Cell-Cycle Dependent Appearance of Murine Leukemia-Sarcoma Virus Antigens

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Normal rat kidney (NRK) cells, NRK cells infected with Rauscher murine leukemia virus, and NRK cells infected with Kirsten murine sarcoma-leukemia virus (NRK-K) were synchronized by a double thymidine block. At intervals after release from thymidine blockage, the cells were examined for the presence of viral antigens in the cytoplasm and on the cell surface by immunofluorescent microscopy by using goat anti-Rauscher murine leukemia virus and goat anti-Moloney leukemia virus (Tween-ether disrupted) sera. Detection of viral antigens in the cytoplasm was periodic during the cell cycle. Antigens were detected first during the S phase, increased during the G₂ phase, and disappeared during the M and G₁ phases. A similar pattern of surface immunofluorescence was observed. Infectious virus was detected in culture fluids from synchronized cells during the M phase. Surface immunofluorescence was detected in NRK-K cells with anti-Rauscher murine leukemia virus and may represent the presence of group-specific antigens on the cell surface. Control, uninfected NRK cells, which did not normally fluoresce, showed weak immunofluorescence during the S and G₂ phases after synchronization. Synchronization can be used to amplify latent oncornavirus expression.

The details of oncornavirus replication are still poorly understood. In chronically infected cells, production of virus is stopped by inhibitors of DNA synthesis and RNA transcription (5, 19). Recently, we reported that the release of infectious murine leukemia-sarcoma virus from chronically infected cells is coordinated to the cell cycle and occurs concomitant with mitosis (12). Since infectious virus release occurs at one time during the cell cycle, it seemed likely that other events in virus replication might also be temporarily restricted. We have therefore used synchronized cells to examine the sequential events in oncornavirus replication. The appearance of virion-associated antigens in synchronized tissue culture cells chronically infected with murine leukemia-sarcoma viruses was investigated by using immunofluorescent techniques.

MATERIALS AND METHODS

Virus and cell culture. A continuous cell line from normal rat kidney (NRK) was infected with 4×10^4 focus-inducing units of Rauscher murine leukemia virus (RMuLV) obtained from JLSV9 cells. The cell line chronically infected with RMuLV will be designated NRK-R cells. NRK cells were also infected with 5×10^4 focus-forming units of Kirsten murine sarcoma virus (KiMSV) obtained from an erythroblas-

totic spleen filtrate (12). The virus inoculum also contains Kirsten murine leukemia virus (KiMuLV). The cell line chronically infected with KiMSV-KiMuLV will be referred to as NRK-K cells. The virus released from this line has been shown to contain both leukemia and sarcoma viruses in a ratio of 50 KiMSV:1 KiMuLV as determined by in vitro assays (12). Cells were grown with minimal essential medium with Earle salts containing 10% fetal calf serum supplemented with 100 units of penicillin per ml and 100 μ g of streptomycin per ml in an atmosphere of 5% CO₂ at 37 C. All cell lines were subcultured weekly with trypsin-EDTA for about 30 in vitro passages as previously described (12, 13). Cell cultures were monitored weekly for mycoplasma contamination; only mycoplasma-free cultures were used for these experiments (16).

Cell synchronization. NRK, NRK-R, and NRK-K cells were plated in 15 ml of growth medium at a density of 10^5 cells/ml in 100-mm petri dishes containing several glass cover slips (9 by 50 mm) or in 4 ml of medium in 30-mm Falcon petri dishes. The cells grew logarithmically after 2 to 3 days. Synchronization was achieved by a double thymidine block as previously described (10). Briefly, cell division was inhibited by incubating cells in growth medium containing 0.002 M thymidine for 16 h. Cell division was allowed to proceed for 8 h in medium containing 0.00001 M deoxyguanosine, deoxycytosine, and deoxyadenosine. Incubation with 0.002 M thymidine was repeated for another 16 h. The thymidine-rich medium was re-

moved after the second incubation period, this was considered 0 h postrelease.

Cover slips were removed from petri dishes at varying intervals postrelease to determine the extent of cell synchrony. The cells on a piece of each cover slip were swollen for 15 min in 0.075 M KCl, treated with cold Carnoy's fixative (3 methanol:1 glacial acetic acid), and stained with Giemsa stain, and at least 500 cells were examined to determine the percentage of cells in metaphase (11). Pilot experiments established that at least 60% of the cells passed through mitosis between 8.5 and 10 h postrelease. Seven-day growth curves and [³H]thymidine uptake studies were used to determine the phases of the cell cycle. The cell cycles for NRK, NRK-R, and NRK-K cells were $S + G_2/M/G_1 = 8.5 \text{ h}/0.5 - 1 \text{ h}/2.5 = 12.0 \text{ h}$ as determined by the method of Peterson and Anderson (15).

Virus assays. Medium from synchronized cultures of NRK-R and NRK-K cells was removed and replaced with fresh growth medium at 2-h intervals postrelease. Each 2-h medium collection was passed through a membrane filter (45-nm pore size; Millipore Corp.) and stored at -70°C. Medium was assayed for MuLV by focus induction in sarcoma virus-positive, leukemia virus-negative cells (1); MSV was titered by focus formation in NRK cells (6).

Sera. Immune sera were obtained from Roger Wilsnack through the Virus Cancer Program, National Cancer Institute. According to information provided by Wilsnack, these sera have been characterized by immunodiffusion, counter-electrophoresis, and neutralization studies. The sera used were (i) goat antiserum to intact RMuLV of mouse cell origin (serum no. 2S-295). This antiserum showed cross-reactivity with antigens of Moloney leukemia virus (MoMuLV) in immunodiffusion tests as well as antigens of Gross, Moloney, and feline leukemia virus antigens by counterelectrophoresis. The 2S-295 serum also neutralized *in vitro* infectivity of RMuLV but not of KIMSV(KiMuLV); (ii) goat antiserum to Tween-ether disrupted MoMuLV of mouse cell origin (serum no. 1S-166). The 1S-166 serum was prepared by Tween-ether extraction of MoMuLV, which results in a mixture of viral nucleoids, internal virion proteins, and some disrupted envelope fragments as antigenic determinants. This antiserum showed cross-reactivity with antigens to feline leukemia virus and Gross-MuLV by determination in immunodiffusion and counterelectrophoresis tests. The 1S-166 serum also neutralized infectivity of MoMuLV *in vitro* at a dilution of 1:1,280 and of RMuLV and KuMSV-(KiMuLV) at a dilution of 1:300; (iii) control goat serum was purchased from Grand Island Biological Co., Grand Island, N.Y.

Sera were inactivated at 56°C for 30 min and stored undiluted in samples at -20°C. Prior to use, sera were diluted with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.003% sodium azide, and were passed through a membrane filter (45-nm pore size; Millipore Corp.). Goat sera and goat anti-RMuLV were used at dilutions of 1:50. Goat anti-MoMuLV was used at a dilution of 1:100. These dilutions were chosen because immune sera gave barely detectable staining with unsynchronized

NRK-K and NRK-R cells and no staining with NRK cells. Normal goat serum did not stain NRK, NRK-R, and NRK-K cells at a dilution of 1:50.

Cytoplasmic immunofluorescence. NRK, NRK-R, and NRK-K cells were grown on cover slips and removed from petri dishes at time intervals from 0 to 24 h postrelease. Cells were washed three times with PBS, fixed for 10 min in acetone-dry ice, and stored at -20°C. Cells from each experiment were reacted with sera, stained with fluorescein conjugate, and read on the same day (7). Staining was performed in three steps. Step 1: Cover slips with cells were placed on glass beads in petri dishes in a humidified environment. Cells were incubated for 30 min with immune sera and then washed three times for 5 min each with PBS. Step 2: Cells were incubated for 30 min with rabbit anti-goat IgG-fluorescein conjugate (Hyland, Costa Mesa, Calif.) diluted 1:20 with PBS-BSA and washed three times for 5 min with PBS. Step 3: Cells were counterstained with 0.005% Evan's blue for 15 min, washed three times for 5 min each with PBS, and mounted on glass microscope slides in Elvanol, pH 8.3 (17). Cells were evaluated under code, and in most cases the slides were read independently by two persons. Cells (200 to 500) were evaluated on each specimen. The overall brightness of each slide was judged on a 1+ to 4+ scale where 1+ fluorescence represented barely detectable staining and 4+ fluorescence was very bright. An American Optical Microstar microscope fitted with a vertical UV illuminator was used. We used a BG12 exciter filter, a OG515 barrier filter, and a 500-nm dichroic dial setting.

Membrane immunofluorescence. Membrane staining was performed in suspension after washing monolayers three times with PBS and then scraping cells from petri dishes with a rubber policeman. Cells were further washed three times by centrifugation at $500 \times g$ in a Sorvall RC-3 refrigerated centrifuge. The staining protocol was the same as that described for cytoplasmic staining except that all washes were done by centrifugation. In addition, a counter stain was not used and the cells were mounted in PBS diluted 1:1 with glycerin. Cells were also stained for membrane immunofluorescence attached to cover slips in humidified dishes. The protocol was identical to that for cells stained in suspension, except that washing was done by aspiration.

RESULTS

Cytoplasmic immunofluorescence. NRK, NRK-K, and NRK-R cells were grown on cover slips for 2 days, synchronized by a double thymidine block, and examined for the percentage of cells in mitosis at various times after release from thymidine blockage. The three cell lines were also examined for cytoplasmic immunofluorescence with control goat, goat anti-RMuLV, and goat anti-MoMuLV sera. Immunofluorescence was detected as diffuse granular staining throughout the cytoplasm but was not

FIG. 1. *Cytoplasmic immunofluorescence. NRK-R cells attached to cover slips were fixed with acetone and dry ice. Cells were stained with goat anti-RMuLV sera and then with rabbit anti-goat IgG-fluorescein conjugate as described. After counterstaining with 0.005% Evans blue, cells were examined under UV illumination. Fluorescence was granular and apple green in color. Fluorescence was distributed diffusely throughout the cytoplasm. Nuclei stained red. All positive cytoplasmic fluorescence reactions appeared as above. Magnification $\times 210$.*

detected in nuclei (Fig. 1). Control sera did not cause immunofluorescence. In early experiments we observed staining in at least 80% of the cells or not at all. Therefore, slides were scored as positive or negative. The intensity of fluorescence of a positive slide was judged 0, $\frac{1}{2}+$, +, 2+, 3+, or 4+ on the basis of the brightness of 80% of the fluorescent cells. Culture fluids from synchronized cell cultures were also sampled for the presence of infectious virus. The results are summarized in Fig. 2 and Table 1.

Cytoplasmic immunofluorescence and virus release were periodic and coordinated to the cell cycle in NRK-R and NRK-K cells. The greatest cytoplasmic fluorescence was detected in both cell lines 1 h prior to mitosis and release of infectious virus (Fig. 2A and Table 1). Periodicity of cytoplasmic immunofluorescence in NRK-R and NRK-K cells was reproduced in six separate experiments by using anti-RMuLV and anti-MoMuLV sera. Two types of variation between experiments were detected: (i) variation in the time of peak mitosis, 8.5 to 10 h;

and (ii) variation in the range of intensity of fluorescence, 0 to 3+ fluorescence in one experiment versus $\frac{1}{2}+$ to 4+ fluorescence in another. These differences were minimized by preparing composites from all experimental data. The data of each experiment were first expressed relative to peak mitosis at 9 h after release from thymidine blockage, and then the intensities of fluorescence of comparable time points were averaged. The average intensities were plotted to form the composite. The composite data for six cytoplasmic fluorescence experiments including NRK-R and NRK-K cells are presented in Fig. 2A. Staining was detected immediately following release from thymidine blockage and disappeared by 4 h. Cytoplasmic fluorescence then increased until 7 to 8 h postrelease and disappeared by 9 h postrelease, i.e., during peak mitosis and virus release. Staining was not again detectable until cells reentered the S phase.

Control NRK cells were monitored for the presence of C-type virus throughout the course of these experiments. NRK cells are free of

FIG. 2. Composite data of cytoplasmic and membrane immunofluorescence in synchronized cells. Cytoplasmic and membrane immunofluorescence were performed on NRK-K, NRK-R and NRK cells as described in Fig. 1 and 3. The data from each time point of replicate experiments were averaged and the composite results were plotted. When necessary, the data were normalized so that peak mitosis was 9 h after release from thymidine blockage. The sera used were control goat (○), goat anti-RMuLV (▲), and goat anti-MoMuLV (●). (A) Composite data from six cytoplasmic fluorescence experiments with both NRK-K and NRK-R cells; (B) composite data from five membrane fluorescence experiments with both NRK-K and NRK-R cells; (C) composite data from five cytoplasmic fluorescence experiments with NRK cells; and (D) composite data from four membrane fluorescence experiments with NRK cells.

TABLE 1. Release of infectious virus from synchronized cell cultures

Time (h) ^a	NRK-K cells		NRK-R cells	
	FFU/ml ^b	MI ^c	FIU/ml ^d	MI
2	0	0	240	0.5
4	0	0	240	0.5
6	1,000	2.4	260	1.0
8	2,700	10.0	320	4.5
10	8,500	20.0	1,280	17.0
12	2,000	8.5	140	5.0
14.25	1,000	7.0	240	2.0
16.25	1,300	3.5	480	1.5
18.25	500	5.0	620	1.5
20.25	4,000	13.0	860	1.5
22.25	6,200	15.0	1,140	6.0
24.25	5,000	6.5	480	2.0

^a Hours after release from the second thymidine block.

^b Focus forming units of KiMSV per milliliter of growth medium; fresh growth medium was incubated with cells for 2 h, filtered through a membrane filter (45-nm pore size; Millipore Corp.), and assayed for infectivity.

^c Mitotic index, the percentage of cells in metaphase.

^d Focus inducing units of RMuLV per milliliter of growth medium; fresh growth medium was incubated with cells for 2 h, filtered through a Millipore filter, and assayed for infectivity.

C-type virus as judged from (i) the absence of virus-like particles in electronmicrographs, (ii) the absence of sarcoma-virus or leukemia-virus activity in tissue culture fluids, and (iii) the absence of particles in culture fluids which incorporate [³H]uridine and have a buoyant density of 1.16 to 1.18 g/ml in sucrose (14; Panem and Stefanski, unpublished data). However, NRK cells harbor a latent C-type virus inducible by treatment with 5-bromodeoxyuridine and express low levels of murine group-specific antigens (3, 8, 9, 14). Therefore, NRK cells were synchronized and examined for cytoplasmic fluorescence with anti-RMuLV and anti-MoMuLV sera. Culture fluids of synchronized NRK cells were also tested for release of infectious virus. No infectivity in sarcoma virus or leukemia virus assays was detected.

Cytoplasmic immunofluorescence was detected periodically in NRK cells. Figure 2C shows the composite data from five experiments. Fluorescence was diffuse and granular as seen in the infected cells. Staining was absent immediately after release from thymidine blockage, occurred with both sera by 6 h post-release, and disappeared during mitosis at 8 h post release. The intensity of fluorescence did not exceed 1+ in NRK cells, whereas infected cells sometimes displayed 3+ to 4+ fluores-

cence at the same serum dilutions. Immunofluorescence was detected in synchronized cells only during S+G₂, and we could not demonstrate immunofluorescence in unsynchronized NRK cultures.

Membrane immunofluorescence. Synchronized NRK, NRK-R, and NRK-K cells were stained for membrane immunofluorescence in suspension or attached to cover slips. By using both procedures, fluorescence was detected as a beaded ring at the cell surface (Fig. 3). No staining of nuclei or cytoplasm was found, and fluorescence was not obtained with control sera. Cells mounted on slides were 100% viable as judged by their ability to exclude trypan blue. When staining was performed on cells attached to cover slips, fluctuations in fluorescence were detected, but a reproducible periodicity was not seen. It was considered that incomplete removal of virions from the cell surface obscured any periodic patterns from being detected and that further washing by centrifugation might remove absorbed virions. Reproducible patterns of immunofluorescence were obtained after the cells were washed by centrifugation and stained in

suspension. Furthermore, virions were not detected at the cell surface when NRK-R and NRK-K cells which had been stained in suspension were examined by electron microscopy, whereas cells stained on cover slips showed virions. Therefore, all subsequent membrane fluorescence tests were done in suspension and surface staining was considered to represent cell surface components and not intact virions. Surface staining was detected periodically in 2 consecutive cell cycles. A composite of five experiments with both NRK-R and NRK-K cells is presented in Fig. 2B. Fluorescence appeared immediately after release from thymidine blockage, decreased between 2 to 3 h after release, and increased to maximum intensity between 6 and 7.5 h. Staining intensity declined and disappeared during mitosis and was not again found until the cells reentered the S phase when cytoplasmic staining reoccurred. Staining disappeared during the second round of mitosis and virus release. The patterns produced by anti-RMuLV and anti-MoMuLV were the same.

The appearance of surface immunofluores-

FIG. 3. *Membrane immunofluorescence.* NRK-R cells were stained in suspension with goat anti-RMuLV and then with rabbit anti-goat IgG-fluorescein conjugate as described. Cells were examined under UV illumination. Fluorescence was detected as apple green beading at the cell surface. All positive reactions appeared as above. Negative cells were not visible under UV illumination. Magnification $\times 210$.

cence in synchronized control NRK cells from four separate experiments is shown in Fig. 2D. The periodic pattern of surface immunofluorescence is the same as that seen in NRK-R and NRK-K cells. As in cytoplasmic immunofluorescence experiments, staining of synchronized control cells was less intense than in synchronized infected cells, and immune staining of NRK cells was not observed in nonsynchronized cultures. It should be noted that with both infected and control cultures, when membrane and cytoplasmic immunofluorescence tests were performed on the same cultures, we observed the patterns presented here.

DISCUSSION

The experiments presented here indicate that the production of virion-associated antigens and the release of infectious murine oncornavirus are coordinated to the cell cycle. The patterns of antigen appearance and release of infectious virus are repeated in consecutive cell cycles and can therefore not be interpreted as artifacts of the double thymidine block. Since the strongest cytoplasmic and membrane immunofluorescence occurred just prior to virus release, we are probably detecting the synthesis of virion proteins and their subsequent insertion into mature virions.

The distribution of murine *gs* antigens in cultured cells as detected by immunofluorescence and immunoperoxidase reactions has been described as diffuse staining throughout the cytoplasm (4, 7). Although the antigens in these experiments are not well characterized, murine *gs* antigens are probably being described. In synchronized cells, peak fluorescence was found during the S and G2 phases, but was not detected during mitosis and the early G1 phase. The absence of detectable immunofluorescence may result from diminished antigen synthesis and depletion of antigen pools. It seems premature to conclude that there are times in the cell cycle when virion proteins are absent from the cytoplasm of synchronized cells. Nevertheless, with a single antiserum dilution, we have demonstrated that immunofluorescence varies periodically with the cell cycle.

The pattern of surface immunofluorescence in synchronized NRK-R and NRK-K cells follows closely the pattern observed in the cytoplasm. Although membrane fluorescence was sometimes detected immediately after release from the thymidine block, all staining disappeared during mitosis and virus release. In the second mitotic cycle, surface staining did not reappear until the S phase, coincident with

cytoplasmic immunofluorescence. The periodic absence of surface fluorescence probably reflects the depletion of virion antigens due to virus maturation. We interpret this staining of cell surfaces immediately after release from thymidine blockage to represent residual virion antigens not utilized for virion production during the cell synchronization protocol. We suggest that virion proteins newly synthesized in the cytoplasm are rapidly inserted into plasma membranes from which they are removed during virus maturation.

Anti-RMuLV serum did not neutralize KiMSV(KiMuLV) but did cause membrane immunofluorescence of NRK-K cells under conditions where virions have been removed from the cell surface. This finding may be explained as the reaction of anti-RMuLV serum with (i) virion envelope components on the cell surface which are not involved in virus neutralization, (ii) internal virion components which are present at nonbudding sites on the cell surface, or (iii) nonvirion cell surface components which contaminated the virus used to produce anti-RMuLV serum. This last explanation seems unlikely as anti-RMuLV serum does not react with fetal calf serum or normal mouse cells and because the RMuLV used for antiserum production was grown in mouse cells (R. Wilsnack, personal communication). We favor the second explanation because preliminary immunoelectronmicroscope findings indicate that anti-RMuLV serum stains nonbudding plasma membrane sites of NRK-K cells but does not stain intact KiMSV(KiMuLV) virions (Panem, unpublished data). Furthermore, Yoshiki et al. recently showed that serum prepared against feline leukemia virus caused staining of nonbudding plasma membrane sites on cells infected with Gross leukemia virus (20) in immunoelectronmicroscopy. These authors concluded that an oncornavirus interspecies antigen was present on the cell surface. Further experiments on the appearance of internal virion components on cell surfaces are in progress.

Synchronized control NRK cells periodically expressed oncornavirus antigens in the cytoplasm and on the cell surface, whereas unsynchronized NRK cells did not stain with immune sera. Synchronization therefore appears to amplify the expression of a latent C-type virus. The antigens detected in NRK cells are probably murine *gs* antigens because the immune sera used here were made against virus of mouse cell origin. Further, the latent rat helper virus found in NRK cells does not share cross-reacting neutralizing antigens with Moloney or Rauscher mouse leukemia viruses (8). The detection of

weak fluorescence in NRK cells confirms recent reports that murine gs antigen can be detected at low levels by sensitive radioimmunoassays in NRK and other "virus-free" cells (3, 14, 18). Periodic expression of latent virus information explains the staining of mitotic control cells reported by several workers (4; R. B. Fritz, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 217, 1973).

The experiments presented here were done to explain the temporal restriction on the release of infectious murine leukemia-sarcoma viruses (12). It is now demonstrated that the level of virion antigens varies with the cell cycle, and we interpret these findings as temporarily restricted antigen synthesis. This interpretation would be strengthened by studies on the inhibition of protein synthesis in synchronized cells.

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