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Evidence for a Stable Intermediate in Leukemia Virus Activation in AKR Mouse Embryo Cells

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Analysis of the requirement for serum in the activation of the endogenous leukemia virus expression in AKR mouse embryo cells by 5-iododeoxyuridine shows that activation can be dissociated into two discrete serum-dependent events. The first involves incorporation of 5-iododeoxyuridine into DNA and results in the formation of a stable "activation intermediate" resembling the provirus formed during infection of stationary mouse embryo cells with exogenous leukemia virus. The second event, resulting in expression of the activation intermediate as synthesis of virus proteins, requires DNA replication but not 5-iododeoxyuridine.

In recent years much genetic and biochemical evidence has accumulated indicating that the genomes of murine leukemia viruses (MuLV) exist in an unexpressed form in all cells of a number of strains of mice (2, 7, 10, 14, 15). The cellular factors that determine the frequency of spontaneous expression of the viral genome are not known, but it is clear that this frequency differs among various mouse strains (15, 17) and that expression can be significantly enhanced by treatment of appropriate cells with such chemicals as 5-bromodeoxyuridine (BrdUrd) or 5-iododeoxyuridine (IdUrd) (1, 3, 11, 18). As an approach toward the molecular mechanism governing expression of the virus genome, we have analyzed some factors affecting activation by IdUrd of the leukemia virus of cultured embryonic cells from AKR mice, and have correlated our findings with events known to be significant in the replication of exogenous RNA tumor viruses.

MATERIALS AND METHODS

Viruses and cell cultures. Moloney leukemia virus was originally obtained from the laboratory of Wallace P. Rowe, National Institutes of Health. Stocks of virus were prepared in secondary Swiss (NIH strain) mouse embryo cell cultures grown in Eagle minimum essential medium (EMEM) containing 10% fetal calf serum. The cultures were maintained without antibiotics and were free of mycoplasma. Virus pools were prepared by collecting fluid from cultures infected at least 6 days and not more than 24 h after addition of fresh medium. Immediately after collection the virus was stored in a liquid nitrogen freezer.

AKR mouse embryo cells (passage number 44) were obtained from the laboratory of Wallace P. Rowe (15). The cultures were grown in EMEM with 10% fetal calf serum. For induction of virus synthesis, semiconfluent cultures were exposed to IdUrd (Schwarz/Mann) at 100 μg/ml for 24 h.

Virus assays. Activation of endogenous AKR virus was assayed by immunofluorescent techniques, which have been shown to be more sensitive than conventional XC plaque assays (6). Cover slips containing cells to be examined were fixed in cold acetone and stored at −20 C. The cells were subsequently incubated with rat antiserum to MuLV (1:40 dilution) for 30 min at 37 C, washed in phosphate-buffered saline (PBS), incubated for 30 min at 37 C with fluorescein-labeled antibody to rat globulin and lissamine-rhodamine counterstain, and washed again in PBS. The fluorescent cells were counted with a Zeiss fluorescence microscope equipped with a B612 exciter filter and a 470-nm barrier filter. The immunofluorescent reagents were provided by the Office of Program Resources and Logistics, National Cancer Institute. Results are presented as percentages of fluorescent cells of the total, determined by counting at least 10 microscopic fields and a minimum of 2,000 total cells. Confidence intervals were determined by using a confidence level of 95% and standard techniques to determine the confidence interval for a mean of normal distribution with unknown variance.

When immune precipitates of the viral antigens detected with the reagents were analyzed on polyacrylamide gels containing sodium dodecyl sulfate, essentially all of the major viral proteins reacted with the antisera; thus, the pattern seen with immune precipitates differed only quantitatively from that observed with unreacted lysates of the virus.

Although the percentage of cells activated varied with individual experiments, within each set of experiments duplicate samples generally varied less than the confidence intervals given with the data.

RESULTS

Effect of serum deprivation on exogenous virus expression. Temin (8, 19) has shown that infection of stationary chick fibroblasts with

exogenous avian sarcoma virus results in the formation of a provirus intermediate in which the viral genome is present as DNA. This intermediate is stable and will ultimately initiate expression of viral functions when the cells are allowed to divide. This observation suggests that regulatory functions occurring during the cell cycle are required to initiate virus expression from the viral DNA (8). The results shown in Table 1 suggest that a similar requirement is demonstrable with exogenous murine leukemia virus. When NIH/3T3 cells are deprived of serum, DNA synthesis, as detected by [^{3}H]thymidine incorporation, is completely inhibited after 24 h (data not shown). When cells which have been serum starved for 24 h are infected with Moloney leukemia virus, no virus expression can be detected by immunofluorescent techniques for up to 4 days after infection. However, when serum is added to the cultures at 1, 2, 3, or 4 days after infection, virus expression is evident 48 h after the readdition of serum. The requirement for cellular DNA synthesis and/or cell division is further suggested by the fact that cytosine arabinoside at concentrations that completely inhibit [^{3}H]thymidine incorporation (data not shown) can completely block virus expression when added at the time of readdition of serum.

TABLE 1. *Effect of serum deletion and cytosine arabinoside on exogenous virus infection*[a]

Treatment	Percent fluorescent cells + C.I.[b]	
	− Cytosine arabinoside	+ Cytosine arabinoside
No serum	0	0
Serum at day 1	74.1 ± 2.1	0
Serum at day 2	72.3 ± 1.7	0
Serum at day 3	69.8 ± 2.4	0
Serum at day 4	57.1 ± 3.1	0

[a] Approximately 10^5 NIH/3T3 cells were plated into 35-cm^2 wells containing duplicate cover slips. Twenty-four hours later the medium was removed, and the cells were washed three times with medium lacking serum and then refed medium lacking serum. After 24 h (at which time [^{3}H]thymidine incorporation is minimal as determined in control experiments), the cells were incubated with DEAE-dextran (25 μg/ml) in medium lacking serum for 1 h, and then were infected with MLV for 2 h in the absence of DEAE-dextran. The cells were then washed twice with medium containing no serum and refed medium without serum. Serum (10%) and cytosine arabinoside (0.1 mM) were subsequently added back at the indicated times. After 48 h the medium was removed, the cells were washed twice with PBS, and the cover slips were fixed in acetone at 4 C for 20 min. The cover slips were air-dried and stored at −20 C until they were stained for the presence of virus-induced proteins as described in Materials and Methods.

[b] C.I. = 95% confidence interval.

Effect of serum deprivation on activation by IdUrd. The above results suggest that during virus infection a stable intermediate is formed, the expression of which is dependent upon some cellular functions occurring during the cell cycle. We next examined activation of endogenous virus to determine whether a similar requirement might exist. Since activation by IdUrd requires DNA synthesis for incorporation of IdUrd and since this incorporation is required for activation, as suggested by the data of Teich et al. (18) as well as experiments presented below, we reasoned that activation might require two cell cycles for complete virus expression. Such a requirement is indicated by experiments (Fig. 1) in which we examined the ability of IdUrd to activate virus expression when added at various times after the removal of serum. When AKR cells are deprived of serum (Fig. 1A), the rate of DNA synthesis as measured by [^{3}H]thymidine incorporation initially remains constant for approximately 18 h, after which it begins to decline. Finally, by 30 to 36 h no further [^{3}H]thymidine incorporation is demonstrable. Since the doubling time of these cells is approximately 12 h (data not shown), at most two to three doublings could occur between the time when serum is removed and the time when no further DNA synthesis is detectable. Therefore, if IdUrd is added at various times after serum removal, the cells will be exposed to IdUrd for various numbers of cell cycles.

The ability of IdUrd to activate virus expression when added at different times after serum removal is shown in Fig. 1B. Serum was removed from the cultures at 0 h, and IdUrd was added at the times indicated. In all cultures, IdUrd was left on the cells for 24 h and then removed, and the cells were allowed to recover in medium with or without serum but in the absence of IdUrd. The percentage of cells activated to express viral antigens was subsequently determined after 48 h by fluorescent antibody techniques. When IdUrd was added at various times after serum removal and the cells were allowed to recover in the absence of serum, only when IdUrd was added immediately at the time of serum removal did a small percentage of cells express viral antigens. When IdUrd was added at later times, no activation was observed. As noted above, under these conditions, between the time of serum removal and when DNA synthesis is no longer detectable, the majority of cells should have gone through one

cell cycle and some cells through two cell cycles. These data therefore suggest that more than a single cell cycle is required for activation, and the small but significant activation observed when IdUrd is added at the time of serum removal suggests that perhaps two cell cycles are required. Furthermore, since these cells were maintained in the absence of serum from the time of IdUrd treatment throughout the experiment, activation of viral expression can occur in the absence of serum provided the cells can complete the cell cycles required to activate expression.

In contrast to the above results, when IdUrd was added at various times after the removal of serum and the cells were allowed to recover after a 24-h IdUrd treatment in the presence of serum, the ability of IdUrd to activate virus expression roughly paralleled the ability of the cells to synthesize DNA (and thus incorporate IdUrd) after the removal of serum. These results therefore confirm the demonstration by Teich et al. (18) that incorporation of IdUrd is required for activation, since the ability to activate correlates with the ability to incorporate IdUrd as a function of time after serum removal. These data further suggest that a second cell cycle is necessary to initiate virus expression, which may be unrelated to IdUrd incorporation.

Stability of the activation intermediate. The above data suggest that, in cells treated with IdUrd at least 7 h after serum removal, there may exist an "activation intermediate" that subsequently will initiate virus expression after the readdition of serum. To explore this possibility further we determined whether a stable activation intermediate could be demonstrated (Table 2). AKR cells were deprived of serum for 8 h and treated with IdUrd for 24 h. The IdUrd was subsequently removed, and the cells were refed medium lacking serum. At various times serum was added back, the cells were allowed to recover for 48 h, and the percentage of cells activated was determined. As seen in Table 2, a stable activation intermediate could be demonstrated; cells treated with IdUrd could be held in the absence of serum without evidence of virus expression, but this expression could be elicited as long as 4 days after IdUrd treatment by restoration of serum.

Effects of various inhibitors on activation. To analyze further the physiological requirements for activation we examined the effects of various inhibitors when added either with IdUrd (first phase) or with the readdition of serum (second phase). Thymidine can completely block activation of virus expression by IdUrd at concentrations that do not block DNA

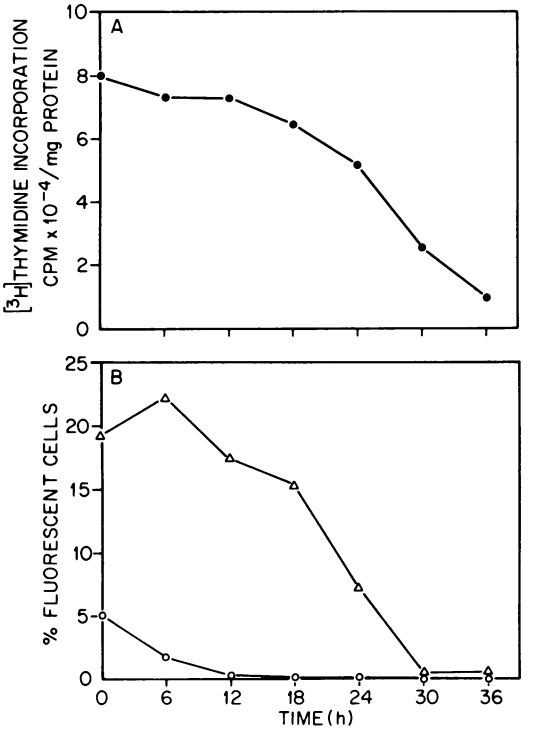

FIG. 1. *Requirements for DNA synthesis in activation of AKR virus by IdUrd. Approximately 10^5 AKR cells were plated in 35-cm² wells either not containing cover slips (A) or containing duplicate cover slips (B). Twenty-four hours later the medium was removed, and the cells were washed three times with medium lacking serum and then refed medium lacking serum. (A) At the indicated times, 5 μCi of [³H]thymidine (21.4 Ci/mmol) was added per ml and the cells were incubated with isotope for 1 h. The cells were then washed three times with PBS, scraped into 1 ml of PBS, and frozen at −20 C. Incorporated radioactivity was determined by sonically treating the cell suspension and spotting samples on Whatman 3MM disks. The disks were subsequently washed five times, 20 min each, in 5% trichloroacetic acid at 4 C, twice in ethanol:ether (1:1), and once in ether. Radioactivity was determined by scintillation counting. Protein was determined by the method of Lowry et al. (12). (B) At 0 h and at subsequent times indicated, IdUrd (100 μg/ml) was added to the cells and the cells were incubated in the presence of IdUrd for 24 h. After IdUrd treatment, the cells were washed twice with medium lacking serum and were refed medium with 10^{-6} M hydrocortisone either lacking serum (○) or containing serum (△). Hydrocortisone can increase the number of cells scored as positive by increasing the intracellular concentration of viral proteins but has no effect on the activation processes per se (J. N. Ihle et al., manuscript in preparation). Therefore, we have used it here to increase the sensitivity of the immunofluorescent technique. The cells were then incubated an additional 48 h and the cover slips were harvested and treated as in Table 1.*

TABLE 2. *Stability of activation intermediate*[a]

Treatment	Percent fluorescent cells ± C.I.[b]
Serum throughout	4.8 ± 0.4
Serum at day 1	3.7 ± 0.3
Serum at day 2	3.1 ± 0.5
Serum at day 3	4.1 ± 0.3
Serum at day 4	2.2 ± 0.4
No serum at day 1, 2, 3, or 4	0

[a] Approximately 10^5 cells were plated into 35-cm^2 wells containing duplicate cover slips. Twenty-four hours later the medium was removed from all cells and the cells were washed three times with medium lacking serum. Each well except the control (serum throughout) was refed medium lacking serum. After 8 h IdUrd (100 μg/ml) was added to all wells. After 24 h IdUrd was removed and the cells were washed twice with medium lacking serum, and medium lacking serum was refed to the cells. At appropriate times the cells were refed medium containing serum (controls were fed medium lacking serum), the cells were cultured an additional 48 h, and the cover slips were harvested and treated as in Table 1.

[b] C.I. = 95% confidence interval.

synthesis as measured by [^{3}H]deoxycytidine incorporation (data not shown). This inhibition is presumably due to competitive blocking of the incorporation of IdUrd (18). We therefore examined the effect of thymidine on activation, using the above techniques, to determine whether IdUrd incorporation was required during both cell cycles. As shown in Table 3, thymidine blocked activation only if added at the time of IdUrd addition and not when added during the second cell cycle, at the time of serum addition. Therefore, only during the first phase is the incorporation of IdUrd necessary, which in turn suggests that only single-strand incorporation of IdUrd into DNA may be required for activation.

In contrast to the above results, when cytosine arabinoside was used at concentrations that block DNA synthesis as measured by [^{3}H]thymidine incorporation (data not shown), virus expression was completely inhibited when added either with IdUrd or with serum. Since cytosine arabinoside reversibly inhibits DNA synthesis in AKR cells, the cells recovering 80% of their capacity to replicate after 12 h (18), these results suggest that both events are required for complete virus expression and both events require DNA synthesis. However, from the above data only the first requirement is related to incorporation of IdUrd into DNA. These data therefore suggest that the second requirement may be related to a regulatory event governing virus expression, which may be similar or identical to that observed with exogenous virus infection; and it is possible that the physical state of the provirus is identical to that of the activation intermediate.

Poly[2′-*O*-methyladenylate] [poly(Am)] and polyadenylate [poly(A)] are competitive inhibitors of the viral enzyme reverse transcriptase (21, 23). These polymers block infection of cells by exogenous virus, apparently by inhibition of the reverse transcriptase reaction (21, 22). We examined the effect of poly(Am) added at various times during infection and activation. As shown in Table 4, poly(Am) inhibited exogenous infection when added with the virus but not after the provirus intermediate had been formed. This result is consistent with the conclusion that the provirus is a DNA intermediate formed by reverse transcription of the added viral RNA (4, 20). Poly(Am) had no effect at either stage of virus activation, indicating that reverse transcriptase is not required for formation of the activation intermediate and, like the provirus, is not required for subsequent expression of this intermediate.

TABLE 3. *Effect of cytosine arabinoside and thymidine on activation*[a]

Treatment	Percent fluorescent cells + C.I.[b]	
	Exp 1	Exp 2
Control	12.6 ± 1.7	8.5 ± 1.6
Thymidine (25 μg/ml)		
With IdUrd only	0	0
With serum only	18.4 ± 2.1	10.3 ± 1.3
With IdUrd and serum	0	0
Control	10.3 ± 0.4	6.7 ± 0.7
Cytosine arabinoside (0.5 mM)		
With IdUrd only	0	0
With serum only	0	0
With IdUrd and serum	0	0

[a] Approximately 10^5 cells were plated into 35-cm^2 wells containing duplicate cover slips. Twenty-four hours later, the medium was removed from the well and the cells were washed three times with medium lacking serum, then refed medium lacking serum. After 8 h IdUrd (100 μg/ml) was added with or without the inhibitors and the cells were incubated for 24 h. IdUrd was then removed and the cells were refed medium containing serum with or without the inhibitors. The cells were harvested after 48 h and treated as described in Table 1.

[b] C.I. = 95% confidence interval.

TABLE 4. *Effect of poly(Am) on exogenous virus infection and activation*[a]

Treatment	Percent fluorescent cells ± C.I.[b]
Exogenous virus:	
Control	23.3 ± 2.1
Poly(Am) with virus only	1.4 ± 0.5
Poly(Am) with serum only	27.1 ± 1.3
Activation:	
Control	8.2 ± 1.6
Poly(Am) with IdUrd only	9.2 ± 1.3
Poly(Am) with serum only	8.5 ± 1.3

[a] Exogenous virus infections were performed as in Table 1. Poly(Am) at 10 μg/ml was added with virus during infection or at the time of serum restoration. When added with serum, the cells were treated with DEAE-dextran (25 μg/ml) for 1 h prior to the addition of serum and poly(Am). Poly(Am) was removed after 24 h and fresh medium containing serum but no poly(Am) was refed to the cells. Activation experiments were performed as in Table 3, with the exception that DEAE-dextran was added for 1 h prior to IdUrd treatment with or without poly(Am) or for 1 h prior to the readdition of serum with or without poly(Am). In both cases poly(Am) (10 μg/ml) was left on the cells for 24 h.

[b] C.I. = 95% confidence interval.

DISCUSSION

Iododeoxyuridine is effective in activating AKR virus only if the cells are actively synthesizing DNA when exposed to the analogue, and activation does not occur if incorporation of IdUrd into DNA is blocked by competing thymidine. These results are consistent with the conclusion that IdUrd must be incorporated into the cellular DNA in order to effect virus activation, and they confirm previous work by Teich et al. (18).

Our results further show that incorporation of IdUrd into DNA results in the formation of a stable cellular alteration, which we have termed the "activation intermediate." After formation of the intermediate in a time span encompassing probably one cell cycle, IdUrd is no longer necessary and the cells, although not expressing viral antigens, retain the capacity for virus expression for at least several days. The molecular nature of the activation intermediate is not known, but it appears to be comparable and perhaps identical to the provirus formed after infection of avian (8, 19) and mouse cells with exogenous oncornavirus. Provirus formation requires reverse transcription of the exogenous viral RNA, a result consistent with the now accepted view that the provirus is DNA (8). Inhibition of the reverse transcriptase function, however, does not affect formation of the activation intermediate. This indicates that, if the intermediate (like the provirus) is some form of viral DNA, its formation does not proceed via synthesis from viral RNA. Thus the available data indicate only that formation of the activation intermediate requires alteration of the cellular DNA, an alteration that can be brought about by incorporation of halogenated pyrimidines into DNA. A suggestion of greater complexity in intermediate formation is available in the demonstration by Wu et al. (24) of a transient period early in the activation process during which activation is blocked by 3'-deoxyadenosine (cordycepin). Since this compound blocks the formation of functional messenger RNA (5, 13), this important result suggests the involvement of cellular mRNA(s), and perhaps the protein(s) transcribed from them, in bringing the virus genome into an expressible form. Their results might also indicate inhibition of a portion of viral RNA synthesis, since some of the viral 34S RNA subunits contain polyadenylate regions (9).

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