Proc. Nat. Acad. Sci. USA
Vol. 70, No. 8, pp. 2206-2210, August 1973

Temperature-Dependent Expression of Transformation by a Cold-Sensitive Mutant of Murine Sarcoma Virus

(normal rat kidney cells/Moloney leukemia virus)

KENNETH SOMERS AND SAUL KIT

Division of Biochemical Virology, Baylor College of Medicine, Houston, Texas 77025

Communicated by Jerome Vinograd, May 7, 1973

ABSTRACT **Morphological transformation of normal rat kidney cells by a murine sarcoma virus was found to be cold-sensitive. Cells transformed by the virus expressed their transformed phenotype at the permissive temperature (39°) but not at the nonpermissive temperature (33°), as judged by the criteria of morphological changes and colony-forming ability on monolayers of normal rat kidney cells. Cold-sensitive expression of transformation was specific for focus-derived normal rat kidney cells transformed by the virus, readily reversible, and not lost during serial propagation of the cells. The genome of the murine sarcoma virus can be rescued by superinfection with Moloney leukemia virus at the permissive or nonpermissive temperature, and the rescued virus exhibited the same cold-sensitive properties as the original transforming virus. These results suggest that maintenance of the transformed state is continuously dependent on a cold-sensitive viral function.**

Clonal isolation in normal rat kidney (NRK) cells of murine sarcoma virus (MSV) from the MSV–Moloney leukemia virus (MSV–MoLV) complex has been described (1). Two types of MSV-transformed NRK cells were obtained using an agar overlay cloning method: (*a*) NRK(MSV-1)-transformed cells produced a virus, *MSV-1(NRK)*, that could transform NRK cells but failed to produce progeny virus in the newly infected cells; and (*b*) NRK(MSV-6)-transformed cells produced a virus, (MSV-7(NRK), that failed to transform or replicate in any cell tested. MSV-1(NRK) contains genetic information for transformation, but has lost replication functions. These replication functions can be supplied by MoLV (1). MSV-6(NRK) has lost both transformation and replication functions and is deficient in DNA polymerase activity (2). MSV-1(NRK) provides a useful tool for examination of events leading to transformation of NRK cells, since the virus is blocked in replication functions. The data to be presented demonstrate that maintenance of transformation of NRK cells induced by MSV-1(NRK) is cold-sensitive.

MATERIALS AND METHODS

Cell Cultures and Viruses. Uninfected NRK cells and MSV-transformed or MoLV-infected NRK cells were grown in Auto Pow minimal essential medium (Flow Laboratories, Rockville, Md.) supplemented with 10% fetal-bovine serum as described (1). Viral pools were made from supernatant fluids of chronically infected cell cultures.

Abbreviations: NRK, normal rat kidney; MSV, murine sarcoma virus; MoLV, Moloney leukemia virus.

Viral Infection and Assay. Infection procedures using DEAE–dextran to enhance infectivity have been described (1, 3). MSV was assayed by focus formation in NRK cells (1), and MoLV was assayed by the XC cell plaque method (4).

RESULTS

Effect of Temperature on Focus Formation Induced by MSV-1(NRK) or MSV-MoLV. MSV-1(NRK) transforms NRK cells, but fails to replicate in the newly transformed cells (1). Infectious progeny has not been detected as tested by [^{3}H]-uridine labeling of the supernatant fluids of transformed cultures, assay for focus-forming activity, or the XC cell assay for plaque-forming leukemia virus. In contrast, the parental MSV–MoLV complex transforms and replicates in NRK cells.

Experiments were initiated to search for spontaneous temperature-sensitive viruses in MSV-1(NRK) and MSV-MoLV-(NRK) stocks. Therefore, MSV-1(NRK) and MSV-MoLV-(NRK) were titrated in NRK cells at 39, 36, and 33°. Table 1 presents the virus titers obtained after incubation at the three temperatures. Unexpectedly, focus formation by both MSV-1(NRK) and MSV-MoLV(NRK) appeared to be cold-sensitive. In repeated experiments with different pools of MSV-1(NRK), titers at 33° were consistently 1–2 logarithmic units less than titers at 39°, even if the cultures at 33° were held for up to 10 days to allow for cell-growth differences at the two temperatures. The finding that focus formation by the uncloned mixture MSV-MoLV was reduced at 33° was not surprising, since MSV-1(NRK) was originally isolated from such parental stocks. Presumably, the uncloned MSV-MoLV contains defective MSV comparable or identical to MSV-1-(NRK).

Effects of Temperature Shifts on Morphology of NRK Cells Infected with MSV-1(NRK). A large focus of NRK cells transformed by MSV-1(NRK) virus at 39° was isolated by trypsinization, with a stainless steel cloning cylinder. The isolated focus was grown in monolayer culture and was serially passaged at 39°. This culture has been designated NRK(MSV-1b), and cytologically consists of predominantly round, highly refractile cells that grow at 39° in piled-up clumps or clusters and tend to detach from the surface of the vessels. In contrast, the morphological appearance of NRK-(MSV-1b) cells grown at 33° is strikingly different. The cells mimic control NRK cells in that they form a confluent monolayer of low refractibility, grow in close contact with neigh-

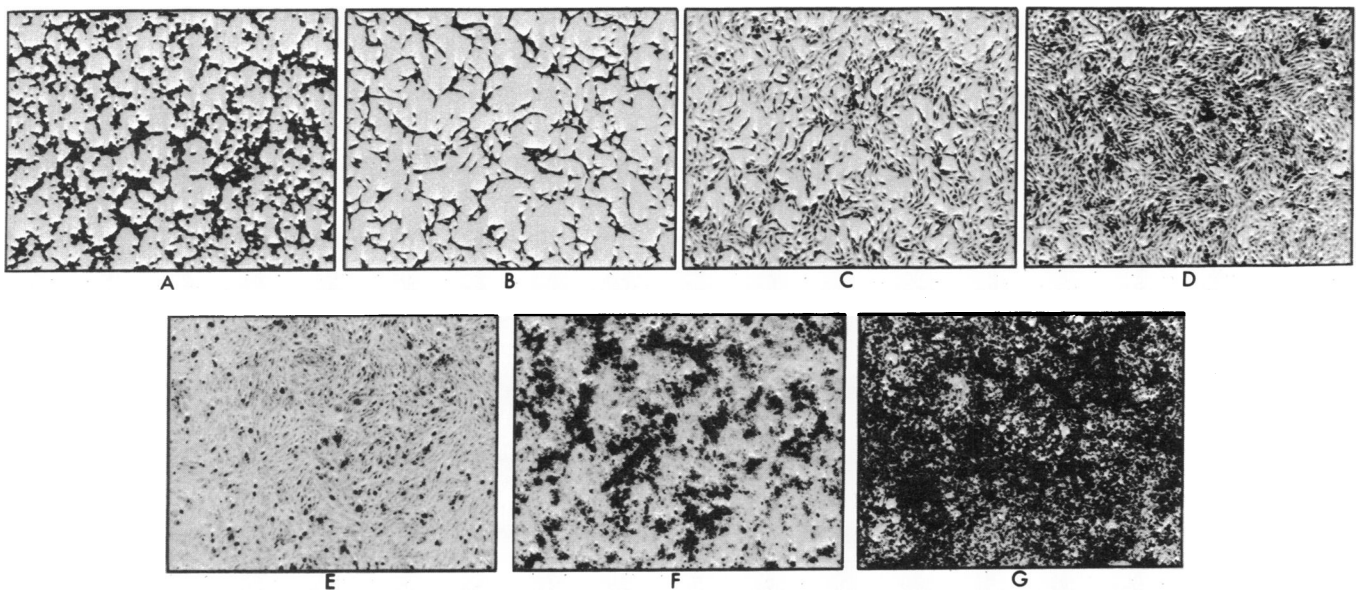

FIG. 1. Photomicrographs demonstrating the effects of temperature shifts on the morphology of NRK(MSV-1b) cells. (*A*) Transformed NRK(MSV-1b) culture kept at 39° for 4 days. (*B–D*) MSV-1(NRK)-infected cultures kept at 33° for 1, 2, and 3 days, respectively, after shift-down in temperature from 39°. (*E*) NRK(MSV-1b) culture kept at 33° for 4 days. (*F* and *G*) MSV-1(NRK)-infected cultures kept at 39° for 3 and 6 days, respectively, after the temperature shift from 33°.

boring cells, and do not form clusters with poor adhesive properties.

Two types of experiments were done to examine the effect of reciprocal temperature shifts on the morphological phenotype of MSV-1(NRK)-transformed cells. First, to examine the effect of reciprocal temperature changes on the morphological phenotype, NRK(MSV-1b) cells grown at 39 and 33° were plated into 60-mm petri dishes and cultivated at 39 and 33°. 1 Day later, half of the dishes at 39° were shifted to 33°, and reciprocally. At successive days thereafter duplicate dishes were stained and photographed. Fig. 1*A* and *E* represent NRK(MSV-1b) cultures kept for 4 days at 39 and 33°, respectively. As presented in Fig. 1*B–D*, the change from round, refractile cells to flat elongated cells could be recognized 24 hr after the cultures were shifted from 39 to 33°. By 2 days nearly the entire cell population appeared morphologically normal. The morphological change was completely reversible (Fig. 1*F* and *G*), but the change from normal back to the transformed state took place more slowly (requiring about 2–3 days) after the shift from 33 to 39°.

To determine the specificity of this temperature-dependent change, we examined the morphological phenotype of other NRK cell lines infected by sarcoma and/or leukemia viruses at 39, 36, and 33° (Table 2). It should be emphasized that these cell cultures have various degrees of viral genome expression. The NRK(MSV-MoLV) cell culture was derived by infection of NRK cells with the MSV-MoLV complex; the culture produces both MSV and MoLV. Similarly, the NRK(MoLV) cell culture was derived by MoLV infection of NRK cells and the cell line produces MoLV. NRK(MSV-1) and NRK-(MSV-6) cell lines were derived by agar cloning of NRK cells transformed by MSV, a method specifically designed to eliminate cross-infection by MoLV (1). Both NRK(MSV-1) and NRK(MSV-6) cell cultures produce viral particles, but these viruses differ in their ability to reinfect NRK cells (1). In NRK(MSV-1b) cell cultures derived from NRK cells infected by MSV-1(NRK), viral genetic information is incompletely expressed; no viral particles are produced at 33, 36, or 39° and the genetic information for transformation is not expressed at 33° (Table 2). At the intermediate temperature (36°), the NRK(MSV-1b) cell line consisted of a mixed population of transformed and normal cells. Table 2 demonstrates that none of the other cell lines exhibited temperature-dependent morphological changes.

Reversibility of transformation is not lost by NRK(MSV-1b) cells after prolonged culture at either temperature, indicating that the temperature-dependent property is quite stable. However, we have observed some leakiness at both temperatures in that occasionally NRK(MSV-1b) cells grown at 39° will segregate variant cells that appear morphologically like normal cells. Likewise, NRK(MSV-1b) cells grown for many passages at 33° will spontaneously segregate transformed cells with low frequency.

TABLE 1. *Titration of MSV-1(NRK) and MSV-MoLV(NRK) at various temperatures in NRK cells*

Virus	Titer (FFU/ml) in NRK cells at 33°	36°	39°	Ratio of titers (33°/39°)
MSV-1(NRK)	1.0	2.4×10^2	7.7×10^2	0.0013
MSV-MoLV(NRK)	1.3×10^4	2.3×10^5	4.8×10^5	0.027

60-mm Dishes were inoculated with 2×10^5 NRK cells. 1 Day later they were exposed for 1 hr at 36° to growth medium containing 25 μg/ml of DEAE–dextran. The cells were washed with serum-free medium and adsorbed with 0.2 ml of MSV for 2 hr at 36°. After adsorption, triplicate plates at each dilution were incubated at 33, 36, or 39°. Dishes were stained 5–6 days after infection, and foci were counted. *FFU*, focus-forming units.

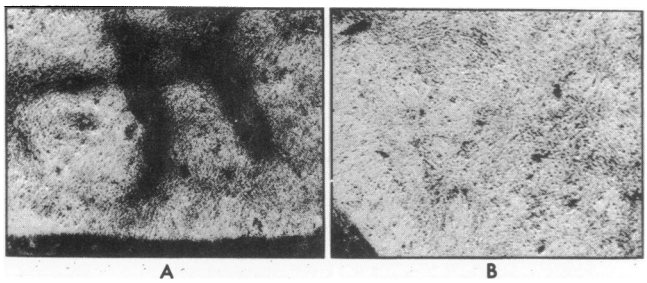

FIG. 2. Effect of temperature shift on the appearance of individual foci induced in NRK cells by MSV-1(NRK). (*A*) A focus of NRK cells transformed by MSV-1(NRK) after 10 days at 39°. (*B*) The same focal area on the sixteenth day, 6 days after the shift to 33°. *Pencil markings* on culture dish serve to identify individual foci.

In a second type of experiment to demonstrate the effect of temperature shift on focus formation by MSV-1(NRK), replicate NRK dishes were infected with 5-fold serial dilutions of virus and incubated at 39°. 10 Days later macroscopic foci were circled at random and photographed. Duplicate dishes at each dilution were shifted to 33°. The remaining cultures were kept at 39°. 6 Days after the temperature shift to 33°, the dishes were stained. The fate of individual foci induced by MSV-1(NRK) is recorded in Fig. 2.

At the time of the temperature shift, foci induced by MSV-1(NRK) were easily recognizable as discrete areas of transformed cells (Fig. 2*A*). When held at 39°, these foci increased in size. However, after the shift to 33°, the foci appeared arrested 3 days later, and 6 days later the cells within the focus took on the characteristics of normal-appearing cells (Fig. 2*B*). In addition, total foci were counted in the dishes at 39 and 33°, and the combined data are presented in Table 3. Nearly all of the foci induced by MSV-1(NRK) were cold-sensitive. The few foci that did remain after the shift to 33° were generally smaller than comparable dishes held at 39° and may represent some leakiness at 33°.

Colony-Forming Ability on NRK Monolayers. The ability to form colonies on a confluent monolayer of contact-inhibited cells has been used to determine normal or malignant growth behavior of different cell lines (5). The colony-forming ability of the cold-sensitive transformant was determined on NRK monolayers at different temperatures.

Cells were plated on confluent NRK monolayers and incubated at 33 and 39°. Cultures at 39 or 33° were fixed and stained for colony counting 7 or 10 days later, respectively. Table 4 demonstrates that the mutant cell line has a much higher colony-forming ability at 39° than at 33°. The colony-forming ability of the control NRK cell line transformed by MSV, NRK(MSV-6), was about equal at both temperatures.

Rescue of the MSV Genome from "Nonproducer" Temperature-Sensitive Transformants. NRK cells transformed by MSV-1(NRK) do not produce or contain infectious MSV. Superinfection of these cells by MoLV or other murine leukemia viruses results in the pseudotype rescue of MSV (1). To test whether NRK(MSV-1b) cells grown at either 39 or 33° contained the MSV genome, we superinfected them with MoLV and assayed the supernatant fluids for focus-forming virus in NRK cells. Table 5 shows that MSV could be rescued from NRK(MSV-1b) cells grown at either 39 or 33°. The rescued virus exhibited the same temperature-sensitive properties as the original transforming virus. The lower yield at 39° of rescued MSV from MoLV-infected NRK(MSV-1b) cells is most likely explained by the fact that NRK(MSV-1b) cells at 39° tend to grow in clusters or clumps and are easily lost during experimental manipulations.

TABLE 2. *Effect of incubation temperature on the morphological phenotype of transformed cell cultures*

Cell line	C-type particles produced	Morphological phenotype at 39°	36°	33°
NRK(MSV-MoLV)	+	T*	T	T
NRK(MSV-1)	+	T	T	T
NRK(MSV-6)	+	T	T	T
NRK(MSV-1b)	−	T	T & N†	N‡
NRK(MoLV)	+	N	N	N
NRK-2	−	N	N	N

NRK cell lines to be tested were grown in replicate 4-oz bottle cultures at 39, 36, and 33° and examined morphologically for two passages.

* Transformed morphology.

† Mixed phenotype of transformed and normal cells.

‡ Normal morphology.

TABLE 3. *Effect of temperature shift on the fate of foci induced by cold-sensitive MSV-1(NRK)*

MSV-1(NRK) dilution	Individually scored foci after incubation at 39° → 39°	39° → 33°	Total foci after incubation at 39° → 39°	39° → 33°
1:1	17/17*	4/38*	TNTC†	9
1:5	5/6	1/16	24	2
1:25	2/2	0/3	6	0

NRK cells grown in 60-mm dishes were infected with dilutions of MSV-1(NRK) and incubated at 39°. 10 Days after infection at 39°, representative foci were circled at random, and dishes were shifted to 33° or held at 39°. 6 Days later all dishes were stained, and the fate of individually scored foci and total foci was examined microscopically.

* Number of foci after incubation at 39 or 33° per number of foci initially scored at 39°.

† Too numerous to count.

TABLE 4. *Colony-forming ability of cold-sensitive NRK(MSV-1b) cells on NRK monolayers*

Cells	% of cells plated yielding colonies at 33°	39°	Ratio 33°/39°
NRK	0	0	—
NRK(MSV-1b)	10.8	57.5	0.187
NRK(MSV-6)	22.0	29.5	0.753

200 and 2000 cells were plated on fully confluent monolayers of NRK cells in 60-mm petri dishes and incubated at 39 and 33°. Medium was changed every 3–4 days. 7 Days later at 39° and 10 days later at 33°, the cells were fixed and stained for colony counting.

Of considerable interest was the finding that MoLV superinfection of morphologically normal NRK(MSV-1b) cells at 33° resulted in formation of focal areas of transformed cells. MoLV infection of control NRK at either 33 or 39° does not induce focus formation. This finding provides a potential tool for assay of murine leukemia viruses. Furthermore, morphological alteration of cells at the nonpermissive temperature of 33° by MoLV infection suggests that MoLV provides some function(s) necessary for expression of the transformed state.

DISCUSSION

Several temperature-sensitive mutants of avian sarcoma viruses have been described (6–11). Recently, the isolation of temperature-sensitive murine leukemia virus was reported (12). In this paper we described the properties of a cold-sensitive murine sarcoma virus. The demonstration that transformation by MSV-1(NRK) is cold-sensitive represents the first report of a cold-sensitive tumor virus mutant, although cold-sensitive mutants of bacteriophage (13–17), bacteria, and yeast (18–22) have been reported. Isolation of MSV mutants that are cold-sensitive for maintenance of the transformed state permits an examination of MSV gene expression at the level of transcriptional and translational control. Elucidation of how the expression of proviral genetic information is controlled in transformed cells could lead to the understanding of tumor development and would increase the general knowledge of gene regulation in mammalian cells.

Cells infected with MSV-1(NRK) expressed their transformed phenotype at the permissive temperature (39°) but not at the nonpermissive temperature (33°) by the criteria examined, which included morphological changes and the ability to form colonies on monolayers of NRK cells. These morphological findings have recently been extended to include a study on the effect of temperature shifts on the uptake of labeled 2-deoxyglucose. Uptake of labeled 2-deoxyglucose was directly correlated with expression of the transformed phenotype (23). The results indicate that cellular transformation induced by MSV-1(NRK) in NRK cells is cold-sensitive and suggest that maintenance of the transformed state requires some viral genetic function. The possibility was considered that the observed temperature-dependent alterations were due to a cellular, rather than a viral, function, as reported for SV40-transformed 3T3 cells (24). However, this possibility seems unlikely since the virus rescued from the phenotypically normal NRK(MSV-1b) cells at 33° exhibits the same temperature-dependent properties as the virus originally used to transform the cells. The ability to rescue the MSV genome from these phenotypically reverted cells even after prolonged serial passage at 33° indicates that the MSV genome is associated with these cells in a stable manner. The nature of this association remains to be determined, but it is different from that reported for spontaneous revertants of MSV-transformed mouse 3T3 cells, which exhibited morphological and functional changes characteristic of normal cells but from which the MSV genome could not be rescued (25).

Maintenance of the transformed phenotype was cold-sensitive only in focus-derived NRK cells transformed by MSV-1(NRK) particles. NRK cells chronically infected with MoLV[NRK(MoLV)] and NRK cell clones transformed by MSV, NRK(MSV-1) and NRK(MSV-6), which produce MSV-1(NRK) and MSV-6(NRK) virus particles, respectively, demonstrated no temperature-dependent morphological changes (Table 2). This result would be expected if temperature-dependent maintenance of transformation was specific for NRK cells transformed by MSV-1(NRK) virus particles. Another independently isolated focus-derived cell line transformed by MSV-1(NRK) exhibited the same temperature-dependent morphological changes as NRK-(MSV-1b) cells (J. May, personal communication).

TABLE 5. *Pseudotype rescue of the MSV genome from NRK(MSV-1b) cells grown at the permissive and nonpermissive temperature*

Cell line and temperature of culture	Superinfecting virus	FFU/0.2 ml in NRK at 33°	36°	39°
NRK(MSV-1b) 33°	None	0	0	0
	MoLV*	49	256	TNTC†
NRK(MSV-1b) 39°	None	0	0	0
	MoLV*	3	31	55
NRK 33 or 39°	MoLV	0	0	0

2×10^5 NRK(MSV-1b) cells grown in 60-mm dishes at either 33 or 39° were either mock-infected or superinfected with MoLV under standard conditions. The cultures were fed 3 days later. Supernatant fluids were collected 6 days after infection and stored at −70°. The supernatant fluids were assayed undiluted in NRK cells at 33, 36, and 39°.

* 2.3×10^5 PFU (plaque forming units)/ml by 3T3-XC cell test.

† Too numerous to count.

One problem relevant to the present finding is how this MSV-1(NRK) virus can replicate in NRK(MSV-1) cells in the absence of any *demonstrable* helper virus with the cells being equally transformed at 33 and 39°, and then fail to replicate in newly infected NRK cells, while transforming them to a cold-sensitive phenotype. Although the solution to this problem requires further study, the following hypotheses merit consideration: (*a*) During passage of NRK(MSV-1) cells, a rat leukemia virus was activated and provided a helper function enhancing expression and production of MSV-1(NRK) particles. However, few rat leukemia virus particles were produced or the leukemia virus was very poorly infectious for normal NRK cells. It should be noted that MSV-1(NRK) particles are positive for the MSV-MuLV gs antigen and are neutralized by antisera to MoLV (26). (*b*) NRK(MSV-1) cells were initially transformed by a helper-independent MSV particle, but the virus produced by these cells is a defective segregant lacking the ability to replicate in normal NRK cells. The segregation process might occur by a mechanism analogous to the production of nontransforming particles by RSV-transformed cells (27).

The finding that MoLV superinfection of NRK(MSV-1b) cells grown at 33° could induce morphological changes comparable to that observed upon temperature-shift to 39° was of considerable interest. This observation is potentially applicable as another means of assaying leukemia viruses. A similar assay system using the parameter of focus induction in a 3T3 cell line transformed by MSV, containing the sarcoma genome in the absence of replicating leukemia virus (S^+L^- cells) has been described (28). Preliminary results demonstrated no significant differences between assays for MoLV

infectivity by either NRK(MSV-1b) focus induction at 33° or the XC plaque test. Similar findings were reported for MoLV infectivity assays with S^+L^- cells or XC indicator systems (29). It is conceivable that the phenotypically reverted NRK(MSV-1b) cell system at 33° will be useful for detection or isolation of rat leukemia viruses and murine leukemia viruses with altered host ranges. Furthermore, the availability of this indicator cell system should be valuable in attempts to isolate host-range mutants of leukemia virus capable of replicating in NRK(MSV-1b) cells but incapable of replication in NRK cells.

Induction of transformed cells by MoLV infection of NRK(MSV-1b) cells at 33° suggests that MoLV provides or induces some function that permits the expression of transformation at the nonpermissive temperature. Evidence in the mouse-cell system indicates that induction of MSV from S^+L^- cells requires replication of murine leukemia virus (30), and that the ability of murine leukemia virus to rescue MSV from S^+L^- cells and its ability to replicate are equally sensitive to UV irradiation (29). It is not known whether rescue of MSV from nonproducer NRK(MSV-1b) rat cells requires replication of the helper virus. Evidence suggesting that the MSV genomes present in nonproducer rat cells and S^+L^- cells differ in their degree of expression would favor the prediction that the function(s) provided by murine leukemia virus that are required for MSV rescue from nonproducer rat cells might also be different from that observed in the mouse-cell system (31). Alternatively, the MSV genome in NRK(MSV-1b) cells might be genetically different from the MSV genome present in nonproducer rat cells transformed by the Kirsten strain of MSV (31). Genetic differences between different MSV genomes, detectable by RNA–DNA hybridization, might be reflected by variable genomic expressions in transformed cells.

We thank Grace Chan for able technical assistance. This investigation was aided by Public Health Service Grants (CA-06656-10 and 1-K6-AI2352) from the National Cancer Institute and the Institute of Allergy and Infectious Diseases and by Research Grant Q-163 from the Robert A. Welch Foundation.

1. Somers, K. & Kit, S. (1971) *Virology* **46**, 774–785.
2. May, J. T., Somers, K. D. & Kit, S. (1972) *J. Gen. Virol.* **16**, 223–226.
3. Somers, K. D. & Kirsten, W. H. (1968) *Virology* **36**, 155–157.
4. Rowe, W. P., Pugh, W. E. & Hartley, J. W. (1970) *Virology* **42**, 1136–1139.
5. Aaronson, S. A., Todaro, G. J. & Freeman, A. E. (1970) *Exp. Cell Res.* **61**, 1–5.
6. Toyoshima, K. & Vogt, P. K. (1969) *Virology* **39**, 930–931.
7. Martin, G. S. (1970) *Nature* **227**, 1021–1023.
8. Biquard, J.-M. & Vigier, P. (1970) *C.R. H. Acad. Sci. Ser. D* **271**, 2430–2433.
9. Temin, H. M. (1971) in *The Biology of Oncogenic Viruses*, ed. Silvestri, L. (North-Holland, Amsterdam), pp. 176–187.
10. Kawai, S. & Hanafusa, H. (1971) *Virology* **46**, 470–479.
11. Bader, J. P. & Brown, N. R. (1971) *Nature New Biol.* **234**, 11–12.
12. Stephenson, J. R., Reynolds, R. K. & Aaronson, S. A. (1972) *Virology* **48**, 749–756.
13. Edgar, R. S. & Lielausis, I. (1964) *Genetics* **49**, 649–662.
14. Christensen, J. R. & Saul, S. H. (1966) *Virology* **29**, 497–499.
15. Dowell, C. E. (1967) *Proc. Nat. Acad. Sci. USA* **58**, 958–961.
16. Scotti, P. D. (1968) *Mutat. Res.* **6**, 1–14.
17. Cox, J. H. & Strack, H. B. (1971) *Genetics* **67**, 5–17.
18. O'Donovan, G. A. & Ingraham, J. L. (1965) *Proc. Nat. Acad. Sci. USA* **54**, 451–457.
19. Guthrie, C., Nashimoto, H. & Nomura, M. (1969) *Proc. Nat. Acad. Sci. USA* **63**, 384–391.
20. Tai, P. C., Kessler, D. P. & Ingraham, J. (1969) *J. Bacteriol.* **97**, 1298–1304.
21. Bayliss, F. T. & Vinopal, R. T. (1971) *Science* **174**, 1339–1341.
22. Reid, P. (1971) *Biochem. Biophys. Res. Commun.* **44**, 737–744.
23. May, J. T., Somers, K. D. & Kit, S. (1973) *Int. J. Cancer* **11**, 377–384.
24. Renger, H. C. & Basilico, C. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 109–114.
25. Fischinger, P. J., Nomura, S., Peebles, P. T., Haapala, D. K. & Bassin, R. H. (1972) *Science* **176**, 1033–1035.
26. Somers, K. & Kit, S. (1972) *Abstracts Annu. Meeting Amer. Soc. Microbiol.*, p. 186.
27. Vogt, P. K. (1971) *Virology* **46**, 939–946.
28. Bassin, R. H., Tuttle, N. & Fischinger, P. J. (1971) *Nature* **229**, 564–566.
29. Nomura, S., Bassin, R. H., Turner, W., Haapala, D. K. & Fischinger, P. J. (1972) *J. Gen. Virol.* **14**, 213–217.
30. Peebles, P. T., Bassin, R. H., Haapala, D. K., Phillips, L. A., Nomura, S. & Fischinger, P. J. (1971) *J. Virol.* **8**, 690–694.
31. Aaronson, S. A., Bassin, R. H. & Weaver, C. (1972) *J. Virol.* **9**, 701–704.