Analysis of Simian Virus 40-Induced Transformation of Hamster Kidney Tissue in Vitro

VIII. Induction of Infectious Simian Virus 40 from Virogenic Transformed Hamster Cells by Amino Acid Deprivation or Cycloheximide Treatment

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Clones of virogenic simian virus 40 (SV40)-transformed hamster kidney cells were exposed to medium deficient in the essential amino acids leucine, arginine, or methionine. Infectious virus was induced after deprivation periods of from 24 to 32 hr. The highest yields of infectious SV40 were obtained from cultures deprived for 3 to 4 days. Infectious virus was also induced in cells that were treated with the metabolic inhibitor cycloheximide. Pulse labeling experiments revealed that both protein synthesis and deoxyribonucleic acid (DNA) synthesis were inhibited by concentrations of cycloheximide which were effective for virus induction. It is suggested that inhibition of protein synthesis by either amino acid deprivation or by cycloheximide was responsible for the induction of infectious virus from virogenic cells. We postulate that the inhibition of protein synthesis caused a temporary inhibition of DNA synthesis which resulted in the induction of infectious virus.

Virogenic mammalian cells transformed by simian virus 40 (SV40) contain the complete viral genome(s) integrated into their deoxyribonucleic acid (DNA; reference 27). Only a portion of the viral genome is expressed, and infectious virus is not spontaneously synthesized. However, infectious virus may be induced from cloned cell populations by several methods including co-cultivation with indicator cells and treatment with exogenous agents such as mitomycin C (9, 10), bromodeoxyuridine, caffeine, and ultraviolet (UV) or X-irradiation (25; J. C. Kaplan et al., Proc. 9th Can. Cancer Conf., *in press*). These agents all have in common the ability to inhibit DNA synthesis and are also inducers of lysogenic bacteria. However, the mechanism of induction is not completely understood. It has been reported that the inhibition of protein synthesis in mammalian cells by amino acid deprivation or treatment with metabolic inhibitors like cycloheximide or puromycin results in an inhibition of DNA synthesis (5, 12, 17, 20, 31). It was therefore of interest to determine the effect of these conditions on the inducibility of SV40-transformed hamster cells, since rescue of virus by a new method might help to elucidate the molecular events of induction.

In the present study, we report that deprivation of virogenic transformed hamster kidney cells of the essential amino acids leucine, arginine, or methionine caused the induction of infectious

SV40 virus. In addition, treatment of these cells with metabolic inhibitors which depress protein synthesis, such as cycloheximide and puromycin, also resulted in induction of infectious virus. A comparative study of the rates of protein synthesis and DNA synthesis, after treatment with cycloheximide, was carried out in an attempt to determine whether inhibition of protein synthesis preceded the inhibition of DNA synthesis.

MATERIALS AND METHODS

Cell cultures. The T8-AP1 cell line, an established clone of transformed hamster kidney cells, was used in the present studies. Its origin and history have been described previously (6, 9). Cells of this line are virogenic; they contain the complete SV40 genome(s) in that infectious virus can be rescued by co-cultivation with indicator cells (9) or treatment with chemical or physical agents (9, 10, 25). Cells were maintained in Eagle's minimal essential medium (Grand Island Biological Co.) which contained a fourfold concentration of amino acids and vitamins (MEM × 4), 10% fetal calf serum (FCS; Microbiological Associates), penicillin (250 units/ml), and streptomycin sulfate (250 µg/ml). Cultures were maintained at 37°C in a humidified atmosphere which contained 5% CO₂ in air. All experiments were conducted with T8-AP1 cells between their 7th and 15th passages in tissue culture after cloning. These cells were free from *Mycoplasma* contamination.

Amino acid deprivation. Amino acid deprivation experiments were carried out as follows. Medium was

removed from confluent monolayer cultures of T8-AP1 cells in 60-mm plastic petri dishes (Falcon Plastics, Inc.), and the monolayers were washed twice with phosphate-buffered saline, pH 7.2 (PBS). Thereafter, the cultures were maintained for various lengths of time (24 to 168 hr) in medium (MEM $\times$ 4–10% dialyzed FCS) deficient in either leucine, arginine, or methionine. Complete medium (5 ml) was returned to the monolayers for an additional 3 days. The cultures were then frozen and thawed three times, and a cell-free extract was made by sonically treating the suspension in a Raytheon Sonifier (3 amp for 60 sec).

Cycloheximide, puromycin, and canavanine treatment. Confluent monolayers of T8-AP1 cells were exposed to various concentrations of cycloheximide (Nutritional Biochemicals Corp.), puromycin-HCl (Nutritional Biochemicals Corp.), or canavanine sulfate (Calbiochem. Corp.) for 8, 16, and 24 hr. At the end of each time period, the metabolic inhibitor was removed, the monolayers were washed, and fresh medium was added for an additional 3 days. Extracts were prepared as described for the amino acid deprivation experiments.

Assay of SV40 infectivity. Cell-free extracts were tested for SV40 infectivity by a modification of a previously described plaque assay (7). BSC-B monkey kidney cells and Dulbecco's medium were used in the present studies. Portions (0.2 ml) of each sample were inoculated onto three replicate monolayers of BSC-B cells. Plaques were developed with neutral red (0.01%) on the 12th day and read on days 13 and 14.

Measurement of DNA and protein synthesis. Cell monolayers were incubated for 15 min or 1 hr in 5 ml of MEM $\times$ 4–10% FCS containing either ^3H -thymidine (New England Nuclear Corp., NET-027X, specific activity, 20.2 Ci/mmol) at a final concentration of 1.0 $\mu\text{Ci/ml}$ or leucine-4,5- ^3H (ICN, specific activity, 29.8 Ci/mmol) at a final concentration of 5.0 $\mu\text{Ci/ml}$. Labeled monolayers were washed twice with cold PBS, scraped from the dish, collected by centrifugation, washed twice with cold PBS, and sonically treated for 3 min. The nucleic acids and proteins were precipitated from half of the sonically treated material with cold 5% trichloroacetic acid, and the precipitate was collected on glass-fiber discs (Reeve Angel Co.). The discs were washed three times with cold 5% trichloroacetic acid and dried. Radioactivity was determined by counting the discs in toluene scintillation fluid, containing 4 g of Omnifluor per liter (New England Nuclear Corp.), in a Packard 3320 liquid scintillation counter. Total protein was estimated on the original solution by using the procedure of Lowry et al. (21).

RESULTS

Amino acid deprivation. Deprivation of T8-AP1 cells of leucine, arginine, or methionine resulted in induction of infectious virus in all cases (Fig. 1). Infectious SV40 was synthesized when leucine was withdrawn from the medium for at least 24 hr, and virus yield peaked after 3 days of deprivation. Thereafter, approximately the same yields of virus were obtained from cultures deprived of leucine for 4 to 6 days (Fig. 1A). Beyond 6 days of amino acid withdrawal, the cell monolayers were

completely destroyed in all deprivation experiments.

The highest yields of infectious virus obtained from T8-AP1 cells deprived of arginine occurred after a deprivation period of 3 to 4 days (Fig. 1B). The amount of infectious virus decreased between 4 and 7 days of deprivation. The reason for the

FIG. 1. Virus yield after amino acid deprivation of T8-AP1 cells. Cell cultures were treated as described in Materials and Methods. Virus yield in plaque-forming units (PFU) is plotted against time of amino acid deprivation (days). Each value represents the average of two to three experiments. Each cell extract (5 ml) was equivalent to approximately 10^6 cells. Although points on the abscissa indicate actual time of deprivation of the particular amino acid, in all cases complete medium was added for an additional 3 days before harvest.

observed decline in infectivity is not known but could possibly be related to the small pool of arginine in mammalian cells.

T8-AP1 cells were deprived of methionine from 1 to 6 days (Fig. 1C). Significant amounts of virus did not appear unless the cells were deprived for at least 3 days. Methionine deprivation beyond 3 days yielded the same quantities of virus.

Since amino acid deprivation resulted in virus induction, the effect of substitution of an amino acid analogue on induction of infectious virus was determined. L-Canavanine sulfate is a structural analogue of arginine. It has been reported that the substitution of canavanine for arginine in hamster and mouse cells causes an inhibition of protein, ribonucleic acid (RNA), and DNA synthesis (15). Only trace amounts of virus were induced from T8-AP1 cells treated with 0.22 mM canavanine for 24 hr. This analogue was extremely toxic to the cell monolayers. At least 50% of the cell sheet was destroyed during a 24-hr exposure utilizing this concentration of analogue. Cells treated with lower concentrations of canavanine did not produce detectable amounts of infectious virus.

Induction of virus by cycloheximide treatment.

Since the inhibition of protein synthesis in mammalian cells is generally accompanied by a depression of DNA synthesis and since amino acid deprivation causes virus induction in T8-AP1 cells, the effect of a chemical inhibitor of protein synthesis, cycloheximide, on inducibility was determined. Induction of infectious virus was achieved with cycloheximide concentrations ranging from 5 to 25 $\mu\text{g/ml}$ with exposure for 16- and 24-hr periods (Table 1). Within the effective concentration range, the virus yields were not directly related to the concentration of cycloheximide used. Virus yields were lower than those obtained after amino acid deprivation. Although the drug did not produce visible toxicity to the cell monolayers, it is conceivable that the extent of inhibition of protein synthesis was enough to induce T8-AP1 cells but was also too extreme to allow for maximum virus production. Another inhibitor of protein synthesis, puromycin, was also used to induce T8-AP1 cells at a concentration of 10 $\mu\text{g/ml}$. Although a trace of infectious SV40 virus was released, in general this drug caused severe toxicity to the cell monolayers.

Inhibition of protein and DNA synthesis by cycloheximide. Cycloheximide is a potent inhibitor of protein synthesis in mammalian cells. It is possible that particular proteins are required for the initiation and maintenance of DNA replication. We therefore wished to determine whether cycloheximide treatment inhibited both protein and DNA synthesis in T8-AP1 cells under the conditions of the induction experiments and whether inhibition of protein synthesis preceded inhibition of DNA synthesis. Therefore, T8-AP1 cell monolayers were treated with various concen-

TABLE 1. *Virus yield after cycloheximide induction^a*

Cycloheximide ($\mu\text{g/ml}$)	Exposure (hr)	Virus yield (PFU) ^b
0	8	0
	16	0
	24	0
2	8	0
	16	0
	24	0
5	8	0
	16	425
	24	325
10	8	0
	16	400
	24	600
25	8	0
	16	110
	24	200

^a Cell cultures were treated as described in the Materials and Methods.

^b Virus yield expressed as plaque-forming units per sample. Each cell extract (5 ml) contained approximately 10^6 cells. Each value represents the average of two to three experiments.

trations of cycloheximide (5 to 25 $\mu\text{g/ml}$) for either 8-, 16-, or 24-hr time periods. At the end of each interval, the cycloheximide was removed and the monolayers were labeled with either ^3H -thymidine or ^3H -leucine as described above.

Figure 2A shows that protein synthesis was inhibited at all of the time periods and concentrations of cycloheximide used. Maximum inhibition was 87% after a 24-hr exposure to 25 μg of cycloheximide per ml. Similarly, Fig. 2B shows that DNA synthesis was also markedly depressed in the presence of cycloheximide. A 92% inhibition was observed when the monolayers were incubated with 25 μg of cycloheximide per ml for 24 hr. Maximum virus yields were obtained when the cultures were exposed to cycloheximide at concentrations of 10 $\mu\text{g/ml}$ for 16- and 24-hr periods (Table 1). Under these conditions, protein synthesis was inhibited by 77 and 85%, and DNA synthesis was inhibited by 70 and 92% at 16 and 24 hr, respectively.

Since inhibition of protein and DNA synthesis occurred to approximately the same extent after an 8-hr exposure to cycloheximide, a kinetic experiment was carried out to determine whether the inhibition of protein synthesis preceded or paralleled a depression of DNA synthesis during the first 7 hr of cycloheximide exposure. It was observed that even as early as 30 min after cycloheximide treatment both protein synthesis and DNA synthesis were inhibited to approximately

FIG. 2. Effect of cycloheximide on macromolecular synthesis of T8-AP1 cells. The cells were treated with cycloheximide and pulse-labeled for 1 hr with either ^3H -leucine or ^3H -thymidine at the end of 8-, 16-, and 24-hr periods. Different concentrations of cycloheximide were used for 8 hr ($\bullet$), 16 hr ($\circ$), or 24 hr ($\times$), respectively.

the same extent (Fig. 3). Thereafter, inhibition of DNA synthesis paralleled inhibition of protein synthesis. However, DNA synthesis was inhibited to a greater degree than protein synthesis.

DISCUSSION

These studies demonstrate that the deprivation of transformed virogenic cells of some essential amino acids results in infectious virus induction. The first amino acid to be withdrawn from the T8-AP1 cell medium was leucine, a general component of all proteins. It is likely that leucine removal primarily caused an inhibition of protein synthesis, since a unique relationship has not been established between this amino acid and the control of DNA synthesis. Arginine is found in high concentrations in histones which have been implicated in the control of DNA synthesis (8). The pattern of induction with arginine deprivation was different from the one observed subsequent to leucine deprivation. Infectivity increased until 4 days of deprivation but declined greatly from 5 to 7 days, whereas it had leveled off in the leucine-deprived cultures. The reason for the observed decline of infectivity is unknown but could be related to the small pool of arginine in mammalian cells (24). As a result, the effects of its depletion on virus synthesis have been realized sooner than with leucine. Arginine deprivation has been found to cause an inhibition of both viral DNA (23, 33) and protein synthesis (14). In addition, arginine is necessary for the assembly of certain viruses (23, 26, 33). The amino acid methionine was also withheld from T8-AP1 culture medium. Again, infectious SV40 virus was induced in a pattern similar to that of leucine deprivation. It is not known whether the effects of methionine removal are due to an inhibition of

FIG. 3. Effect of cycloheximide on macromolecular synthesis of T8-AP1 cells. The cells were treated with cycloheximide at a final concentration of $10 \mu\text{g/ml}$ and pulse-labeled for 15 min at the indicated time periods with either ^3H -thymidine ($\bullet$) or ^3H -leucine ($\circ$). Percent inhibition of either ^3H -thymidine incorporation or ^3H -leucine incorporation into DNA and protein, respectively, is plotted against time of cycloheximide treatment (hours).

protein synthesis or instead a direct inhibition of DNA synthesis. It has been shown that methylation of DNA is involved in the control of DNA replication in mammalian cells in that DNA synthesis is inhibited after methionine deprivation (11). The induction of infectious virus is thought to be composed of several molecular events. Initially, the genome is induced from its integrated state in cell DNA; it is then replicated and encapsidated. Amino acid deprivation could act by favoring the early phase of induction as infectious SV40 is never made unless complete medium is returned to T8-AP1 monolayers after the deprivation period.

Cycloheximide is a potent inhibitor of protein synthesis in mammalian cells. Since it was possible to induce infectious SV40 from T8-AP1 cells with this agent, it indicates that a direct inhibition of protein synthesis led to virus induction. Virus yields were lower after induction by cycloheximide than by amino acid deprivation. The reason is unknown but could be due to the dependence of the virus maturation process on the presence of a cycloheximide-sensitive protein. Kit et al. have shown that cycloheximide inhibits SV40 DNA replication when it is added early or late after infection (18). Puromycin, another inhibitor of protein synthesis, caused the induction of only trace amounts of infectious SV40. However, this metabolic inhibitor was extremely toxic to the cell monolayers.

Pulse labeling experiments were done to follow the incorporation of tritiated thymidine into DNA and tritiated leucine into protein under the conditions of the induction experiments. It was found that both protein and DNA synthesis were inhibited, but inhibition of both processes occurred so rapidly, after exposure of the cells to cycloheximide, that a temporal relationship between inhibition of the two phenomena could not be established. It has been demonstrated that an inhibition of protein synthesis preceded an inhibition of DNA synthesis in synchronized mammalian cell populations. Treatment of cells in the G₁ phase of the cycle with either cycloheximide or puromycin prevented the cells from entering the S or DNA synthetic phase (17, 20). It is therefore likely that a particular protein(s) is involved in the initiation of DNA synthesis. It has also been shown in certain mammalian cells that withdrawal of isoleucine from the medium caused the cells to arrest in G₁. When isoleucine was returned to the medium, the cells then entered the S phase in synchrony (31). Protein synthesis has been shown to be essential for the initiation of DNA synthesis in bacteria (19).

If the end result of protein synthesis inhibition by either amino acid deprivation or cycloheximide treatment is an inhibition of DNA synthesis, then all of the agents found thus far which induce infectious SV40 from T8-AP1 cells have in common the ability to effect a temporary inhibition of DNA synthesis. Until now, all exogenous agents found to induce virogenic cells have been mutagens. They cause structural damage to the DNA molecule, either phosphodiester bond breakage (X ray), cross-linking (mitomycin C), base substitution (bromodeoxyuridine), or base damage (UV). Amino acid deprivation is probably a more physiological method of induction since no agent is added to the cells.

Although the molecular events responsible for virus induction are not known, most of the available evidence suggests that the viral genome is excised from its integrated state. In the case of

bacterial lysogeny, a viral coded endonuclease has been described which could be responsible for genome excision (28). Some agents that have successfully rescued SV40 virus cause DNA breakage either directly (X ray) or indirectly (UV or mitomycin C). It has been demonstrated in mammalian cells that amino acid deprivation can result in chromosomal breakage as well (13). It is not known whether DNA breakage is responsible for genome excision and virus induction or whether random chromosome breakage is incidental to virus induction.

An alternative hypothesis is that inhibition of protein synthesis dilutes the number of repressor molecules in the cell, causing genome derepression and virus induction. Factors responsible for repression of the viral genome in transformed mammalian cells are currently unknown, yet a repressor substance, of protein nature, has recently been described which is specified by SV40 virus (29). An activity has also been described in adenovirus 12-transformed BHK cells which inhibits plaque formation in permissive cells (W. Strohl and W. Schlessinger, *personal communication*). The inhibition of macromolecular synthesis could cause a metabolic imbalance that is physiologically conducive to genome derepression. It has been reported that certain virogenic transformed cells, when fused with permissive cells, yield greater amounts of infectious virus if they are treated with the metabolic inhibitors iododeoxyuridine or 8-azaguanine (32). Such agents could be influencing the coding for and synthesis of repressor molecules in the cell.

The discovery of yet another technique for inducing infectious virus is important in helping to elucidate the molecular events involved. Amino acid deprivation as a method for induction of infectious virus has not previously been described for mammalian cells. However, arginine deprivation of Burkitt's lymphoma cells has resulted in the expression of EBV antigens in a greater proportion of cells (16). In addition, in bacteria it has been demonstrated that colicins may be induced by amino acid deprivation and puromycin treatment (3, 4).

The studies presented here may have relevance for investigating cells transformed by oncogenic RNA viruses. Since the discovery of a reverse transcriptase enzyme, it has been postulated that genomes of oncogenic RNA viruses are integrated into cellular genomes via DNA copies (2, 30). This suggests that agents capable of inducing DNA virus-transformed cells should similarly induce oncogenic RNA viruses from cells. In fact, it has been shown recently that the agents iododeoxyuridine and bromodeoxyuridine can induce infectious RNA leukemia virus (22) or virus markers (1) in normal mouse cells. Ultimately one would like to investigate human malignant cells with techniques which successfully induce

viruses from animal cells. Therefore, it is important to study and have available as many methods for virus induction as possible.

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ADDENDUM IN PROOF

Pulse-labeling experiments designed to determine whether protein or DNA synthesis, or both, were inhibited after amino acid deprivation of T8-AP1 cells revealed that the rates of both protein and DNA synthesis were inhibited. In these studies, protein synthesis was inhibited to a greater degree than DNA synthesis after deprivation of arginine, methionine, or leucine.

Infectious virus has also been induced after amino acid deprivation from another clone isolated from a different virogenic SV40-transformed kidney cell line. The kidney cells were established from an inbred strain of hamsters (LSH/ssLAK) which were obtained from the Lakeview Hamster Colony. At least 1 log more virus was recovered when transformed cells were deprived of either methionine or arginine.

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