p53-Plus-*ras*-Transformed Rat Embryo Fibroblasts Express Tumor-Specific Transplantation Antigen Activity Which Is Shared by Independently Transformed Cells

ALAN B. FREY† AND ARNOLD J. LEVINE*

Department of Biology, Princeton University, Princeton, New Jersey 08544-1014

Received 10 July 1989/Accepted 5 September 1989

p53-plus-*ras*-transformed rat cell lines express a tumor-specific transplantation antigen that is common to a number (85%) of independently derived p53-plus-*ras*-transformed cell lines. This has been shown by immunizing rats with irradiated p53-plus-*ras*-transformed cells and demonstrating protection of these animals by subsequent live-cell tumor challenge. Several *c-myc*-plus-*ras*-transformed cell lines (54% of the lines tested) and one adenovirus E1a-plus-*ras*-transformed cell line (9% of those tested) were shown to share a common tumor-specific transplantation antigen by their ability to immunize a rat against a p53-plus-*ras* cell line challenge. Several experimental approaches have been used to fractionate and identify the antigen common to these cell lines. The experimental results reported here make it clear that the p53 protein common to most of these transformed cell lines is not likely to be the tumor-specific transplantation antigen.

Hamsters that bear simian virus 40 (SV40)-induced tumors produce antibodies directed against the virus-encoded large and small tumor antigen (T antigen) (1) and the cellularly encoded gene product p53 (9). Similarly, mice immunized with methylcholanthrene-induced tumor cells produce antibodies directed against p53 protein, which is expressed at high levels in these cells (4). Indeed, anti-p53 antibodies have been detected in humans with several different types of cancers (3). For all of these reasons, p53 has been termed a tumor antigen. In the case of the SV40 large tumor antigen, it has also been shown to be a tumor-specific transplantation rejection antigen (TSTA) (13). Immunizing animals with SV40 large T antigen can protect against subsequent challenges with live tumorigenic SV40-transformed cells (13). Mutant genomic and cDNA clones of p53, when cotransfected into rat embryo fibroblasts (REFs) with the T24 *ras* oncogene, produce transformed cell foci (7, 8). These foci are tumorigenic in immature syngeneic rats (6, 8). These observations provided the opportunity to investigate (i) whether p53-plus-*ras*-transformed cells express a TSTA activity, (ii) whether such an activity was common to all p53-plus-*ras*-transformed cell lines or even other cell lines transformed by different oncogenes, and (iii) whether such a common TSTA activity, if present, was the p53 protein itself or another entity. The results of the experiments presented here demonstrate that (i) p53-plus-*ras*-transformed cell lines do express a TSTA activity, (ii) such an activity is common to most p53-plus-*ras*-transformed cell lines and even some *myc*-plus-*ras*-transformed cell lines, and (iii) this TSTA activity is most likely not the p53 protein itself.

MATERIALS AND METHODS

Vector construction and cell lines. All p53 expression vectors have been described previously (7, 8, 12). The adenovirus E1A expression plasmid (pSVE1A) and the *myc* expression plasmid (pLTR*myc*), which has the human *c-myc* gene under the regulation of the murine sarcoma virus long

terminal repeat, have been described previously (10, 11). The T24 *ras* gene construct has also been described previously (7, 8, 12). Preparations of REFs, DNA transfections of these cells, and the cloning and isolation of foci have all been described previously (7, 8). The properties of these cell lines have been characterized (7, 8).

Western (immuno-) blot analysis of soluble proteins derived from REF cell lines. Frozen cell pellets were thawed in 0.3 ml of hypotonic lysis buffer (1% Nonidet P-40, 25 mM Tris hydrochloride, 1 mM EDTA, pH 8.0, containing 100 U of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride). Cell pellets were gently vortexed until suspended, allowed to incubate on ice for 15 min, and then sonicated three times for 10 s each at setting 5 on a Branson sonicator. Disrupted cells were centrifuged for 10 min at gMax in an Eppendorf centrifuge, and the extracted proteins were mixed with an equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer (10% sodium dodecyl sulfate, 30% glycerol, 5% mercaptoethanol). Samples were electrophoresed and blotted to nitrocellulose by using a Hoeffer electroblot apparatus. p53 protein was detected by using monoclonal antibody pab421 diluted 1:1 with 4% bovine serum albumin, and *ras* protein was detected by using sheep anti-human *ras* antipeptide antibody (Oncogene Sciences) diluted 1:100 in bovine serum albumin. After overnight incubation at 4°C, filters were washed with phosphate-buffered saline (PBS), and bound antibody was detected by incubation with 1 μ Ci of ¹²⁵I-protein A. Filters were again washed and then exposed to film for autoradiographic detection.

Immunization of rats. Five-week-old Fisher rats were anesthetized with halothane for subcutaneous injection between the shoulder blades with viable or irradiated cells or with cell extracts in a maximum volume of 0.3 ml. All injections were conducted on several animals, as indicated in the figure legends. After receiving injections (scheduled as indicated in the figure legends), rats were examined for tumor development at 3- to 5-day intervals. Different routes of immunization were investigated but no differences in results were noted. For some experiments, rats were immunized with antigens prepared in Freund adjuvant. In these

* Corresponding author.

† Present address: Department of Cell Biology, New York University Medical Center, 550 First Ave., New York, NY 10016.

cases, injections were administered intraperitoneally. For irradiation of cells, tissue culture plates of subconfluent cells were sealed with Parafilm and given 10,000 rads of gamma irradiation with a Gammacell 40 cesium source. Nonirradiated control cells were left sealed at 25°C for the equivalent time. Cells were then recovered by gentle trypsinization and washed twice in PBS.

Preparation and properties of cell lines. Primary REF were prepared from 14- to 15-day-old Fisher rat embryos or from the back skins of 19-day-old Fisher 344 rat embryos. A total of 2.8×10^5 secondary or tertiary REF cells were seeded in 60-mm dishes and 24 h later were cotransfected with 1.25 μ g of the T24 plasmid containing Ha-*ras* and 1.25 μ g of pLTRp53cG (7, 8), a murine mutated cDNA-genomic hybrid clone under the control of the Harvey murine sarcoma virus long terminal repeat, by using the modified calcium phosphate procedure (7, 8). The cells were transfected for 17 to 18 h, refed with Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, and allowed to recover for an additional day before being passaged at a 1:3 dilution. Cells were refed at 4- to 5-day intervals. Morphologically distinct foci were visible 7 to 10 days after subculture, and selected foci were cloned and expanded into cell lines. All of these cell lines had a high saturation density and produced tumors in syngeneic animals (7, 8).

Cell fractionation of TSTA activity. Localization of immunogenic activity in p53-plus-*ras*-transformed cell line B3 was performed as follows. Confluent monolayers of B3 cells were harvested by dislodging them with a gentle stream of cold PBS, and cells were collected by centrifugation. Cells were suspended in hypotonic buffer containing protease inhibitors and allowed to swell in ice for 20 min. Cells were then lysed by Dounce homogenization, and the cell debris and unbroken cells were removed by centrifugation at $500 \times g$ for 10 min. Nuclei were then pelleted at $1,500 \times g$, and the postnuclear supernatant (which contained total cellular membrane vesicles) was recovered by centrifugation at $150,000 \times g$ for 60 min. The resultant pellet of total cellular membranes was suspended by gentle homogenization in PBS and washed by centrifugation as described above. Total cellular membranes were suspended at a concentration of 1 to 2 mg/ml in 0.1 M sodium carbonate, pH 11.5, and incubated on ice for 30 min. Membranes were recovered by ultracentrifugation for 60 min at $100,000 \times g$ and suspended in 0.1 M Tris hydrochloride, pH 7.5, containing protease inhibitors. The extracted fraction was neutralized by the addition of 3 M Tris hydrochloride, pH 7.5, and frozen in liquid nitrogen. The supernatant resulting from high-speed centrifugation of the total cellular homogenate was frozen in N_2 . Proteins contained in the nuclear fraction were extracted by stirring with 0.6 M NaCl for 30 min followed by centrifugation at $20,000 \times g$ for 30 min. This supernatant contained extracted nuclear proteins and was frozen in liquid nitrogen.

After protein concentration determination, the different subcellular fractions (total cellular homogenate, nuclear extract, alkali-extracted total cellular membranes, and cytosolic proteins) were used for tumor protection studies. Pairs of Fisher rats were immunized twice at 7-day intervals with 200 μ g of protein from each fraction. Rats were then challenged with 2.5×10^3 B3 cells, and tumor development was monitored as previously described.

This experiment was repeated two times with pairs of rats and fresh extracts derived from different preparations of B3 cells. Rats received total cell homogenate, nuclear extract, total cell membranes, or cytosolic extract which had, in some cases, been purified over an anti-p53 immunoaffinity

column (2). Material which was not retained on the affinity column (see Fig. 2, lane b) and material specifically eluted from the affinity column (see Fig. 2, lane c) were both used to inoculate rats (see Table 3).

RESULTS AND DISCUSSION

The experiments described here were carried out to determine whether cell lines transformed by the oncogenes p53 plus *ras* (6-8, 10) expressed a TSTA, and, if so, to characterize the nature of this activity. To address these questions experimentally, a p53-plus-*ras*-transformed Fisher rat cell line, called B3 (8), was characterized in some detail before it was adopted as a standard cell line in these studies. Inoculation of 10^5 B3 cells subcutaneously into a 5-week-old Fisher rat produced a palpable tumor (0.5 cm) in 10 to 14 days. The kinetics of tumor growth in rats are presented in Fig. 1a. As few as 100 B3 cells inoculated into a rat produced a detectable tumor in 35 to 70 days. The kinetics of tumor growth as a function of the inoculum size (100 , 200 , 2×10^3 , 2×10^4 , or 2×10^5 cells) is presented in Fig. 1b. The B3 cell line is the most tumorigenic line of 15 p53-plus-*ras* REF cells (based upon this cell number assay) studied to date. Most p53-plus-*ras* REF cell lines will produce tumors in rats with 10^5 to 10^6 cells in 15 to 35 days. Finally, the B3 cells do indeed express a TSTA-like activity. A total of 2×10^6 B3 cells were irradiated with 10,000 rads and injected into rats. The challenge injection with live B3 cells 7 days later was carried out with increasing concentrations of cells (2×10^3 to 2×10^6 cells). The kinetics of tumor growth observed in this experiment are shown in Fig. 1c. No tumors were observed when 2,000 B3 cells were used as the challenge dose. Significant delays in the kinetics of tumor formation and growth were observed at higher challenge doses of B3 cells. This experiment demonstrates that B3 cells express a TSTA that can protect against itself. Fisher rats injected with 2×10^6 irradiated B3 cells one, two, or three times (at 7-day intervals) were all protected from B3 cell-induced tumors (zero of six rats in each case) when 2×10^3 cells were used as a challenge dose (Table 1). When phosphate-buffered saline or normal REF cells (10^7 REF cells irradiated as described above) were used in the immunizing injection (one or three times), 100% of the rats (six of six rats in each case) developed palpable tumors in 21 days when 2×10^3 B3 cells were used as the challenge dose (Table 1). The minimum number of irradiated B3 cells able to confer protection from tumor formation by 2×10^3 B3 cells was determined to be between 2×10^4 and 2×10^5 irradiated B3 cells.

Having quantified all of these variables and determined the parameters for the transplantation rejection assay, the next question could be answered. Do all p53-plus-*ras*-transformed cell lines share a common TSTA activity or are they unique in this respect? Thirteen independently derived p53-plus-*ras*-transformed REF cell lines (7, 8) were irradiated and used to immunize at least two rats for each cell line. Each rat was challenged with a dose of live B3 cells (2×10^3 cells) which produced tumors in 100% of the unimmunized (or control REF-immunized) rats. Table 2 shows that 85% (11 of 13) of the p53-plus-*ras* cell lines protected rats from B3-induced tumors. When a similar experiment was carried out with *myc*-plus-*ras*-transformed REF cell lines or E1A-plus-*ras* REF cell lines, 54% (7 of 13) and 9% (1 of 11) of these cell lines, respectively, protected rats from B3-induced tumors. In all, about half of these cell lines tested (19 of 37 p53, *myc*, or E1A-plus-*ras* cell lines) protected rats from a B3 cell line tumor challenge. There was some specificity

FIG. 1. (a) Tumorigenicity of p53-plus-ras-transformed REF cell line B3 in syngeneic rats. Pairs of Fisher 344 rats were immunized subcutaneously with 2×10^5 cells (▲) as described in Materials and Methods. Control animals (●) received an equivalent number of untransformed REF cells or PBS buffer only. Animals were inspected at 3- to 5-day intervals for tumor development. (b) Titration of tumorigenic dose of B3 cells. Pairs of Fisher rats were injected with a single dose of different amounts of B3 cells (▲) as described in Materials and Methods. The dosages given each pair were as shown in the graph. Control animals (●) received 10^6 untransformed REF cells only. (c) Effect of immunization on B3 tumor development. Pairs of rats were immunized three times at 7-day intervals with 2×10^6 irradiated B3 cells (10,000 rad) as described in Materials and Methods. Control animals (●) received irradiated untrans-

TABLE 1. TSTA activity of p53-plus-ras transformed cells^a

Cell line	No. of immunizations	No. of animals with tumors/total (%)	Time (days) of tumor appearance
B3	1	0/6 (0%)	
B3	2	0/6 (0%)	
B3	3	0/6 (0%)	
REF	3	6/6 (100%)	21
REF	1	6/6 (100%)	21
REF	3	6/6 (100%)	21

^a Fisher rats were injected at 7-day intervals with 2×10^6 B3 cells which were irradiated with 10,000 rads, 10^7 REF cells, or PBS as described in Materials and Methods. After the final immunization, animals were challenged with 2×10^3 viable B3 cells, and the development of tumor formation was monitored at 5-day intervals. Two separate experiments were added to obtain a total of six rats in each row. Tumor development was noted by the appearance of a palpable tumor of at least 0.5 cm on the day indicated.

evident in that the great majority of p53-plus-ras cell lines expressed this (or these) TSTA, while very few of the E1A-plus-ras cell lines had this activity. Rat-1 cells, transfected with the same p53 gene used to cooperate with the *ras* oncogene in transformed REF cell lines, failed to immunize rats against B3 cell challenges. p53 protein is expressed at high levels in these Rat-1 cell lines (5), which suggests that p53 protein does not protect against the B3 cell challenge. Primary REF cells and SV40-transformed REF cells also failed to protect rats from tumors induced by an injection of B3 cells. These data demonstrate that (i) most p53-plus-ras REF cell lines share a common TSTA, and (ii) some other transformed cell lines express a similar or identical TSTA that can confer tumor protection against the p53-plus-ras REF B3 cell line. These results are not unique to a B3 cell line challenge in that some of these same p53-plus-ras cell lines can protect against tumor challenges by other p53-plus-ras-transformed cell lines (data not shown).

One of the common features of many transformed cell lines is that the levels of p53 protein are elevated in most cases because of the enhanced stability of the protein (7, 8). Thus, p53 protein itself might be a reasonable candidate for the TSTA activity detected in the transformed REF cells. To test this idea, B3 cell extracts were fractionated after cell lysis. Differential centrifugation was used to obtain fractions rich in nuclei, total cellular membranes, or a soluble cytosolic component. Both the membrane fraction and the cytosolic or soluble fraction could confer protection from a B3 cell tumor challenge upon rats immunized with these fractions (Table 3). Nuclear fractions failed to confer such protection (Table 3). Extraction of the membrane fractions at alkaline pH (11.5) removes most of the proteins that are attached to membranes but are not integral membrane proteins. Treatment of this membrane fraction with alkali failed to remove the TSTA activity conferred upon rats by these B3 cell membranes. This observation suggests that at least one TSTA activity is due to an integral membrane protein. Western blotting with p53 antibodies that recognize native and denatured p53 protein (8) failed to detect any p53 protein in this alkaline-treated membrane fraction that contained TSTA activity.

formed REF cells or PBS buffer only. (Tumor production in control animals in this experiment followed the curve graphed for animals challenged with 2×10^3 cells.) Other animals were challenged with various doses of B3 cells (▲) 7 days after the last immunization and were inspected at 5-day intervals for tumor development. Challenge doses were as shown in the graph.

TABLE 2. TSTA cross-protection between cell lines^a

Cell line (no. of animals with tumors/no. of animals tested)		
REFs (8/8)		
p53 plus <i>ras</i> ^b	<i>myc</i> plus <i>ras</i> ^c	E1A plus <i>ras</i> ^d
A1 (0/4)	<i>myc</i> 1 (2/2)	E1 (2/2)
A2 (0/4)	<i>myc</i> 2 (0/4)	E2 (2/2)
A3 (0/2)	<i>myc</i> 3 (2/2)	E3 (2/2)
A4 (0/4)	<i>myc</i> 4 (4/4)	E4 (4/4)
B1 (0/4)	<i>myc</i> 5 (0/4)	E5 (2/2)
B2 (0/4)	<i>myc</i> A (2/2)	E6 (2/2)
B3 (0/6)	<i>myc</i> B (0/2)	E7 (2/2)
B4 (4/4)	<i>myc</i> C (2/2)	Ea (2/2)
T36 (0/2)	<i>myc</i> D (0/2)	Eb (0/4)
C1 (0/2)	<i>myc</i> E (0/2)	Ec (2/2)
C2 (0/2)	<i>myc</i> F (0/2)	
C3 (0/2)	<i>myc</i> I (0/2)	
C4 (4/4)	<i>myc</i> X (2/2)	

^a Fisher rats were immunized twice at 7-day intervals with 2×10^6 to 5×10^6 cells which had received 10,000 rads of irradiation as described in Materials and Methods. Animals were challenged with 2×10^5 B3 cells 10 days after the last immunization, and tumor production was monitored at 5-day intervals. Note that protection from the appearance of a tumor was always complete (two or four animals) and not partial for each cell line tested.

^b Of 13 cell lines tested, 11 (85%) offered cross-protection.

^c Of 13 cell lines tested, 7 (54%) offered cross-protection.

^d Of 11 cell lines tested, 2 (9%) offered cross-protection.

The total cell homogenate cytosolic soluble proteins contained some TSTA activity (Table 3) and also were shown to contain large levels of p53 protein by Western blot analysis (Fig. 2, lane a). The p53 protein in this fraction could be removed by passing these soluble proteins through a p53 antibody immunoaffinity column, which removed all of the detectable p53 protein in this preparation (Fig. 2, lane b). This cytosolic fraction without detectable p53 retained its ability to protect rats from the B3 cell challenge (Table 3). Finally, p53 was purified by immunoaffinity chromatography, as described previously (13), from several sources: (i) *Escherichia coli* expressing a p53 cDNA clone (13), (ii) B3 cells, and (iii) A1 cells (8). In each case, p53 was used to immunize rats in a transplantation rejection assay. Even though the rats produced high titers of antibody to p53 protein, p53 failed to protect these animals from a tumor challenge with B3 REF cells. Clearly, p53 is not the TSTA activity found in the cytosolic soluble fraction of B3 cells.

These studies demonstrate that REF cells transformed by p53 plus *ras* share a TSTA that is found in most (85%) of these cell lines. Some *myc*-plus-*ras* (54%) and E1A-plus-*ras* (9%) cell lines also express a TSTA that can protect against a p53-plus-*ras* REF tumorigenic cell challenge in rats. In the

TABLE 3. Cell fractionation of TSTA activity^a

Cell fraction	No. of tumors/no. of animals tested
Intact REF cells (nontransformed)	4/4
Irradiated B3 cells (intact)	0/4
Total B3 cell homogenate	2/4
Nuclear extract	4/4
Total cell membranes	0/4
Cytosolic extracts	
Soluble cytosolic extract	2/4
Flowthrough of anti-p53 column	0/4
Affinity-purified p53 antigen	4/4

^a See Materials and Methods for details.

FIG. 2. Adsorption of p53 from B3 soluble protein extracts. B3 cells were fractionated by hypotonic lysis as described in Materials and Methods. Soluble proteins were fractionated by DEAE chromatography, and the flowthrough fraction (which contained p53 and the TSTA activity) was recirculated over an immunoaffinity resin containing anti-p53 monoclonal antibody PAb421 as described previously (2). The adsorbed and nonadsorbed fractions were used for immunization of rats as described in Materials and Methods. Animals were challenged with 2.5×10^5 B3 cells, and tumor production was monitored. An immunoblot of column fractions analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously (8) and is shown here. Lanes a, p53 immunoreactive protein in soluble extract; b, material not adsorbed by the anti-p53 antibody resin; c, p53 protein eluted from the immunoaffinity resin after elution of bound hsc70 with ATP as described previously (2). +, TSTA activity; -, no TSTA activity. The immunizing dose of protein not adsorbed by the affinity resin was similar to that used in experiments with crude subcellular fractions or partially purified proteins (0.1 to 0.2 mg).

B3 cell line, there may be two different entities that demonstrate TSTA activity in rats. One of these appears to be an integral membrane protein, while the second one (related or not) fractionates with the soluble proteins from the cytosolic compartment. Thus, it appears that p53, the tumor antigen expressed at high levels and which is common in all of these transformed cell lines, does not have the ability to confer TSTA activity and protect rats from the B3 cell tumor challenge.

ACKNOWLEDGMENTS

We thank A. K. Teresky and Robin Lufkin for excellent technical assistance and K. James for her efforts in preparation of the manuscript.

A. Frey was an Anna Fuller Fund Fellow. This work was supported by Public Health Service grants 1 P01 CA41086-04 and -05 from the National Cancer Institute.

LITERATURE CITED

- Black, P. H., W. P. Rowe, H. C. Turner, and R. J. Huebner. 1963. A specific complement-fixing antigen present in SV40 tumor and transformed cells. *Proc. Natl. Acad. Sci. USA* 50:1148-1152.
- Clarke, C., K. Cheng, A. Frey, R. Stein, P. Hinds, and A. J. Levine. 1988. Purification of complexes of nuclear oncogene p53 with rat and *Escherichia coli* heat shock proteins: In vitro dissociation of hsc70 and dnaK from murine p53 by ATP. *Mol. Cell. Biol.* 8:1206-1215.
- Crawford, L. V., D. C. Pim, and R. D. Bulbrook. 1982. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. *Int. J. Cancer* 30:403-408.

4. DeLeo, A. B., G. Jay, E. Appella, G. C. Dubois, L. W. Law, and L. J. Old. 1979. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc. Natl. Acad. Sci. USA* **76**:2420–2424.
5. Eliyahu, D., D. Michalovitz, and M. Oren. 1985. Overproduction of p53 antigen makes established cells highly tumorigenic. *Nature (London)* **316**:158–160.
6. Eliyahu, D., A. Raz, P. Gruss, D. Givol, and M. Oren. 1984. Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. *Nature (London)* **312**:646–649.
7. Finlay, C., P. Hinds, A. Frey, and A. J. Levine. 1987. Mutations which activate p53 transformation with *ras* produce an altered p53 protein that preferentially binds to a heat shock protein, hsc70, p. 126–132. *In* F. W. Alt, E. Harlow, and E. B. Ziff (ed.), *Current communications in molecular biology, nuclear oncogenes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. Finlay, C., P. Hinds, T.-H. Tan, D. Eliyahu, M. Oren, and A. J. Levine. 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell. Biol.* **8**:531–539.
9. Linzer, D. I. H., and A. J. Levine. 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40 transformed cells. *Cell* **17**:43–51.
10. Parada, L., H. Land, R. Weinberg, D. Wolf, and V. Rotter. 1984. Cooperation between gene encoding p53 tumour antigen and *ras* in cellular transformation. *Nature (London)* **312**:649–651.
11. Ruley, E. 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature (London)* **304**:602–606.
12. Tan, T.-H., J. Wallis, and A. J. Levine. 1986. Identification of the p53 protein domain involved in formation of the simian virus 40 large T antigen p53 protein complex. *J. Virol.* **59**:574–583.
13. Tevethia, S. S., R. S. Greenfield, D. Flyer, and M. J. Tevethia. 1980. SV40 transplantaion antigen: relationship to SV40-specific proteins. *Cold Spring Harbor Symp. Quant. Biol.* **44**:235–242.