept for an observation period of 4 months.

Antisera

Antiserum to the major envelope antigen gp85 of avian myeloblastosis virus (AMV) and an SR-RSV-induced tumor-bearing rabbit (TBR-B1) serum were kindly provided by Dr. R. Ishizaki (Nippon Medical School, Tokyo, Japan). The characteristics of the TBR-B1 serum were described in detail previously [15]. Antiserum to SR-RSV *gag* gene products, including $Pr76^{gag}$, was donated by Dr. H. Mitsui. To obtain antisera to tumor-specific antigens, syngeneic rats were immunized by subcutaneous injection of both 5×10^4 viable NY8-3Y1 cells and 5×10^6 mitomycin C-treated cultured NY8-3Y1 cells. They subsequently received 20 weekly subcutaneous or intraperitoneal challenges with increasing numbers of viable cultured cells (1×10^5–1×10^7). Rats that remained tumor-free throughout the immunization period were bled individually from the retro-orbital sinus 4 days after the final intraperitoneal immunization, and their sera titered against the immunizing tumor cells. The

highest titered antisera were pooled and used for analysis of surface antigens.

Complement-dependent cytotoxicity (CDC) assays

A microassay was used: 2 μl of serial dilutions of antiserum from 1:10 in dMEM were injected under paraffin oil into wells of a Terasaki microplate (Falcon No. 3034) with an automatic Hamilton syringe. One thousand target cells in 1 μl were added to each well, and the plates were incubated for 20 min at 37°C. Subsequently, 1 μl of rabbit complement was added at dilutions of 1:3–1:12. Plates were further incubated for 45 min. Thereafter, 0.5 μl trypan blue was added to each well and the results were read microscopically. Cytotoxic index (CI) was calculated as follows: $CI = 1 - t/c$ (t = the percentage of unstained cells in the test sample, c = the percentage of unstained cells in the control sample). A CI exceeding 0.20 was regarded as positive.

Immunoprecipitation and polyacrylamide gel electrophoresis

Rat cells, grown in 60-mm culture dishes, were incubated for 14 hr in labeling medium containing 50 μCi of [^{35}S]-methionine (800–1200 Ci/mmol; Amersham Corp., Arlington Heights, IL, U.S.A.) per ml. Cells were washed in STE buffer (150 mM NaCl, 50 mM Tris-hydrochloride, 1 mM EDTA, pH 7.2) and lysed in RIPA buffer [150 mM NaCl, 50 mM Tris-hydrochloride, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% aprofinine, 1 mM phenylmethylsulfonyl fluoride, pH 7.2]. Lysates were incubated for 15 min at 4°C with 125 μl of normal rat serum and the fixed Cowan I strain of *Staphylococcus aureus* was added as described by Kessler [16]. The adsorbed complexes were then clarified by centrifugation at 100,000 *g* for 30 min at 4°C. For immunoprecipitation, samples of the labeled cell extract were incubated for 30 min at 4°C with either 5 μl of anti-NY8-3Y1 sera or 1 μl of TBR-B1 serum. The resulting immune complexes were removed from solution by the addition of the fixed Cowan I strain of *S. aureus*. The adsorbed complexes were washed and subsequently eluted into 80 μl of sample buffer (0.07M Tris-hydrochloride, 11% glycerol, 3% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol, pH 6.8) by heating for 5 min in a boiling water bath. Analyses of such eluted immunoprecipitates were carried out by gel electrophoresis using SDS gels (10% acrylamide) by the method of Laemmli [17]. A low-molecular-weight calibration kit (Pharmacia), containing phosphorylase b (94k), bovine serum albumin (67k), ovalbumin (43k) and carbonic anhydrase (30k), was used for the molecular weight determination. The gels were fixed and stained in 50% methanol, 10% acetate containing 0.05% Coomassie brilliant blue, destained and dried. Gels containing [^{35}S]-methionine-labeled proteins were prepared for photofluorography [18] and exposed to Kodak XS film.

RESULTS

Transplantation experiments

To examine the relationship between tumor-specific transplantation antigen (TSTA) and RSV *src* gene expression, transplantation experiments were undertaken (Table 2). When an RSV-induced BALB/c mouse tumor CSA1M was subcutaneously inoculated into syngeneic mice, the tumor grew and killed the hosts, the mean survival time being 34 days. All mice immunized with CSA1M or CSA9F, another RSV-induced BALB/c mouse tumor, rat fibroblasts transformed by wild type RSV (SR-3Y1) or an *env* gene deletion mutant of RSV (NY8-3Y1) rejected the CSA1M cells totally. The mice immunized with rat fibroblasts transformed by an *src* gene temperature-sensitive mutant of RSV, ts68-3Y1, showed reduced rejection activity against CSA1M. Ten of 19 such mice did not reject CSA1M and were killed by tumor growth, the mean survival time being 57 days. No immunity to CSA1M challenge was acquired by mice immunized with a chemically induced mouse tumor, a murine leukemia or non-infected 3Y1 cells.

Table 2. Transplantation resistance against an SR-RSV-induced BALB/c mouse tumor (CSA1M) in syngeneic mice immunized with mouse or rat tumors

Cell lines used for immunization*	Incidence of lethal growth of CSA1M† in immunized hosts	Mean survival (days)
Mouse RSV tumors		
CSA1M	0/7	
CSA9F	0/6	
Other mouse tumors		
MCSA4M	6/6	40
RL♂1	3/3	37
Rat RSV tumors		
SR-3Y1	0/8	
NY8-3Y1	0/9	
ts68-3Y1	10/19	57
Normal rat cells		
3Y1	5/5	39
None	5/5	34

*5×10^6 cells were inoculated.

†1×10^5 CSA1M cells were challenged 22 days after immunization.

Serological analysis

To assess the expression of ASV structural components at the cell surface, ASV-induced tumor cells were examined by CDC assays with rabbit antiserum to AMVgp85 and rat antiserum to SR-RSV $Pr76^{gag}$ (Table 3). All cells transformed by SR-RSV or its mutants were insensitive to these antisera. B77-3Y1 reacted with the anti-AMVgp85 serum, with a cytotoxic titer of 160, but not with the anti-SR-RSV $Pr76^{gag}$ serum. NY8-3Y1 cells could induce cytotoxic antibodies to themselves in syngeneic rats (Table 3, Fig. 1). With the antiserum, SR-3Y1, 3Y1 transformed by B77 ASV (B77-3Y1) and cultured cell lines of SR-RSV-induced mouse tumors were assayed for the expression of a common TSSA by CDC assays. The anti-NY8-3Y1 serum reacted with SR-3Y1, B77-3Y1 and all SR-RSV mouse tumors. The antiserum was cytotoxic for 3Y1 transformed by an *src* gene temperature-sensitive mutant ts68 of SR-RSV (ts68-3Y1) cultivated at the permissive temperature, but not at all when it was cultivated at the non-permissive temperature. The anti-serum was not reactive with a spontaneously induced rat tumor Sp6, non-infected 3Y1 cells, methylcholanthrene-induced mouse tumors, murine leukemias, mouse embryo cells and normal spleen cells.

Table 3. Cytotoxic sensitivity of rat and mouse cells to antisera against ASV structural components and syngeneic antiserum against a rat fibroblast cell line transformed by an env *gene deletion mutant of SR-RSV (NY8-3Y1)*

Cells	Cytotoxic titer* with antisera against: AMVgp85^{env}	RSVPr76^{gag}	NY8-3Y1
Rat ASV tumors			
SR-3Y1	—	—	320
NY8-3Y1	—	—	640
ts68-3Y1 (p)†	—	—	80
ts68-3Y1 (np)‡	—	—	—§
B77-3Y1	160	—	20
Other rat tumor			
Sp6	—	—	—
Normal rat cells			
3Y1	—	—	—
Mouse RSV tumors			
CSA1M	—	—	40
CSA9F	—	—	20
CBr2F	—	—	80
S908D2	—	—	40
B6SA1M	—	—	80
B6SA3F	—	—	80
C3SA1F	—	—	40
Other mouse tumors			
MCSA1F	—	—	—
MCSA4M	—	—	—
RL♂1	—	—	—
BW5147	—	—	—
Normal mouse cells			
BALB/c embryo cells	—	—	—
BALB/c splenocytes	—	—	—

*Reciprocal of serum dilution producing more than 0.20 of cytotoxic index. Mean value of three repeated experiments.
†Cultivated at the permissive temperature.
‡Cultivated at the non-permissive temperature.
§No positive reaction at 1:10 serum dilution.

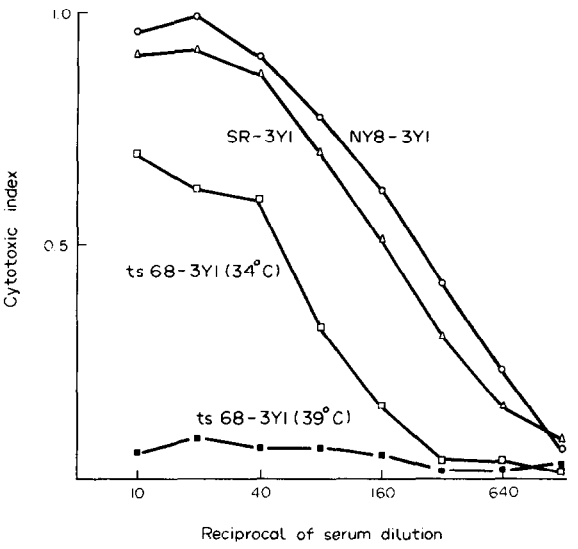

Fig. 1. Titration of syngeneic anti-NY8-3Y1 serum with SR-3Y1 (Δ——Δ), NY8-3Y1 (O——O), ts68-3Y1 cultivated at the permissive temperature (34°C) (□——□) *or ts68-3Y1 cultivated at the non-permissive temperature* (39°C) (■——■).

Polyacrylamide gel electrophoresis

An RSV-induced tumor-bearing rabbit (TBR-B1) serum immunoprecipitated a 60,000-dalton polypeptide (p60) from [^{35}S]-methionine-labeled extracts of both SR-3Y1 and NY8-3Y1 but not from labeled extracts of non-infected 3Y1 cells (Fig. 2). In addition, the TBR-B1 serum immunoprecipitated a 76,000-dalton (p76) and a 105,000-dalton polypeptide from labeled extracts of NY8-3Y1, but not from labeled extracts of both SR-3Y1 and non-infected 3Y1. Two out of three anti-NY8-3Y1 sera which detected common TSSA immunoprecipitated both 76,000-dalton (p76) and 105,000-dalton polypeptides from labeled extracts of NY8-3Y1 cells but not from labeled extracts of either SR-3Y1 or non-infected 3Y1 cells. Another batch of anti-NY8-3Y1 serum did not precipitate these polypeptides. No anti-NY8-3Y1 sera immunoprecipitated the 60,000-dalton polypeptide from either SR-3Y1 and NY8-3Y1 cells.

DISCUSSION

In our present studies the establishment of transplantation immunity to a syngeneic RSV-induced mouse tumor was observed in mice immunized with rat fibroblasts transformed by a wild type RSV or an *env* gene deletion mutant of RSV, SR-3Y1 and NY8-3Y1 respectively. These findings suggested the presence of a group-

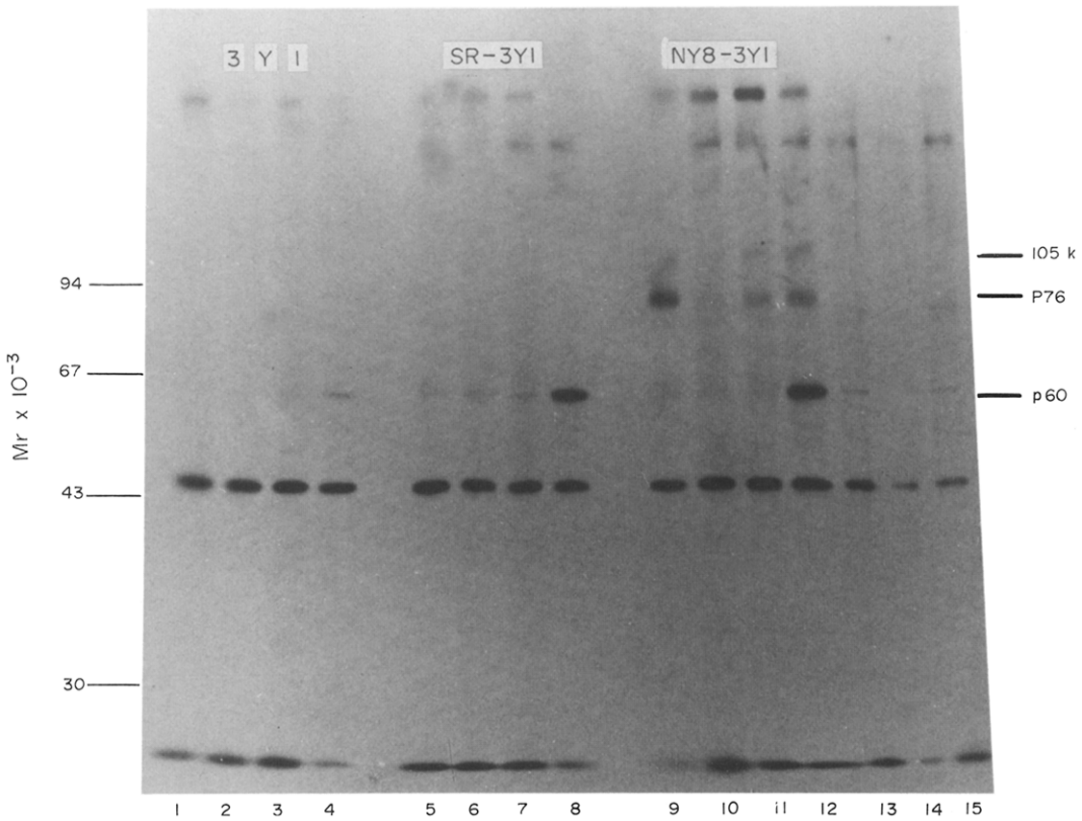

Fig. 2. Immunoprecipitation from 3Y1, SR-3Y1 and NY8-3Y1 labeled by [^{35}S]*-methionine. Tracks* 1–4 *and* 13 *contain immunoprecipitates from non-infected 3Y1 cells; tracks* 5–8 *and* 14 *contain immunoprecipitates from SR-3Y1; and tracks* 9–12 *and* 15 *contain immunoprecipitates from NY8-3Y1. Antisera tested were the following: anti-NY8-3Y1 serum* 1, *tracks* 1, 5 *and* 9; *anti-NY8-3Y1 serum* 2, *tracks* 2, 6 *and* 10; *anti-NY8-3Y1 serum* 3, *tracks* 3, 7 *and* 11; *TBR-B1 serum, tracks* 4, 8 *and* 12; *normal rat serum,* 13–15.

specific TSTA on RSV-induced mouse and rat tumors, and no association between TSTA and RSV *env* gene expression. The transplantation immunity was clearly decreased when recipients were immunized with rat fibroblasts transformed by an *src* gene *ts* mutant of RSV, ts68-3Y1. Since the body temperature of the mice (38°C) is a nearly non-permissive temperature (39–40°C) for the *ts* mutants, the mutant-infected rat cells seem to lose TSTA in the mice because of their restrictive body temperature. These findings suggest that the TSTA expression is correlated with the RSV *src* gene expression.

Further, in this report, using syngeneic antiserum against NY8-3Y1, we have demonstrated a common TSSA detectable by CDC assays at the cell surface of ASV-induced rat and mouse tumor cells, again eliminating a critical association between the TSSA and *env* gene products. Expression of the TSSA is, however, definitely associated with RSV *src* gene expression. These findings are in keeping with several previous reports [19–22]. It is possible, therefore, that the *src* gene codes not only for $pp60^{src}$ but also for TSSA.

Barnekow *et al.* have recently reported that significant amounts of $pp60^{src}$ are exposed on the outer cell surface of chick embryo fibroblasts transformed by RSV and are also present in the supernatant medium [23]. Localization of $pp60^{src}$ on the outer cell surface contrasts with previous reports which showed this molecule to be solely in the cytoplasm [24–27]. They have pointed out that the discrepancy may be the result of variations in experimental procedure [23].

Phillips and Perdue identified a surface neoantigen on rat tumors induced by RSV by immunoprecipitation with IgG from a rat surgically relieved of a transplantable Rous sarcoma [28]. This plasma membrane component had a molecular weight of approximately 60,000 daltons.

We have demonstrated in this report that serum from an RSV tumor-bearing (TBR) animal immunoprecipitated a 60,000-dalton (p60) polypeptide corresponding to $pp60^{src}$ from extracts of both SR-3Y1 and NY8-3Y1 cells. The TBR serum also precipitated a 76,000-dalton polypeptide (p76), corresponding to an RSV core protein precursor $Pr76^{gag}$, and a 105,000-dalton polypeptide of unknown origin only from extracts of NY8-3Y1. Some anti-NY8-3Y1 sera detecting a common TSSA on RSV-induced rat and mouse tumors immunoprecipitated only p76 and 105k polypeptides from extracts of NY8-3Y1 but not from extracts of SR-3Y1. These results suggested that neither p60, p76 nor 105k polypeptide was associated with the TSSA.

Levinson *et al.* reported that the ts68-3Y1 cells which we used produced similar amounts of $pp60^{src}$ at the permissive and restrictive temperatures; the stability of the protein also being unaffected by the restrictive temperature [29]. In contrast, phosphorylation of the protein was greatly reduced at the restrictive temperature [29, 30]. Further, comparison of the thermolability of the purified *src*-specific phosphotransferase activities revealed that the enzyme obtained from the ts68-3Y1 was 7–10 times more thermolabile than the wild-type enzyme [31].

All these findings raised the possibility that the TSSA was not identical to the $pp60^{src}$ protein itself, but was related to functioning products derived from $pp60^{src}$. The *src* protein of the ts68-3Y1 cells may be as functionally inactive as TSSA at the non-permissive temperature despite its presence in the cell, either because of a change in its location within the cell or a change in its antigenic configuration. Alternatively, the TSSA may be a cell membrane molecule modified by the *src*-specific phosphotransferase. In either case, the molecule may be very labile and not preserved in detergent extracts, judging from the fact that we failed to detect immunoprecipitation of the antigen by using syngeneic antisera.

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