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Alterations to Controls of Cellular DNA Synthesis by Adenovirus Infection

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Human adenovirus type 5 and temperature-sensitive mutants *ts*36, *ts*37, and *ts*125 induced cellular DNA synthesis in quiescent rodent cells at both permissive and nonpermissive temperatures. Cellular DNA synthesis induced by adenovirus type 5 or by serum required protein synthesis for both initiation and continuation, whereas viral DNA synthesis was not dependent upon continued protein synthesis once it was initiated. Both cellular and viral DNA replication was induced in adenovirus type 5-infected cells in the presence of dibutyryl cyclic AMP at concentrations which inhibited induction by serum, which suggested that some of the controls of DNA synthesis in serum-treated and virus-infected cells are different. After adenovirus infection of quiescent cells, there was a decrease in the number of cells with G_1 DNA content and an increase in cells with G_2 diploid and greater DNA contents. Thus, adenovirus type 5 induces a complete round of cellular DNA replication, but in some cells, it induces a second round without completion of a normal mitosis. These results suggest that adenovirus type 5 is able to alter cell growth cycle controls in a way which may be related to its ability to transform cells.

Several serotypes of human adenoviruses have been shown to induce cellular DNA synthesis in serum-arrested or confluent cells of several types (23, 24, 31). Adenoviruses thus overcome the "restriction point" controls (19) in the G_1 phase of the cell cycle and cause cells to begin cycling. This phenomenon is not unique to adenoviruses as it has also been observed for several other viruses, including simian virus 40 (7) and Rous sarcoma virus (17). The mechanism by which this induction occurs is not understood, but it is likely that viruses alter cell cycle controls in some way.

Many adenoviruses, including the nononcogenic varieties, transform rodent cells in vitro, as well as a wide range of other cell types (3, 10). In some cases these transformed cells form tumors when inoculated into appropriate animals (10). How this event is caused is also unknown.

Temperature-sensitive (*ts*) (28), deletion (16), and host range (13) mutants of adenovirus type 5 (Ad5), which show different properties with respect to rodent cell transformation, have been isolated (8, 11, 29). The regions which affect transformation frequency are the left-hand 1.1 to 6% of the Ad5 genome (10), the region coding for the gene *N* product (18.5 to 22.0% [6, 29]), and the region coding for the DNA-binding protein (62 to 68% [8]). *ts* mutants in gene *N* (*ts*36 and ts37) are defective for viral DNA replication in human cells and have a reduced ability to transform rat cells at 38.5°C, but behave as does wild-type virus at 32.5°C (29). Mutant *ts*125 is defective in viral DNA replication because of a defect in the DNA-binding protein at 38.5°C (27) and transforms rat cells with a higher frequency than does wild-type Ad5 at all temperatures (8).

Some degree of correlation between transformation frequency and the induction of cellular DNA synthesis has been suggested for simian virus 40 (2, 7, 18, 26). Simian virus 40 T antigen, which has been implicated in transformation (2, 4), has been clearly shown to induce cellular DNA replication (2). These observations led us to investigate further the induction of DNA synthesis in cells infected by Ad5 and some of the mutants defective in viral DNA replication and with altered transformation properties. We report experiments designed to probe the mechanism of this induction with some drugs. We provide evidence that a complete round of cellular DNA synthesis occurs in quiescent cells after infection by Ad5 and that Ad5 induces the formation of cells with higher-than-normal DNA contents.

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MATERIALS AND METHODS

Cells and media. Primary cultures of C57BL mouse embryo fibroblasts and PVG or Wistar rat embryo fibroblasts were prepared as previously described (1). Cultures of all cells were grown in Autopow medium (AP; Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% fetal calf serum (FCS) in 75-cm^2 plastic tissue culture flasks (Falcon Plastics, Oxnard, Calif.).

Virus and virus growth. Ad5, *ts*36, *ts*37, and *ts*125 were grown in KB cells and titrated in HEK cells by the fluorescent cell counting method (21). Virus inocula were stored at −70°C as crude cell lysates. All mutants were grown from stocks originally supplied by J. Williams, Carnegie-Mellon University, Pittsburgh, Pa.

Radioactive isotopes and chemicals. ^{32}P (carrier-free) was obtained from the Australian Atomic Energy Commission, Lucas Heights, Sydney, Australia, and [*methyl*-^{3}H]thymidine ([*methyl*-^{3}H]dThd; 48 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, United Kingdom. Cycloheximide and N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphoric acid (dbcAMP) were from the Sigma Chemical Co., St. Louis, Mo.

Viral DNA preparation. ^{32}P-labeled Ad5 was prepared by incubating infected cells in phosphate-free Eagle medium supplemented with 50 μCi of ^{32}P per ml and 1% FCS. ^{32}P-labeled virus was then purified and DNA was extracted as previously described (30).

Growth and arrest of cells. Primary cells were seeded at a density of 4×10^5 to 7×10^5 cells into 50-mm plastic petri dishes (Kayline Plastics, Thebarton, South Australia), cultured for 1 day in AP–10% FCS, and then arrested by incubation in AP–0.2% bovine serum for 2 days (mouse) or 3 days (rat).

Infection. Cells were infected with Ad5 in 1.0 ml of AP without serum for 1.5 h or with 1.0 ml of AP only for a mock infection. The inoculum was then removed, and either the original medium was replaced (virus-infected and mock-infected cultures) or AP–10% FCS was added (serum-stimulated cultures). In all experiments the multiplicity of infection was between 10 and 20 infectious units per cell.

Intracellular DNA synthesis (method 1). After being labeled with [*methyl*-^{3}H]dThd, both infected and uninfected cell cultures were washed once with phosphate-buffered saline and lysed by incubation for 2 h at 37°C with a solution containing 1.0 ml of 1-mg/ml protease VI (preincubated for 5 min at 56°C and for 30 min at 37°C) per 50-mm petri dish, 0.1 M NaCl, 5 mM EDTA, 10 mM Tris-hydrochloride (pH 8.0), and 0.5% sodium dodecyl sulfate. DNA was extracted twice with phenol (saturated with 0.1 M NaCl–0.1 M Tris-hydrochloride [pH 8.0]–1 mM EDTA) and an equal volume of chloroform-isoamyl alcohol (24:1). DNA was then dialyzed against STE (0.1 M NaCl–50 mM Tris-hydrochloride [pH 7.2]–1 mM EDTA).

Neutral CsCl equilibrium gradient centrifugation of intracellular [^{3}H]DNA. A 0.4-ml amount of each DNA sample extracted and treated as described above (method 1) was mixed with ^{32}P-labeled Ad5 DNA, STE, 0.1% Sarkosyl, and CsCl to give a final volume of 8.0 ml and a density of 1.704 to 1.707 g/ml. The samples were overlaid with paraffin oil and centrifuged in a 50 Ti rotor at 33,000 rpm for 40 h at 20°C. The gradients were fractionated from the bottom, and 80-μl samples were spotted onto Whatman 3MM 2.0-cm filter paper disks. Disks were washed, and the radioactivity was determined as described in method 2. The curves of total [*methyl*-^{3}H]dThd-labeled DNA were analyzed into viral DNA and cellular DNA components by using a PDP 11 computer and principles described previously (31).

Total intracellular DNA synthesis (method 2). After being labeled with [*methyl*-^{3}H]dThd, cultures were washed twice with phosphate-buffered saline and then harvested by treatment with 0.025% trypsin in phosphate-buffered saline. Cells were suspended, and 100-μl samples were removed and spotted onto Whatman 3MM 2.0-cm filter paper disks. Disks were washed twice for 10 min in cold 10% trichloroacetic acid and twice for 10 min in cold ethanol. Filter paper disks were dried, and their radioactivity was measured in a Packard liquid scintillation spectrometer.

Autoradiography. Cells were grown on glass cover slips (diameter, 13 mm) in 50-mm plastic petri dishes. At appropriate times after infection and labeling with [*methyl*-^{3}H]dThd, cover slips were removed from petri dishes, washed several times with phosphate-buffered saline, and fixed in methanol-acetic acid (3:1). Cover slips were mounted on glass microscope slides, dipped in Kodak NTB-2 photographic emulsion (diluted 1:3 with distilled water), exposed for 1 to 3 days at 4°C, and developed with Kodak D19 developer followed by fixation with Ilford Hypam Rapid Fix. Cover slips were then stained with Giemsa, and the proportion of cells showing nuclear grains was scored. At least 400 cells were counted per cover slip.

Tests for viral antigens. Cells were grown on cover slips as described above, fixed in acetone (−20°C), and tested for viral antigens by the indirect fluorescent-antibody test, using rabbit P antiserum (which reacts with the DNA-binding protein and with T antigen [12]) or V antiserum (which reacts with Ad5 virions).

Feulgen microspectrophotometry. Cells were grown on glass microscope slides in 10-mm glass petri dishes, fixed, Feulgen stained, and scanned on a Zeiss microspectrophotometer as described by Gould (9).

Flow microfluorimetry. Cells were grown in 25-cm^2 plastic flasks (Falcon) in AP–10% FCS, arrested in G_1 by serum starvation, and infected, mock infected, or serum stimulated as described above. Nuclei were released from the cells and stained with mithramycin-ethidium bromide, and their DNA contents were analyzed by flow microfluorimetry by the method of Taylor (25).

RESULTS

Induction of cellular DNA synthesis in rodent cells. Rodent cells are semipermissive for replication of the nononcogenic group C adenoviruses (5, 31), although they can be transformed by these and other adenoviruses (8, 29). Thus, it seemed that such a semipermissive system would be useful for studying the induction of cellular DNA synthesis and its possible con-

nection with viral DNA replication and with transformation. Cellular DNA synthesis was induced in serum-starved rat and mouse cells either by the addition of fresh serum or by infection with Ad5 (Fig. 1). The induction was greater with serum than with Ad5. The induction of cellular DNA synthesis by Ad5 occurred before any viral DNA was detectable by equilibrium gradient centrifugation (Fig. 1).

The induction of cellular DNA synthesis by Ad5 was prevented by treatment of the virus with antiserum to Ad5 virions before infection or by UV irradiation (Fig. 2). These data provide evidence that the induction of DNA synthesis is due to a specific effect of virus. The induction of DNA synthesis is also markedly dependent upon the multiplicity of infection and can occur in the complete absence of serum (data not shown). This latter result excludes the possibility that Ad5 is only altering the response of cells to low serum.

Effect of *ts* mutations in Ad5 on the induction of cellular DNA synthesis. The induction of cellular DNA synthesis by the *ts* mutants *ts*36, *ts*37, and *ts*125 (see above) was studied to investigate possible relationships among the induction of cellular DNA replication, viral DNA replication, and transformation.

The three *ts* mutants induced cellular DNA synthesis detectable by CsCl density gradient centrifugation of DNA from rat (Table 1) and mouse (Table 2) cells at the permissive temperature (32.5°C), at which viral DNA was also replicated. Cellular DNA replication was also induced at the nonpermissive temperature (39.5°C), at which viral DNA replication was

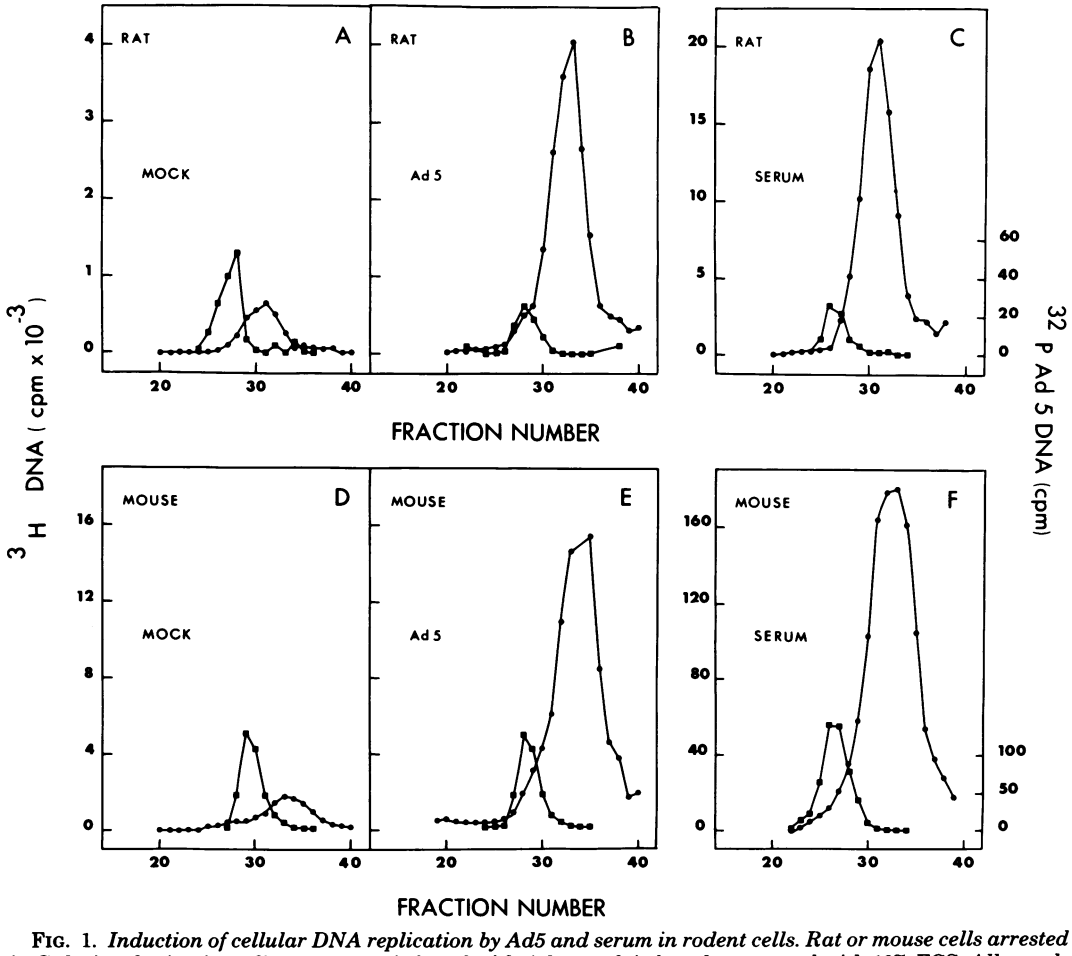

FIG. 1. *Induction of cellular DNA replication by Ad5 and serum in rodent cells. Rat or mouse cells arrested in G1 by incubation in 0.2% serum were infected with Ad5, mock infected, or treated with 10% FCS. All panels represent equilibrium gradient profiles of [methyl-^{3}H]dThd-labeled DNA extracted as described in the text. Cells were labeled 12 to 24 h (rat) and 24 to 48 h (mouse) postinfection. Symbols: ●, [methyl-^{3}H]dThd-labeled DNA from infected cells; ■, ^{32}P-labeled Ad5 DNA marker.*

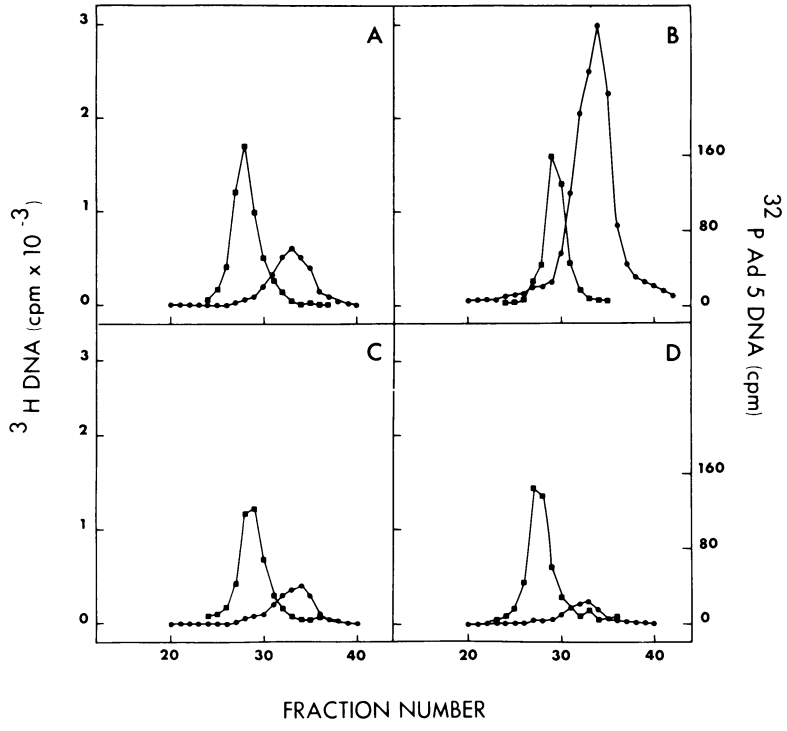

FIG. 2. *Effect of treatment with antibody to Ad5 or UV irradiation on induction of cellular DNA synthesis in Ad5-infected rat cells. The procedure was as outlined in the legend to Fig. 1. (A) Mock infected; (B) Ad5 infected; (C) Ad5, preincubated with antiserum (1:5) to Ad5 at 37°C for 0.5 h; (D) Ad5 pretreated with UV irradiation (800 μW cm^{-2} at 0°C for 0.5 h). Symbols: ●, [methyl-^{3}H]dThd-labeled DNA; ■, ^{32}P-labeled Ad5 DNA marker.*

undetectable (rat cells) or reduced to less than 15% (mean, 9.9%) of the wild-type Ad5 control (mouse cells). Table 1 also shows that the induction of cellular DNA synthesis could be measured by autoradiography as an increase in the proportion of cells synthesizing DNA (Ad5, *ts*36, and *ts*37). This measurement was only strictly valid at 39.5°C when no viral DNA replication was occurring. Under permissive conditions, estimates from equilibrium gradients suggested that up to 16% of the observed DNA synthesis was due to viral DNA replication.

At 39.5°C, no or very little viral DNA synthesis was observed after infection by the *ts* mutants (Tables 1 and 2). Late viral proteins, which are dependent upon viral DNA replication, are reduced by 2 to 3 orders of magnitude (data not shown). Since the induction of cellular DNA synthesis is not reduced, it must be controlled by one or more early viral proteins. However, neither the gene *N* product nor the Ad5 DNA-binding protein can play an essential role in the induction of cellular DNA replication.

Effect of cycloheximide on DNA synthesis in serum-treated and in Ad5-infected cells. Protein synthesis is required for the initiation of adenovirus DNA replication but not for its continuation (14). Furthermore, DNA synthesis in simian virus 40-transformed HEK fibroblasts expressing T antigen is less sensitive to inhibition by cycloheximide than is DNA replication in normal HEK cells (15). In this section, we report on the requirements for protein synthesis of the initiation and of the continuation of DNA synthesis induced by either Ad5 or serum. G_1-arrested cells were infected with Ad5 or were serum treated, and 1.0 μg of cycloheximide per ml was then added. All cultures were subsequently labeled with [*methyl*-^{3}H]-dThd, and DNA was prepared by method 1 and analyzed by CsCl gradient centrifugation. Results from these experiments (Table 3) showed that Ad5-induced and serum-induced DNA replication was inhibited by cycloheximide added immediately after stimulation. These results imply that protein synthesis is necessary for the initiation of cellular DNA replication by Ad5 and by serum. When DNA synthesis was allowed to begin and then cycloheximide (1.0 μg/ml) was added to cultures (36 h after infection or serum

addition), both serum-induced and Ad5-induced cellular DNA syntheses were still inhibited (Table 3). Under these conditions, adenovirus DNA synthesis continued normally (Table 3), as has been reported previously (14). Thus, a continuation of cellular DNA synthesis requires continued protein synthesis after induction by serum or by virus, but viral DNA synthesis becomes independent of protein synthesis once it has begun. Cycloheximide may inhibit the synthesis of a cellular protein which is continuously required for cellular, but not for viral, DNA replication, irrespective of the agent initiation cellular DNA replication.

Effect of dbcAMP on DNA synthesis. The intracellular concentration of cyclic AMP (cAMP) has been shown to increase in some cells when they are deprived of serum and then decrease again when the cells are supplied with fresh serum and begin to progress through the cell cycle (22). Also, transformation of some cells by viruses (e.g., Rous sarcoma virus) will lower intracellular cAMP levels (20), which correlates with the higher growth fraction characteristic of transformed cells. In addition, dbcAMP has been reported to inhibit Ad12-induced cellular DNA replication in serum-arrested BHK-21 cells (32). To determine whether alterations in

TABLE 1. *Induction of cellular DNA synthesis by Ad5, ts36, ts37, and ts125 in G_1-arrested rat cells*[a]

Inoculum	Time of labeling (h)	Temp (°C)	DNA component[b] (cpm)		Degree of stimulation[c]	% of cells with radiolabeled nuclei	Degree of stimulation by autoradiography[d]
			Viral	Cellular			
Mock	20–35	39.5	0	140,815	1.0	7	1.0
Ad5	20–35	39.5	70,386	414,133	2.94	31	4.43
*ts*125	20–35	39.5	0	469,424	3.33	ND[e]	ND
*ts*36	20–35	39.5	0	298,298	2.12	37	5.28
*ts*37	20–35	39.5	0	670,827	4.76	23	3.29
Mock	48–60	32.5	0	37,181	1.0	11	1.0
Ad5	48–60	32.5	54,455	288,414	7.75	29	2.64
*ts*125	48–60	32.5	32,701	250,675	7.67	ND	ND
*ts*36	48–60	32.5	5,239	112,748	3.03	24	2.18
*ts*37	48–60	32.5	33,539	305,355	8.16	23	2.09

[a] Cells were arrested and infected as described in the text.
[b] Total labeled DNA from CsCl gradients was analyzed into viral DNA and cellular DNA components as described in the text.
[c] Radioactivity in the cellular DNA component divided by radioactivity from mock-infected cultures in the same fractions.
[d] Autoradiography results from a separate experiment.
[e] ND, Not done.

TABLE 2. *Induction of cellular DNA synthesis by Ad5, ts36, ts37, and ts125 in G_1-arrested mouse cells*[a]

Inoculum	Time of labeling (h)	Temp (°C)	DNA component[b] (cpm)		Degree of stimulation[c]
			Viral	Cellular	
Mock[d]	29–45	39.5	0	20,181	1.0
Ad5[d]	29–45	39.5	3,640	117,219	5.81
Mock	29–45	39.5	0	7,500	1.0
*ts*125	29–45	39.5	512	34,729	4.63
*ts*36	29–45	39.5	0	45,707	6.09
*ts*37	29–45	39.5	566	44,260	5.90
Mock[d]	24–48	32.5	0	45,568	1.0
Ad5[d]	24–48	32.5	703	165,397	3.63
Mock	24–48	32.5	0	9,214	1.0
*ts*125	24–48	32.5	7,526	273,369	29.67
*ts*36	24–48	32.5	753	152,720	16.57
*ts*37	24–48	32.5	3,775	72,504	7.87

[a,b,c] As for Table 1.
[d] Ad5 induction tested in a separate experiment.

TABLE 3. *Effect of cycloheximide on cellular and viral DNA synthesis induced by Ad5 or serum in G_1-arrested rat and mouse cells*[a]

Cell type	Inoculum	Addition of cycloheximide	Time of cycloheximide addition (h)	DNA component[b] (cpm)		Degree of stimulation[c]
				Viral	Cellular	
Rat	Mock	−		0	4,643	1.0
	Ad5	−		50,886	102,197	22.01
	Ad5	+	0	1,081	500	NS[d]
	Ad5	+	36	50,372	10,595	2.28
	10% FCS	−		0	143,390	30.88
	10% FCS	+	36	0	5,796	1.25
Mouse	Mock	−		0	6,710	1.0
	Ad5	−		5,458	23,782	3.54
	Ad5	+	0	1,600	199	NS
	Ad5	+	36	5,099	3,467	NS
	10% FCS	−		0	44,464	6.63
	10% FCS	+	0	0	1,500	NS
	10% FCS	+	36	0	3,726	NS

[a,b,c] As for Table 1. After infection or serum stimulation, cultures were treated with 1.0 μg of cycloheximide per ml at the indicated times. They were labeled with [*methyl*-^{3}H]dThd from 36 to 48 h later, and DNA was then extracted and analyzed by gradient centrifugation.

[d] NS, No stimulation (counts per minute incorporated less than in mock-infected control).

intracellular cAMP levels are essential for Ad5-induced DNA synthesis, the effect of an exogenous supply of excess cAMP was studied.

Rat cells in 0.2% serum were treated with 10% serum or infected with Ad5 as previously described. dbcAMP was then added immediately to the culture medium. Serum-induced DNA replication was more sensitive than Ad5-induced DNA replication to inhibition by dbcAMP at concentrations of up to 2 mM (Fig. 3A and B). In a further experiment, DNA was extracted from serum-treated and Ad5-infected cells that either had been treated with 1.0 mM dbcAMP or had not been treated, and the results were analyzed by equilibrium gradient centrifugation (Table 4). These results showed that both viral and cellular DNA replication occurred in Ad5-infected cells treated with 1.0 mM dbcAMP. However, cellular DNA replication was completely inhibited in serum-stimulated cells treated with the same concentration of dbcAMP. These results indicated that, in rat cells treated with serum, lowering of intracellular cAMP levels was essential for cell cycle progression. This does not appear to be true for Ad5-infected cells. Consistent with this explanation is that serum appears to increase cAMP phosphodiesterase activity in rat cells, but Ad5 does not (M. M. Appleman and A. W. Braithwaite, unpublished data).

To determine at what point dbcAMP was acting to inhibit cell cycle progression, G_1-arrested cells were restimulated with serum, and dbcAMP was subsequently added at different times. These results (Fig. 3C) showed that dbcAMP inhibited the induction of DNA synthesis at a point between 12 and 14 h after restimulation with serum, as resistance to dbcAMP occurred by 14 h. This result argues that not only can Ad5 overcome the serum arrest point, but it can also overcome a separate arrest point several hours later.

Completion of a round of DNA synthesis after adenovirus infection. The results from preceding sections illustrated that Ad5 and some *ts* variants can overcome restrictions imposed on cells which inhibit their progression through the cell cycle. These data showed only that DNA is synthesized but did not provide evidence for a complete round of DNA replication. In this section we report the results of experiments which showed that a complete round of DNA synthesis does occur after adenovirus infection.

Rat cells were grown on glass microscope slides, arrested by incubation in 0.2% serum, and infected with Ad5 or stimulated by 10% serum. At 55 h after infection, cells were pulse-labeled with [*methyl*-^{3}H]dThd for 15 min, fixed, and stained with Feulgen for DNA content analysis by microdensitometry (see above). They were then treated for autoradiography. Results from this experiment (Table 5) showed that both serum and Ad5 caused an increase in the proportion of cells synthesizing DNA (S phase) as compared with an unstimulated control and an accumulation of cells having G_2 diploid (4*n*) DNA contents. A small proportion of cells from infected cultures were also found to have $>G_2$

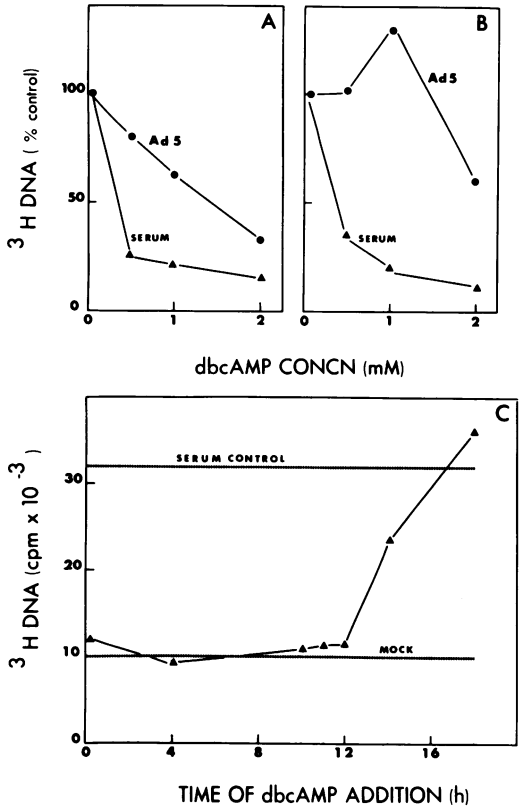

FIG. 3. *Effect of dbcAMP on induction of DNA synthesis in G_1-arrested rat cells infected with Ad5 or treated with 10% FCS. (A and B) Cells were infected or treated with serum, and dbcAMP was then added at the indicated concentrations. Cells were labeled with [methyl-^{3}H]dThd from 12 to 24 h (A) or 48 to 60 h (B) after the addition of virus or serum, and DNA synthesis was measured by method 2 (see text). Results are expressed as percent radioactivity (counts per minute) incorporated into untreated (no dbcAMP), serum-treated, and virus-infected controls. Untreated control DNA synthesis represented 7.8- and 3.5-fold stimulations above mock-infected cells for serum-treated and virus-infected cells for (A) and 4.6- and 2.6-fold stimulations for (B). (C) Cells were serum treated, and 1.0 mM dbcAMP was added immediately or at the indicated times after treatment. Cells were labeled from 14 to 26 h after the serum addition, and DNA synthesis was measured as described for (A) and (B).*

diploid DNA contents. These probably represented aneuploid and polyploid cells.

In a second experiment, cells were grown in small flasks, G_1 arrested, and infected as described above, and their nuclei were then released, stained with ethidium bromide-mithramycin, and analyzed by flow microfluorimetry (25). Results from this experiment (Fig. 4) again showed that in serum-treated and Ad5-infected cultures there was an increase in the proportion of cells exhibiting G_2 diploid DNA contents compared with the mock-infected control. Furthermore, the virus-infected cultures again showed a decrease in cells in G_1, an increase in cells in the S phase, and some cells exhibiting $>G_2$ diploid DNA contents.

The increased DNA contents (G_2 diploid and greater) observed in virus-infected cells cannot be accounted for by replicating viral DNA as the same result has been obtained with a *ts* mutant at the nonpermissive temperature (J. D. Murray, A. W. Braithwaite, I. W. Taylor, and A. J. D.

TABLE 4. *Effect of dbcAMP on induction of cellular and viral DNA synthesis by Ad5 and cellular DNA synthesis by serum in G_1-arrested rat cells*[a]

Inoculum	Addition of dbcAMP	DNA component[b] (cpm)		Degree of stimulation[c]
		Viral	Cellular	
Mock	–	0	10,300	1.0
10% FCS	–	0	70,040	6.80
10% FCS	+	0	1,007	NS[d]
Ad5	–	12,803	24,373	2.37
Ad5	+	6,172	40,276	3.91

[a,b,c] As for Table 1. After the serum addition or Ad5 infection, 1.0 mM dbcAMP was added to the cultures. Cells were labeled with [*methyl*-^{3}H]dThd 48 to 60 h later, and total cellular DNA was extracted and analyzed by CsCl gradient centrifugation.

[d] NS, No stimulation (less than unstimulated control).

TABLE 5. *Cell cycle stages of Ad5-infected and serum-treated G_1-arrested rat cells*[a] *determined by autoradiography*[b] *and microdensitometry*[c]

Inoculum	P-antiserum-positive cells (%)	Approx % of cells in each stage of cell cycle 55 h after serum treatment or infection			
		G_1	S	G_2+M	$>G_2$ diploid DNA content
Mock	0	ND[d]	4.7[e]	ND	ND
10% FCS	0	46.4	28.5	25.1	0
Ad5	40	27.0	35.6	31.0	6.4

[a] Rat cells were arrested and infected as described in the text.

[b] A total of 1,000 cells were scored for the presence of nuclear grains ([*methyl*-^{3}H]dThd incorporation) to estimate the proportion of cells in the S phase.

[c] Fifty cells without nuclear grains were analyzed by microdensitometry to determine the relative proportions of cells with G_1, G_2+M, or $>G_2$ diploid DNA contents.

[d] ND, Not done.

[e] Based on autoradiography data only.

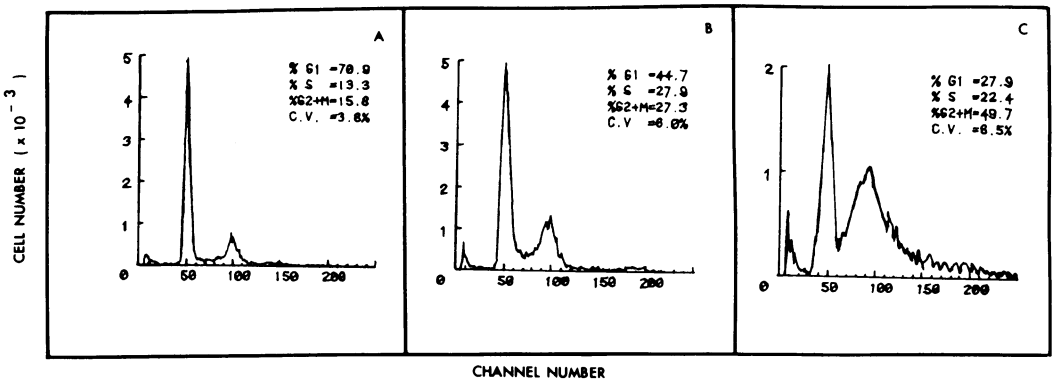

FIG. 4. *DNA contents of Ad5-infected and serum-treated rat cells determined by flow microfluorimetry. Rat cells were arrested in G_1 and infected or serum treated as described in the text. After 48 h, cells were harvested and stained, and their DNA contents were analyzed by flow microfluorimetry (25). (A) Mock infected; (B) serum treated; (C) Ad5 infected (30 to 35% P antiserum positive at this time). "Channel number" indicates the relative DNA content of cells, and the peak in channel 15 represents a chicken erythrocyte DNA marker. C.V., Coefficient of variation of the G_1 peak. (The proportions of cells in different stages of the cell cycle were calculated with a program which does not include cells with DNA contents >G_2 diploid.)*

Bellett, manuscript in preparation). The shift from G_1 to G_2 diploid and greater DNA contents observed above has also been obtained with randomly cycling rat cells infected with Ad5 (J. D. Murray, A. J. D. Bellett, A. W. Brathwaite, L. K. Waldron, and I. W. Taylor, manuscript in preparation). The results obtained from the above experiments, together with data to be published elsewhere (J. D. Murray, A. W. Braithwaite, I. W. Taylor, and A. J. D. Bellett, in preparation), show that a complete round of cellular DNA replication occurs in quiescent rodent cells after infection by Ad5. In addition, the >G_2 diploid DNA contents, which in some experiments involved a third of the cells in virus-infected cultures, provide strong evidence for virus-induced cell cycle abnormalities.

DISCUSSION

In this paper we report evidence that Ad5 and three *ts* mutants defective for viral DNA replication at 39.5°C induce cellular DNA replication in cultures of rat and mouse fibroblasts arrested in the G_1 stage of the growth cycle by incubation in 0.2% serum. This was shown by equilibrium gradient analysis in CsCl and by autoradiography.

The induction of cellular DNA synthesis is a specific effect of Ad5 infection and is sensitive to inhibition of protein synthesis by cycloheximide, as is the initiation of viral DNA replication. That is, the synthesis of a cellular or a viral protein (or both) is required for the initiation of cellular DNA replication by Ad5. Once cellular DNA synthesis has been initiated by Ad5 or by serum, its continuation is still inhibited by cycloheximide, in contrast to viral DNA replication, which once initiated will continue even though further protein synthesis is prevented. Thus, the viral proteins responsible for the continuation of viral DNA replication are not sufficient for the continuation of cellular DNA replication. This suggests that, even when induced by Ad5, continued cellular DNA replication probably requires the continued synthesis of a cellular protein.

The observations that mutants *ts*36, *ts*37, and *ts*125 are not defective for the induction of cellular DNA synthesis at 39.5°C argue against the DNA-binding protein and gene *N* product being responsible for the induction of cellular DNA replication. The results do imply that induction is controlled by gene products transcribed before viral DNA synthesis, which is consistent with the induction by Ad12 of cellular DNA replication in BHK cells, which are nonpermissive for viral DNA replication (24). The Ad5 DNA-binding protein and the gene *N* product are products of early regions 2a and 2b, respectively (6, 23a). The gene(s) responsible for the induction of cellular DNA replication could therefore be located in early region 1, 3, or 4, or it could be one of the other products of region 2b (Stillman et al., in press). Early region 1 is responsible for transformation, which can be established with a fragment of Ad5 DNA containing only this region (10). Early regions 2a and 2b, however, do affect the initiation of transformation as *ts*36, *ts*37, and *ts*125 all have altered transformation frequencies as compared with wild-type Ad5 (8, 29). Cellular DNA replication might be essential for transformation, but early regions 2a and 2b, although not affecting cellular DNA synthesis,

may affect the frequency of transformation through some other mechanism.

The fact that Ad5 can overcome the low-serum arrest point suggests that serum and adenovirus might use different regulatory pathways. Further evidence for this is provided by the fact that Ad5 induces both cellular and viral DNA synthesis in the presence of dbcAMP at concentrations inhibitory to the stimulation of DNA replication by serum. Ad5 is apparently able to bypass a cAMP-sensitive control point between 12 and 14 h after the low-serum arrest point. Thus, Ad5 does not induce cellular DNA replication by lowering the intracellular cAMP concentration. Other evidence that Ad5 may induce DNA synthesis via a pathway different from serum arises from the observation that Ad5 induces DNA synthesis without inducing ornithine decarboxylase (B. F. Cheetham and A. J. D. Bellett, submitted for publication). This enzyme is the first in the biosynthetic pathway for polyamines, the synthesis of which is normally mandatory for DNA synthesis. Ornithine decarboxylase is induced by serum 3 to 8 h after the low-serum arrest point. These data collectively show that Ad5 can bypass three G_1 events essential for normal cell cycle progression. Once DNA synthesis has been initiated by Ad5, a complete round of replication occurs and cells with G_2 diploid ($4n$) DNA contents are formed, as well as a proportion of cells with abnormal ($>G_2$) DNA contents.

Transformation of cells by viruses results in changes in the growth pattern of cells. The results of experiments reported in this paper and elsewhere (Murray et al., manuscript in preparation) provide evidence for the induction of an aberrant cell cycle by adenovirus, which may be a prerequisite for the induction of the transformed state.

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