Specificity of in vitro Binding of Primate Type C Viral RNA and the Homologous Viral p12 Core Protein

Abstract. *The binding of type C viral p12 proteins to purified viral RNA has been examined in vitro with the use of a family of closely related infectious primate type C viruses—the woolly monkey (SSAV) and gibbon (GALV) group. This in vitro protein-RNA binding is type specific. The system should serve as a model for studies of the evolution of nucleic acid binding proteins.*

In both prokaryotic and eukaryotic systems, viral and cellular genes which code for proteins that bind to single- or double-stranded nucleic acids with varying degrees of specificity and affinity have been identified. These nucleic acid binding proteins have regulatory roles in the processes of replication, recombination, genome organization, and gene expression. Mammalian type C viruses code for a phosphoprotein with a molecular weight of approximately 12,000 (p12) (*1*); this phosphoprotein is associated with the RNA genome in the virion core (*2*). Using two different mammalian type C viruses, the Rauscher murine leukemia virus (R-MuLV) and the simian sarcoma-associated virus (SSAV), we demonstrated that viral p12 proteins of R-MuLV and SSAV bind in vitro only to RNA purified from the same virus but that they do not bind to RNA from the heterologous virus (*3*). A group of more closely related type C viruses would allow studies of the relationships between different RNA genomes, the p12 proteins encoded by them and the genome sites to which such proteins bind. In our study on the infectious primate type C viruses related to varying degrees to SSAV, we used direct and competition binding assays, and report that the p12 proteins maintain their specific binding ability preferentially to the viral RNA's which code for them.

Four distinct but related subgroups have been characterized among the infectious primate type C viruses. These are (i) the simian sarcoma-associated virus (SSAV) isolated from a woolly monkey house pet (*4*); (ii) an isolate from a lymphomatous gibbon from a vivarium colony in California (GALV-1) (*5*); (iii) a different isolate from a lymphomatous gibbon from a vivarium colony in Thailand (GALV-SEATO) (*6*); and (iv) a subgroup with three isolates (GBr-1, -2, and -3) from the brains of normal gibbons housed in a colony in Louisiana (*7*). The genomes of viruses of these four subgroups share different extents of overall nucleic acid sequence homology. The RNA genomes of GALV-1 and GALV-SEATO are the most closely related to each other (85 to 90 percent overall sequence homology), while the RNA's of other members of the group share between 55 to 75 percent overall sequence homology in reciprocal crosses between different pairs (*7*). The p12 proteins of SSAV, GALV-1, and GALV-SEATO are also antigenically distinguishable, the p12 proteins of GALV-1 and GALV-SEATO being more closely related to each other than to the p12 protein of SSAV (*8*); the antigenic relatedness of p12 proteins from the GBr isolates to the other three subgroups is not yet established. The infectious primate type C viruses (SSAV/GALV group) are not genetically transmitted, endogenous viruses of primates (*9*); they are closely related to an endogenous type C virus iso-

Table 1. Competition for binding sites on viral RNA's between different p12 proteins. The competition assay included 10 to 20 ng of viral RNA, 20 ng of ^{125}I-labeled p12 protein from the homologous virus and 300 ng of competing unlabeled p12 protein. The products were analyzed, and binding was estimated (see Fig. 1 legend). The ^{32}P radioactivity in the 60*S* region of different gradients was calculated to be $5 \times 10^4 \pm 0.5 \times 10^4$ dpm. The ^{125}I radioactivity migrating in the 60*S* region of various gradients was normalized to a fixed number (5×10^4 dpm) of ^{32}P counts to achieve an accurate estimate of the percentage of displacement. The ^{125}I radioactivity was determined in a Beckman LS-250 liquid scintillation spectrometer at a counting efficiency of approximately 50 percent. Radioactivity is expressed as counts per minute.

Competing p12 protein	[^{125}I]SSAV p12 bound to SSAV RNA	Displacement (%)	[^{125}I]GALV-SEATO p12 bound to GALV-SEATO RNA	Displacement (%)
None	5300		5800	
SSAV	230	96	5500	5
GALV-SEATO	4600	13	400	94
GBr-1	4700	11	5600	3.5
GALV-1	5200	2	2600	55

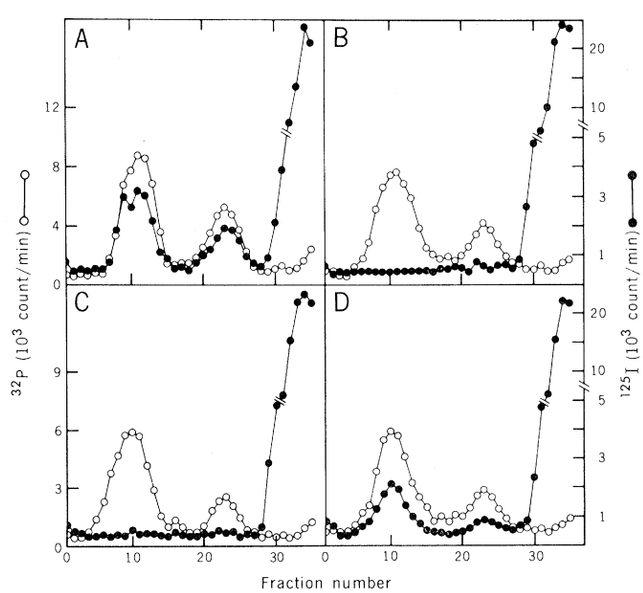

Fig. 1. Neutral sucrose sedimentation profile of p12 protein-viral RNA binding. ^{32}P-Labeled 60*S* and 30*S* viral RNA's and viral p12 proteins were purified as described (*3*). Viral proteins were more than 90 percent homogeneous as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Proteins were labeled with ^{125}I to specific activities of ~5 μc/μg using chloramine T (*11*). Binding was performed by mixing approximately ^{32}P-labeled viral RNA (5×10^4 dpm) and ^{125}I-labeled p12 protein (4×10^5 dpm) in 10 m*M* tris-HCl buffer (*p*H 7.0), 50 m*M* NaCl, and 1 m*M* ethylenediaminetetraacetic acid (EDTA). In homologous systems, these concentrations of p12 protein are in excess of the amount required to saturate the RNA binding sites (*3*). Incubation at 22°C for 2 hours was followed by addition of an equal volume of 12 percent formaldehyde in the above buffer at 4°C. Following formaldehyde cross-linking for 12 hours at 4°C, the products were sedimented through a linear neutral sucrose gradient (5 to 20 percent) in the reaction buffer for 2 hours at 40,000 rev/min at 4°C (Spinco SW50.1 rotor). Approximately 35 fractions were collected from the bottom of each gradient; they were precipitated with trichloroacetic acid and the ^{32}P and ^{125}I radioactivity in the precipitate was counted (*3*). The amount of p12 protein bound was calculated by adding the ^{125}I (in counts per minute) sedimenting with the 60*S* and 30*S* regions of ^{32}P-labeled RNA. The spillover of ^{32}P into the ^{125}I channel was approximately 4 percent and was subtracted when formulating the data. (A) SSAV RNA and SSAV p12 protein; (B) SSAV RNA and GALV-SEATO p12 protein; (C) GALV-SEATO RNA and SSAV p12 protein; (D) GALV-SEATO RNA and GALV-SEATO p12 protein.

lated from the Asian rodent, *Mus caroli* (*10*). The differences between the various subgroups, now replicating in primates, presumably reflect the rapid evolutionary divergence of horizontally transmitted viruses derived from a rodent viral ancestor. Binding assays performed with p12 and RNA molecules purified from these viruses would offer an unusual opportunity to study the specificity of interaction between a rapidly evolving set of nucleic acid genomes and the functionally analogous protein they encode.

Figure 1 shows the results of homologous and heterologous p12 protein-RNA binding assays using components purified from SSAV and GALV-SEATO. The procedures for obtaining purified viral RNA's and p12 proteins and the conditions for the binding assay have been described (*3*) (Fig. 1). While the SSAV p12 protein binds to 50 to 60*S* and 30*S* SSAV RNA (Fig. 1A), it fails to bind to GALV-SEATO RNA (Fig. 1C). The p12 protein isolated from GALV-SEATO binds to its homologous RNA (Fig. 1D) and not to SSAV RNA (Fig. 1B). Approximately 20 percent of the input ^{125}I-labeled protein binds to the homologous viral RNA when subsaturating concentrations of p12 protein are used. These results indicate that there are at least two functionally distinct populations of molecules in the p12 protein preparations; inactivation of p12 proteins during their purification and ^{125}I-labeling as well as the level of phosphorylation of the proteins may limit the extent of the interaction with the homologous viral RNA.

Competition experiments were performed with ^{32}P-labeled 60*S* RNA from either SSAV or GALV-SEATO and ^{125}I-labeled p12 proteins from the homologous viruses. Binding assays were initiated by adding purified RNA to the radiolabeled p12 protein in the presence or absence of unlabeled p12 protein competitor. The extent of competition was determined from the reduction in the ^{125}I-labeled p12 protein cosedimenting with viral RNA in the presence of competing p12 protein compared to the amount bound in the absence of competitor. With ^{125}I-labeled p12 proteins at a specific activity of 2×10^7 disintegrations per minute (dpm) per microgram, a 50 percent reduction in binding is expected with approximately 30 ng of homologous competing protein (that is, at a molar ratio of unlabeled to labeled p12 protein close to 1.0). Virtually complete competition would be seen with approximately 300 ng of the homologous p12 competitor (*3*).

Figure 2A shows the results of competition experiments performed with the RNA and labeled p12 protein from SSAV. Complete competition was seen when 300 ng of unlabeled SSAV p12 protein was used, whereas it was not detected with p12 proteins from the various gibbon type C viruses (Fig. 2A). In a similar experiment performed with radiolabeled components purified from GALV-SEATO, competition was complete with the GALV-SEATO p12 protein, but not with the p12 proteins of SSAV or GBr-1 (Fig. 2B). However, a partial (about 50 percent) displacement of the labeled GALV-SEATO p12 protein was detected with 300 ng of GALV-1 p12 protein. These data are summarized in Table 1.

To confirm that the p12 proteins of GALV-1 can compete for binding sites on GALV-SEATO RNA, assays were performed with varying concentrations of GALV-1 p12 competitor. In contrast

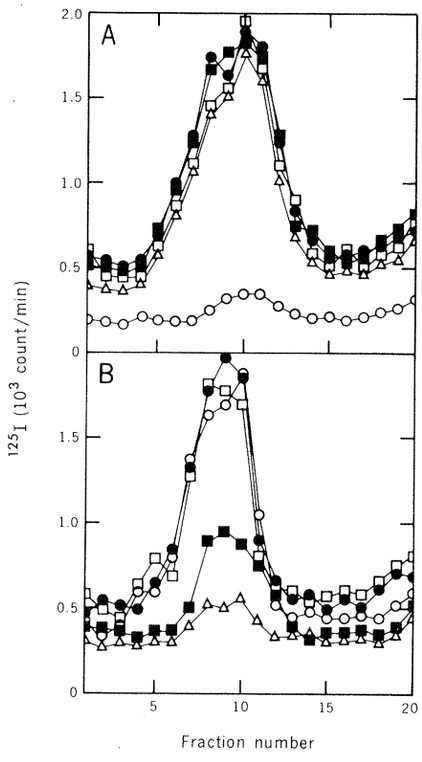

Fig. 2. Neutral sucrose sedimentation velocity profile of bound ^{125}I-labeled p12 proteins in competition binding experiments. All experiments were performed with purified 60*S* viral RNA and with concentrations of ^{125}I-labeled p12 protein in excess of the amount required to saturate the RNA binding sites (*3*). Incubation mixtures contained approximately 5×10^4 dpm of ^{32}P-labeled viral RNA and 4×10^5 dpm of ^{125}I-labeled homologous p12 protein in the presence or absence of 300 ng of unlabeled homologous or heterologous p12 proteins. (A) SSAV RNA and ^{125}I-labeled SSAV p12 protein; (B) GALV-SEATO RNA and ^{125}I-labeled GALV-SEATO p12 protein in the absence of competitor (●) or with 300 ng competing unlabeled p12 protein from SSAV (○), GALV-SEATO (△), GBr-1 (□), or GALV-1 (■). For simplicity, the ^{32}P radioactivity profiles and the top one-third of the gradients are not shown in the figure.

to the homologous competition pattern (see above), less than 10 percent displacement was seen with 70 ng of GALV-1 p12 protein, and 750 ng were required for 85 percent competition. Thus, the GALV-1 p12 protein competes much less efficiently for binding sites on GALV-SEATO RNA than does the homologous GALV-SEATO p12 protein. The ability of an "excess" of GALV-1 p12 protein to compete fully with the ^{125}I-labeled p12 protein of GALV-SEATO shows that both proteins compete for the same binding sites on the viral RNA.

The infectious primate type C viruses of the SSAV/GALV group contain viral reverse transcriptases and major structural proteins (p30) that are antigenically very similar and all viruses of this group interfere with the replication of one another (*7*). Nucleic acid hybridization data indicate that these subgroups represent evolutionary divergence from a common rodent viral ancestor (*10*). Our data demonstrate that a strong specificity in the recognition of homologous viral RNA by viral p12 proteins has been retained in these "natural variants" of the type C viruses. An excess of p12 protein from a heterologous virus does not efficiently compete with the homologous p12 protein for viral RNA binding. While competition was detected between the p12 proteins of the two most closely related viruses, GALV-SEATO and GALV-1, this was observed with concentrations of heterologous competitor 10- to 20-fold greater than that required for a comparable displacement by the homologous competing p12 protein. Thus, in the case of this subset of type C viral genomes, the variation in the coding nucleic acid sequence is not only reflected in the type-specific antigenicity of the functionally analogous p12 proteins but also in the retention of the specificity of the p12 proteins to their homologous viral RNA binding sites.

The remarkable degree of specificity in the binding of type C viral p12 proteins to viral RNA and the fact that only a few (<15) molecules of protein bind to each RNA molecule in vitro (*3*) lead to the conclusion that binding is not fortuitous but reflects a well-conserved physiologic function. If the p12 protein binding sites on the viral RNA genome are distinct from the sequences coding for p12, then at least two sets of sequences must coevolve so that the specificity of the interaction is conserved.

ARUP SEN
GEORGE J. TODARO
Viral Leukemia and Lymphoma Branch, National Cancer Institute, Bethesda, Maryland 20014

References and Notes

1. B. K. Pal and P. Roy-Burman, *J. Virol.* **15**, 540 (1975); B. K. Pal, R. M. McAllister, M. B. Gardner, P. Roy-Burman, *ibid.* **16**, 123 (1975).
2. R. C. Nowinski, E. Fleissner, N. H. Sarkar, T. Aoki, *ibid.* **9**, 359 (1972); J. Lange, H. Frank, G. Hunsmann, V. Moennig, R. Wollmann, W. Schaefer, *Virology* **53**, 457 (1973).
3. A. Sen, C. J. Sherr, G. J. Todaro, *Cell* **7**, 21 (1976).
4. G. H. Theilen, D. Gould, M. Fowler, D. L. Dungworth, *J. Natl. Cancer Inst.* **47**, 881 (1971).
5. T. Kawakami, S. D. Huff, P. M. Buckley, D. L. Dungworth, S. P. Snyder, R. V. Gilden, *Nature New Biol.* **235**, 170 (1972).
6. T. G. Kawakami and P. M. Buckley, *Transplant. Proc.* **6**, 193 (1974).
7. G. J. Todaro, M. M. Lieber, R. E. Benveniste, C. J. Sherr, C. J. Gibbs, Jr., D. C. Gajdusek, *Virology* **67**, 335 (1975).
8. S. R. Tronick, J. R. Stephenson, S. A. Aaronson, T. G. Kawakami, *J. Virol.* **15**, 115 (1975).
9. E. M. Scolnick, W. P. Parks, T. Kawakami, D. Kohne, H. Okabe, R. Gilden, M. Hatanaka, *ibid.* **13**, 363 (1974); R. E. Benveniste, R. Heinemann, G. L. Wilson, R. Callahan, G. J. Todaro, *ibid.* **14**, 56 (1974).
10. M. M. Lieber, C. J. Sherr, G. J. Todaro, R. E. Benveniste, R. Callahan, H. G. Coon, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2315 (1975); G. J. Todaro, R. E. Benveniste, C. J. Sherr, M. M. Lieber, R. Callahan, in *Comparative Leukemia Research 1975*, J. Clemmesen and D. S. Yon, Eds. (Karger, Basel, 1976), pp. 115–120.
11. F. C. Greenwood, W. M. Hunter, J. S. Glover, *Biochem. J.* **89**, 114 (1963).

12. We thank Dr. C. Sherr for many helpful comments and criticisms and B. Comfort, L. Lahey, and R. Nalewaik for technical assistance. Supported in part by the NCI Virus Cancer Program.

15 March 1976

Embryonal Neoplasms in the Opossum: A New Model for Solid Tumors of Infancy and Childhood

Abstract. *Opossums fed the chemical carcinogen ethyl nitrosourea early in postnatal life developed a variety of epithelial and mesenchymal embryonal neoplasms that were closely analogous, in morphology and biological behavior, to tumors of human infancy and childhood for which experimental models in laboratory animals are either imprecise or nonexistent. The embryonal tumors were found in association with, and occasionally at the same sites as, a limited number of malformations.*

Laboratory investigation of embryonal neoplasms (*1*) has been handicapped by the lack of precise experimental models in laboratory animals for the majority of the most important embryonal neoplasms of soft tissue (*2, 3*), bone, and teeth (*1*). Attempts to induce such neoplasms by transplacental exposure of standard laboratory animals to a variety of carcinogens have, with rare exceptions, yielded tumors that do not differ in latency or morphology from nonembryonal tumors induced postnatally with the same agent (*2–4*). The essentially similar response to both transplacental and postnatal carcinogen administration in the traditional laboratory mammals cannot be explained solely on the basis of factors that prevent the carcinogen from reaching the fetus in full concentration or activity—for example, short biological half-life or placental and maternal metabolic alteration or inactivation, or both (*3, 5*). Rice has suggested that another, perhaps more important, reason for the lack of success in induction of embryonal tumors in the common laboratory species, especially for those neoplasms susceptible to endocrine inhibition and immunologic suppression, may be the short interval between the completion of organogenesis and the functional maturation of the reproductive and lymphoid systems (*3*). He also suggests that, in rodents, particularly, the growth of a transplacentally induced tumor would have to be explosive to achieve detectable size before onset of endogenous sex hormone secretion and lymphoid activity.

The postnatal completion of embryonic and fetal development in the opossum (*Didelphis virginiana* Kerr) suggested to us that this species might offer certain advantages over the typical eutherian fetus in utero for experimental oncogenesis and especially for embryonal tumor induction (*6, 7*). At birth, after a gestation of 13 days ± 6 hours (*8*), the opossum represents an amalgam of fetal and embryonic tissue; while determination is largely complete, differentiation has only progressed to the degree necessary for extrauterine survival (*9*). The components of the endocrine system are still in the anlage state (*9*), the gonads are just beginning to differentiate (*10*), the lymphoid system is largely unformed (*11*), and there is no immunologic competence (*12–14*). The first 2½ months of postnatal development in the opossum are roughly equivalent to the last 7 to 8 months of intrauterine development in the human and to the final 8 to 9 days of gestation in the typical rodent (*8–14*). During this period the young opossum is involuntarily attached to the maternal teat within the marsupium, or pouch, where maternal influence is limited to constituents of maternal milk (*15*) and the pouch environment (*16*). Accordingly, when the opossum is used as the experimental animal, it is possible, beginning in some tissues at the anlage state, to expose developing embryonic and fetal tissue to carcinogens in the absence of placental interference, under minimal maternal influence (*6, 7*), and without toxicity to the mother.

Furthermore, the opossum matures slowly in relation to its short life span (about 2½ years) and relatively small body size (about 1.5 to 3 kg). For example, maturation of the lymphoid system requires about 60 days after birth (*11*), and full sexual development is not attained for at least 9 months after birth (*8*). Demonstrated immunologic consequences of the lymphoid maturation rate in the opossum are: (i) the absence of circulating antibody production before 7 days of age (*12*), (ii) an inability to reject skin allografts prior to 12 days of age (*13*), and (iii) an acquired tolerance to soluble and particulate antigens administered before 2 weeks of age (*14*). Thus, there is more time in the opossum than in the typical laboratory mammal for the oncogenic process to proceed prior to the full functional maturation of several factors that may influence or inhibit the oncogenic chain of events.

In two separate experiments performed in successive years, 532 opossums from 72 litters bred in captivity (*17, 18*) and obtained within 17 hours of birth (*18*) were divided by litter into ten groups according to age (< 1 day old and 1, 2, 3, 4, 6, 8, 10, 12, and 16 weeks old). In the first experiment (*7*), animals were given approximately 100 mg of ethyl nitrosourea (ENU) (*19*) per kilogram of body weight as a single dose by mouth. In the second study the same total oral dose of ENU was given every other day in four increments of 25 mg per kilogram of body weight. The ENU was administered within 1 hour of solution in *p*H 4.0 saline phosphate buffer. One or two animals in each litter were given saline phosphate buffer only. Opossums with visible tumors were killed at various ages, depending on the condition of animal and tumor. Animals without grossly manifest tumors were killed either when moribund or 2 years after drug administration.

A variety of mesenchymal and epithelial neoplasms, including embryonal neoplasms of the eye, liver, brain, kidney, muscle, and jaw, developed in the opossums treated with ENU. Three of the embryonal tumors, an intraocular neoplasm (teratoid medulloepithelioma), a tumor of the neuron (ganglioglioma), and an odontogenic tumor of the jaw (myxoma) have not, to our knowledge, been previously induced by systemic administration of a carcinogen. In this report we present preliminary data on the susceptibility of the opossum to induction of embryonal tumors; more detailed statistical and descriptive data on the induced neo-