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Adenovirus Type 2 Activates Cell Cycle-Dependent Genes That Are a Subset of Those Activated by Serum

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We have studied a panel of 10 genes and cDNA sequences that are expressed in a cell cycle-dependent manner in different types of cells from different species and that are inducible by different mitogens. These include five sequences (c-*myc*, 4F1, 2F1, 2A9, and KC-1) that are preferentially expressed in the early part of the G_1 phase, three genes (ornithine decarboxylase, p53, and c-*ras*^{Ha}) preferentially expressed in middle or late G_1, and two genes (thymidine kinase and histone H3) preferentially expressed in the S phase of the cell cycle. We have studied the expression of these genes in nonpermissive (tsAF8) and semipermissive (Swiss 3T3) cells infected with adenovirus type 2. Under the conditions of these experiments, adenovirus type 2 infection stimulates cellular DNA synthesis in both tsAF8 and 3T3 cells. However, four of the five early G_1 genes (c-*myc*, 4F1, KC-1, and 2A9) and one of the late G_1 genes (c-*ras*) are not induced by adenovirus infection, although they are strongly induced by serum. The other sequences (2F1, ornithine decarboxylase, p53, thymidine kinase, and histone H3) are activated by both adenovirus and serum. We conclude that the cell cycle-dependent genes activated by adenovirus 2 are a subset of the cell cycle-dependent genes activated by serum. The data suggest that the mechanisms by which serum and adenovirus induce cellular DNA synthesis are not identical.

Cell cycle-dependent genes are operationally defined here as genes that are preferentially expressed in a specific phase of the cell cycle. A number of animal genes have already been identified whose expression is cell cycle dependent. These include histone genes (2, 22, 57), c-*myc* (13, 28, 35, 49; B. Calabretta, L. Kaczmarek, W. Mars, D. Ochoa, C. W. Gibson, R. R. Hirschhorn, and R. Baserga, Proc. Natl. Acad. Sci. USA, in press), c-*ras* (13, 27, 28), and c-*fos* (18, 29, 38) and genes encoding p53 (48, 60), actin (13, 29, 46), tubulin (46), and thymidine kinase (44). Other cell cycle-dependent genes have been identified as cDNA clones (17, 31, 43) by differential screening of cDNA libraries.

Adenovirus type (Ad2) or Ad5 can stimulate cellular DNA synthesis in G_1-specific temperature-sensitive (ts) mutants of the cell cycle, even when the mutant cells are incubated at the nonpermissive temperature (NPT) (62, 63). These same mutants, tsAF8 and ts13 cells (which are of Syrian hamster origin), enter the S phase when serum stimulated at the permissive temperature (PT) but fail to do so at the NPT (3, 12, 64). The ability of adenovirus to bypass a temperature-sensitive block in G_1 prompted us to ask the question whether it would activate the same cell cycle-dependent genes that are activated by serum. For this purpose, we selected a panel of cell cycle-dependent genes (Table 1) and analyzed their expression in nonpermissive tsAF8 cells at the NPT and in semipermissive Swiss 3T3 cells at 37°C. The expression of these genes after serum stimulation or Ad2 infection was monitored by Northern blots (68). The term expression is used here in one of its accepted usages, that is, as levels of RNA without any prejudice of whether the change is regulated at the level of transcription, processing, or mRNA stability. We have also used serum rather than specific growth factors to make a broader comparison between the two systems.

Our results indicate that the cell cycle-dependent genes activated by adenovirus are a subset of those activated by serum and that adenovirus infection preferentially activates genes whose expression reaches a maximum in the late G_1-S phase.

MATERIALS AND METHODS

Cell line and culture conditions. tsAF8 cells are G_1-specific, temperature-sensitive mutants originally isolated from BHK cells (12); these cells have been extensively characterized (3, 12, 25). Monolayer cultures are routinely maintained in Dulbecco modified Eagle medium containing 10% (vol/vol) donor calf serum at the PT of 34°C. To obtain quiescent (G_0) cultures, tsAF8 cells are plated at 10^6 cells per 100-mm plate, grown to near confluence at 34°C, rinsed twice with prewarmed Hanks salt solution, fed with medium containing 0.5% (vol/vol) donor calf serum, and incubated for an additional 48 h at 34°C. Swiss 3T3 cells were cultured and made quiescent as previously described (47).

Virus propagation, purification, and infection. Ad2 was grown in HeLa suspension cultures maintained in minimum essential modified suspension medium (Flow Laboratories, Rockville, Md.) with 5% fetal calf serum. Virus was purified as described previously (63) with two cycles of CsCl density gradient centrifugations. The virus titer was calculated from the optical density (1 unit of optical density at 260 nm = 3 × 10^{10} PFU).

The cells to be infected were made quiescent as previously described by a 48-h incubation in low-serum medium. The medium was removed, and virus (100 PFU per cell) diluted in Hanks balanced salt solution containing 20 mM $MgCl_2$ was added and allowed to adsorb at 37°C for 1 h (63). tsAF8 cells were then incubated at the NPT. Stimulation of cell DNA synthesis was monitored by autoradiography after [^{3}H]thymidine labeling. Swiss 3T3 cells, after Ad2 infection, were incubated at 37°C in conditioned, low-serum medium.

Plasmids. The plasmids carrying the sequences listed in Table 1 are described in detail in the appropriate references as follows: pKC-1 was described by Cochran et al. (17); p2A9, p2F1, and p4F1 are cDNA clones isolated by differ-

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TABLE 1. Cell cycle-dependent genes and cDNA clones used in these studies

Gene or cDNA clone	Phase of maximum expression	Species of origin (size of RNA kilobases)
c-*myc*	Early G_1	Human (2.4)
4F1	Early G_1	Syrian hamster (1.8)
2F1	Early G_1	Syrian hamster (1.5)
KC-1	Early G_1	Mouse (1.2)
2A9	Mid-G_1	Syrian hamster (0.75)
ODC	middle to late G_1	Mouse (2.2)
p53	middle to late G_1	Mouse (2.0)
c-*ras*^{Ha}	Late G_1	Virus (1.4)
TK	S	Chinese hamster (1.4)
H3	S	Human (0.5)
7B6	Non-cell cycle dependent	Human (0.6)

ential screening of a ts13 (Syrian hamster) library and were described by Hirschhorn et al. (32); the c-*myc* cDNA clone was a gift from Carlo Croce (20, 69); pH1 was a gift from Edward M. Scolnick and contains the Harvey sarcoma virus genome (14); pp53-271, a p53-specific cDNA clone, was a gift from Moshe Oren (54); 7B6, a gift from G. Torelli, is a cDNA clone that is expressed at a constant level in quiescent lymphocytes and for at least 30 h after stimulation. pFO422 (carrying a human histone H3 gene) was a kind gift from Gary Stein (57); p3.2 (carrying an insert from the Chinese hamster thymidine kinase gene) was a gift of John Lewis (41); pODC 934, containing a cDNA of mouse ornithine decarboxylase (ODC), was a gift of Franklin Berger (9). Plasmid DNA was isolated and purified by phenol extraction and Sepharose 2B chromatography (31, 32, 44).

RNA isolation and blot analyses. RNA isolation and blot procedures were previously described (31). Briefly, after extraction RNA was denatured and size fractionated on 1% agarose gels containing 6.6% formaldehyde. Transfer of RNA to nitrocellulose was done as described by Thomas (68). Nick-translated (61) probes (1×10^8 to 2×10^8 cpm/μg) were hybridized to filters and washed as described previously (31, 44). Filters were exposed to Kodak X-ray film with the aid of intensifying screens at $-70°C$. Scans were performed with a Zeineh soft laser densitometer (Biomed Instruments, Inc., Fullerton, Calif.).

TK activity. Assays for thymidine kinase (TK) activity were carried out as described previously (44).

RESULTS

The 11 probes used in these experiments are listed in Table 1. They can be divided into four groups as follows. (i) Four genes (or cDNA sequences) inducible in early G_1 by mitogenic stimuli. They include the oncogene c-*myc* (13, 28, 35, 49, Calabretta et al., in press), two cDNA clones (4F1 and 2F1) (inducible in ts13 and tsAF8 cells by serum stimulation [31] and in human lymphocytes by phytohemagglutinin [Calabretta et al., in press]), and one cDNA clone (KC-1) (inducible in 3T3 cells by platelet-derived growth factor [17]). (ii) Four sequences inducible in the middle or late G_1 by mitogenic stimuli. They include 2A9 (inducible in ts13 and tsAF8 cells by serum stimulation [31]), ODC (also inducible by serum), p53 (inducible by mitogens in 3T3 cells [60] and a well-known transformation-related protein [19, 42, 47, 48, 54]), and the oncogene c-*ras* (which has also been shown to be cell cycle dependent [13, 27, 28]). (iii) Two genes that reach the maximum of expression during the S phase, the TK gene and the histone H3 gene (2, 22, 32, 44, 57). (iv) 7B6, a non-cell cycle-dependent gene, expressed at constant levels throughout the cell cycle (Calabretta et al., in press).

Activation of cellular genes by Ad2 in tsAF8 cells at the restrictive temperature. As mentioned above, tsAF8 cells are blocked in the mid-G_1 phase at the NPT (40°C) and fail to enter the S phase when serum stimulated from quiescence (3). They do enter the S phase, however, even at the NPT, when infected with Ad2 or Ad5 (63). The cells are semipermissive for adenovirus growth at the PT of 34°C, but they are totally nonpermissive at the NPT (52, 63). No viral DNA is made at the NPT, although some early RNAs are produced in decreased amounts (51).

We have confirmed that Ad2 infection stimulates cellular DNA synthesis in tsAF8 cells at the NPT (data not shown), and we show here that thymidine kinase activity increases, albeit not as much as in serum-stimulated tsAF8 at the PT (Table 2). We then investigated the effect that Ad2 infection of tsAF8 at the NPT had on the expression of the genes listed in Table 1. The results obtained are summarized in the composite Northern blot shown in Fig. 1. It is evident that Ad2 infection increases the RNA levels specific for 2F1 (an early G_1 gene) at 8 h postinfection and for H3 and TK (late G_1-S phase genes) between 24 and 40 h postinfection, but has no effect on two other G_1-specific genes, 4F1 and 2A9. All of these genes are strongly inducible by serum in tsAF8 cells at the PT (32, 33, 44).

To evaluate the quantitative aspects of Fig. 1, one should remember that the same blot was used for two or three probes and that every blot was monitored with the 7B6 probe, a non-cell cycle-dependent gene, which is expressed at constant levels throughout the cell cycle (Calabretta et al., in press). Plasmid 7B6 is used to monitor that not only the same amount of RNA has been used for each lane, but that the transfer has also been reasonably uniform. In these experiments, the amounts of 7B6 remained unchanged in tsAF8 cells, regardless of the treatment, for at least 36 h (Fig. 1), indicating that this sequence has a long half-life. A second point is that the blots in Fig. 1 have been deliberately overexposed, so that the G_0 levels are somewhat high. Since in quiescent populations of tsAF8 cells ~8 to 10% of the cells are cycling, it is not surprising that a certain level of expression of G_1 genes is detectable. We wanted, though, to show that at the NPT without Ad2, the expression of some of these genes actually decreased. Although there is some degradation of the RNA in the 4F1 blot (Fig. 1, lane b), it is clear that adenovirus infection does not significantly increase the levels of expression of this cell cycle-dependent cDNA sequence over the time course of our experiment; whereas with 2F1 the levels of expression are increased in adenovirus-infected cells most markedly at 8 h and also (but to a lesser degree) at 24 and 40 h.

TABLE 2. Activity in tsAF8 cells

Time[a] (h)	TK[b] activity (cpm/μg of protein)		
	Serum 34°C	Serum 40°C	Ad2 40°C
0	30.5		
8		29	27
24	893	24	122
40	1,855	59	550

[a] Time after serum stimulation or Ad2 infection. The cells were made quiescent by serum deprivation for 2 days at 34°C (0 time).

[b] TK activity was determined as described in Materials and Methods.

The results suggest that Ad2 infection of tsAF8 cells at the NPT activates a group of genes that are a subset of the cellular genes activated by serum at the PT. Unfortunately, some of the probes listed in Table 1 did not give clear signals in RNA blots from tsAF8 cells, presumably because of poor homologies across species. We therefore extended our studies to Swiss 3T3 cells.

Effect of Ad2 infection on Swiss 3T3 cells. Swiss 3T3 cells are semipermissive for Ad2, and there is considerable cytotoxicity at 24 h postinfection. Our studies were therefore limited to the first 24 h.

First of all, we investigated the effect of Ad2 infection on cellular DNA and RNA synthesis (Table 3). Both serum stimulation and Ad2 infection cause an increase in the fraction of cells with >2*n* amount of DNA. This was confirmed by autoradiography with [^{3}H]thymidine. Quiescent cells had <1% labeled cells, whereas serum-stimulated cells had 68% labeled cells, and Ad2-infected cells had 51% labeled cells. The labeling period was 24 h.

TABLE 3. RNA and DNA content of swiss 3T3 cells[a]

Conditions	Relative fluorescence intensity	
	RNA (red)	DNA (green)
Quiescent	8.2 ± 2.6	35.0 ± 6.0
Serum stimulated (24 h)	16.5 ± 4.6	50.7 ± 12.2
Quiescent + Ad2 (24 h)	8.5 ± 2.3	47.7 ± 11.1

[a] The amount of RNA and DNA per cell was measured in acridine orange-stained cells with a computer-operated microspectrophotometer. The mean RNA content (red fluorescence at >600 nm) and DNA content (green fluorescence at 530 nm) is expressed as arbitrary units per cell with standard deviations for 100 cells measured in each population.

Table 3 shows that the amount of RNA per cell does not increase in Ad2-infected 3T3 cells, although it increases, as expected, in serum-stimulated cells. This confirms in Swiss 3T3 cells our previous finding in tsAF8 cells that Ad2 infection can cause stimulation of cellular DNA synthesis without a concomitant increase in cellular RNA (58). This dissociation between cell DNA replication and growth in size is characteristic of, but not unique to, adenovirus infection (for a review, see reference 5).

Activation of cellular genes by Ad2 in Swiss 3T3 cells. A typical experiment on activation of cellular genes by Ad2 is shown in Fig. 2, a Northern blot of total RNA from serum-stimulated and Ad2-infected 3T3 cells. The probe used was pODC-934, which contains a cDNA for mouse ODC (9). It is clear that both Ad2 and serum increase the levels of ODC RNA; serum acts somewhat faster than Ad2 infection.

For convenience, however, we present other data divided into two groups: Northern blots from serum-stimulated cells and blots from Ad2-infected cells. Figure 3 shows composite Northern blots of RNAs from serum-stimulated 3T3 cells probed with various nick-translated sequences. All of the sequences tested increased in amounts after serum stimulation. The expression of *c-myc*, 4F1, 2F1, and KC-1 (the last not shown) increased within 4 h after serum stimulation, confirming previous results from the literature with either mouse cells (13, 28, 35, 49; Calabretta et al., in press), mouse lymphocytes (35), human lymphocytes (Calabretta et al., in press), rat cells (28, 45), or Syrian hamster cells (31). The expression of 2A9, ODC (Fig. 2), *c-ras*, and p53 increased somewhat later, again in agreement with previous findings in other cell systems (13, 27, 28, 31, 48, 60). Finally, TK and H3 reach a high level of expression 24 h after serum stimulation, also in agreement with the data in the literature (32, 44, 57).

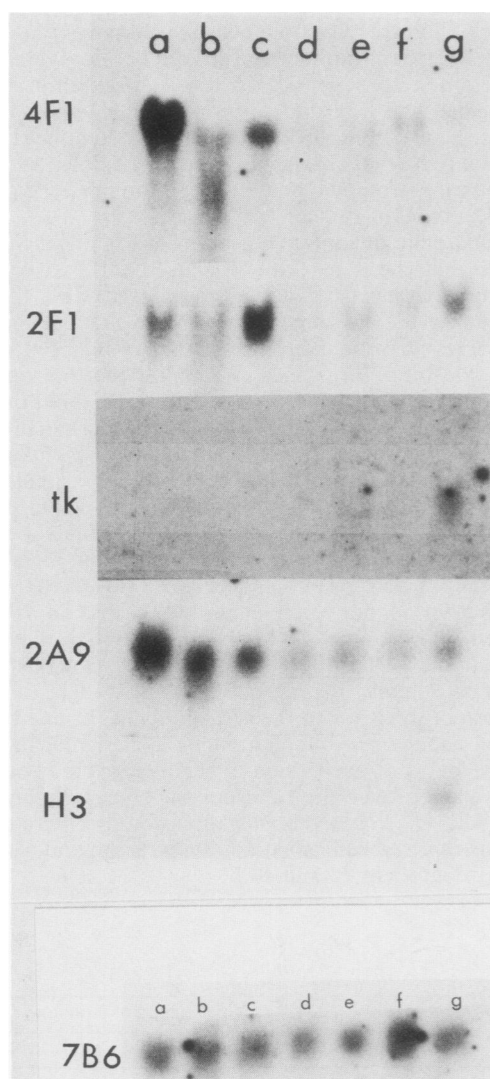

FIG. 1. Northern blots of total RNA from adenovirus-infected tsAF8 cells. Total RNA (10 μg per lane) was size fractionated and blotted as described in Materials and Methods, and the blots were hybridized to the probes indicated to the left. Lanes: a, quiescent cells; b, 8 h at NPT; c, 8 h at NPT after Ad2 infection; d, 24 h at NPT; e, 24 h at NPT after Ad2 infection; f, 40 h at NPT; g, 40 h at NPT after Ad2 infection.

FIG. 2. Northern blot of total RNA from Swiss 3T3 cells. Total RNA (10 μg per lane) was blotted as described in Materials and Methods and hybridized to probe pODC-934 (mouse ODC). Lanes: a and e, quiescent cells; b, c, and d, cells stimulated with serum for 4, 8, and 24 h respectively; f and g, cells stimulated 4 and 8 h, respectively, after infection with Ad-2; h, mock-infected cells at 24 h; i, Ad2-infected cells at 24 h.

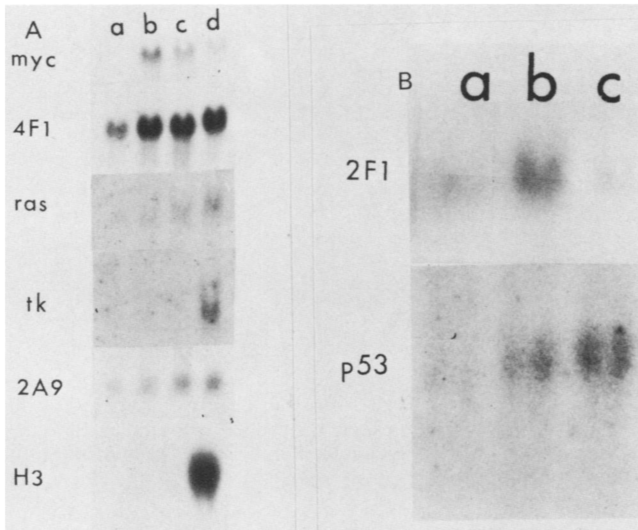

FIG. 3. Composite Northern blots of total RNA from Swiss 3T3 cells. Total RNA (10 μg per lane) was blotted as usual. (A) RNA from serum-stimulated cells hybridized to the probes indicated to the left. Lanes: a, quiescent cells; b, c, and d, cells stimulated with serum for 4, 8, and 24 h, respectively. (B) As in A, except the probes used were as indicated. Lanes: a, quiescent cells; b and c, cells stimulated with serum for 8 and 24 h, respectively.

The results after Ad2 infection are shown in Fig. 4, 5, 6, and 7. Figure 4 shows that the expression of c-*myc* and c-*ras* did not increase after Ad2 infection, although the levels of H3 histone RNA were markedly increased 24 h postinfection. Figure 5 shows a modest increase in the levels of p53 RNA at 24 h after Ad2 infection and a clear increase in the expression of 2F1 at 8 h postinfection, confirming the results obtained with tsAF8 cells. KC-1 expression did not increase above the levels of mock-infected cells. Neither 4F1 nor 2A9 increased after Ad2 infection (Fig. 6), whereas Fig. 7 compares the expression of TK (markedly increased) with a repetition of p53 and *ras*. The sequences tested can be divided into two categories: (i) the expression of 4F1, KC-1, c-*myc*, 2A9, and *ras* is not increased after Ad2 infection, although Swiss 3T3 cells are stimulated to synthesize DNA; and (ii) the expression of 2F1, ODC, p53, TK, and H3 increases after Ad2 infection. All experiments were repeated at least once, and the accuracy of the blots was monitored by using the 7B6 probe as described above.

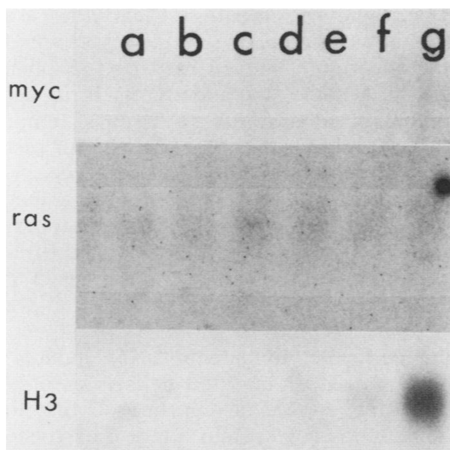

FIG. 4. Composite Northern blot of total RNA from adenovirus-infected Swiss 3T3 cells. Total RNA (10 μg per lane) was blotted and hybridized to the probes indicated to the left. Lanes: a, quiescent cells; b, 4 h after infection with Ad2; c, 6 h after mock infection; d and e, 8 h after infection with Ad2; f, 24 h after mock infection; g, 24 h after infection with Ad2.

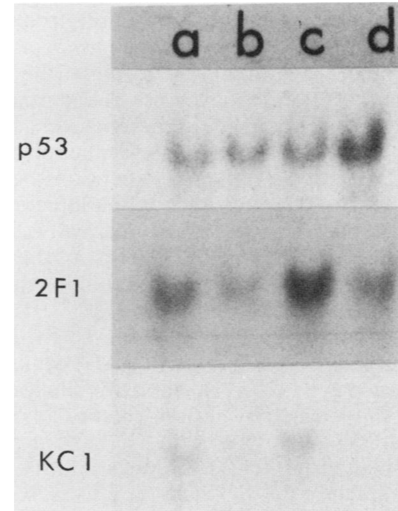

FIG. 5. Northern blot of total RNA from adenovirus-infected Swiss 3T3 cells. Same as in Fig. 4, except the probes are as indicated to the left. Lanes: a, 8 h after mock infection; b, quiescent cells; c, 8 h after infection with Ad2; d, 24 h after infection with Ad2.

DISCUSSION

The main conclusions that can be drawn from these experiments are as follows. (i) In nonpermissive or semipermissive cells stimulated to synthesize DNA by adenovirus infection, the cell cycle-dependent genes activated by Ad2 are a subset of the cell cycle-dependent genes activated by serum. (ii) Although there are exceptions, the trend shows that Ad2 generally activates late G_1- or S-phase genes, whereas early and middle G_1 genes are not affected. To better understand our conclusions, we will discuss our

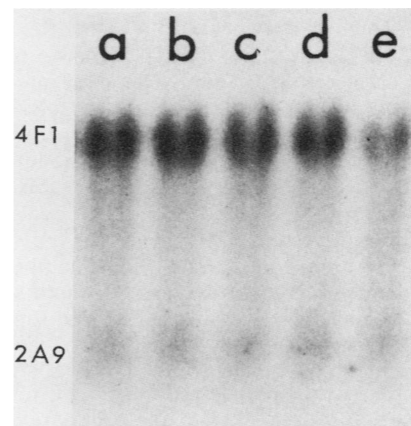

FIG. 6. Same as Fig. 5, except the probes are as indicated to the left. Lanes: a, quiescent cells; b and c, 4 and 8 h, respectively, after infection with Ad2; d, 24 h after mock infection; e, 24 h after infection with Ad2.

results in the context of the data available from the literature.

In the first place, it is important to make the distinction between our data in nonpermissive or semipermissive cells from those that are obtained in permissive cells, where inhibition of macromolecular synthesis is the rule, at least by 24 h postinfection (8, 56), although exceptions occur (4, 50, 70). At any rate, the effect of adenovirus infection on gene expression in permissive cells has been already discussed in several papers (15, 16, 23, 36, 40). The rest of the discussion is therefore limited (unless otherwise noted) to what happens in nonpermissive or semipermissive cells.

Stimulation of cellular DNA synthesis by adenovirus infection has already been reported in tsAF8 cells at the NPT (63) and in mouse cells (10, 11). Such stimulation is clearly different from that caused by serum. First of all, it bypasses the temperature-sensitive block in G_1 (63), a property it shares with simian virus 40 (25), whereas serum or retroviral oncogenes completely fail to do so (3, 25, 33, 63). Second, the stimulation of cellular DNA synthesis is not accompanied by the increase (actually the doubling) in cellular RNA that regularly accompanies serum stimulation (58; present paper) or ODC activity (15, 16). Third, the adenovirus-infected cells synthesize an irregular amount of DNA and do not divide (10, 11). In BHK cells, the stimulation of cellular DNA synthesis by adenovirus is insensitive to low concentrations of actinomycin D, which inhibit serum-stimulated DNA synthesis (39).

Of the genes and sequences tested, only three were previously studied in adenovirus-infected cells: ODC, TK, and histones. TK activity has been reported to be increased in some cells after adenovirus infection (15, 40), but this is the first time that an increase in TK RNA levels is reported. Other viruses, of course, are known to increase TK activity in cultured cells, for instance, simian virus 40 (36, 58), polyomavirus (7), and cytomegalovirus (23). Histone gene expression has only been studied in permissive cells (24). ODC activity was found not to increase in Ad2-infected cells (15, 16). Our data show that the RNA levels do increase. The difference between TK (where both activity and RNA levels are increased) and ODC (increased RNA levels but no increase in activity) is an intriguing hint of the complexity of the regulation of gene expression in adenovirus-infected cells and supports the translation discrimination proposed by Babich et al. (4).

Khalili and Weinmann (36) have analyzed the metabolism of actin mRNA in permissive HeLa cells after adenovirus infection. Their results show that an increase in stability of cytoplasmic actin mRNA occurs after viral infection. They suggest that the increased stability of actin mRNA could be related to impaired transport of new actin mRNA to the cytoplasm or to the rate of translation in the infected cells.

The genes and sequences we selected for this study were previously shown to be expressed in a cell cycle-dependent manner in different systems: 2F1, 4F1, KC-1, c-*myc*, and H3 are inducible in mouse and Syrian hamster fibroblasts and human lymphocytes, by serum, growth factors, or lectins (17, 31, 35; Calabretta et al., in press). The same could be said of the p53 gene, except that in most cases only the protein amount could be determined (48, 60; Mercer and Baserga, submitted for publication).

ODC activity is also increased after a variety of mitogenic stimulations (15, 26, 30, 67), although there are exceptions (53, 55). However, this is the first report in which ODC RNA levels have been examined in the context of the cell cycle. There is some evidence that ODC enzyme levels are related

FIG. 7. Same as Fig. 5, except the probes are as indicated to the left. Lanes: a, 24 h after mock infection; b, 26 h after infection with Ad2.

to growth in size of cells and particularly to the amount of rRNA (for a review, see reference 6). Since adenovirus-infected cells do not accumulate rRNA (58; present paper) even when DNA synthesis is stimulated, one would expect no increase in ODC activity, which is what was reported by Cheetham and Bellett (15). RNA levels of ODC, however, increase. Occasionally, there is a dissociation between ODC activity and rRNA synthesis (21), and this could be another instance. c-*ras* and 2A9 have also been shown to be expressed in a cell cycle-dependent manner (13, 27, 28, 31) in rat liver and cells in culture. The fact that these genes and sequences are expressed in a cell cycle-dependent manner in different cells from different species and stimulated by different mitogens indicates that they are somehow related to the mitogenic process, although there is no evidence in the literature on whether they regulate or are regulated by the cell cycle (with the exception, of course, of the two oncogenes, c-*ras* [33, 66] and c-*myc* [1, 34]).

A search of cellular genes activated by viruses has already begun in a systematic way by differential screening of cDNA libraries (64). Our work is not meant as a systematic approach. We were more interested in determining which genes, among a panel of cell cycle-dependent genes, would be activated by adenovirus infection. Clearly, these are only a subset of the cell cycle-dependent genes activated by the growth factors of serum. The fact that late G_1-S-phase genes are preferentially induced by adenovirus is in agreement with the ability of adenovirus to bypass temperature-sensitive blocks in mid-G_1 (62, 63). Finally, our results and conclusions suggest the following two corollaries: (i) adenovirus stimulates cellular DNA synthesis by a mechanism which is not the same as (although it could be a part of) the mechanism used by serum growth factors; and (ii) adenovirus infection of nonpermissive cells could be used to screen for cell cycle-dependent genes preferentially expressed in the late G_1-S phase.

The mechanism whereby adenoviruses induce cellular DNA synthesis in growth-arrested cells is at present unknown. Spindler et al. (65) have proposed that the early region 1A 243-amino-acid protein alters the physiology of arrested permissive cells to allow maximal viral DNA replication to occur and drives in G_0-arrested nonpermissive cell entry into the S phase. It would be of interest to determine whether mutants defective in this protein are able to activate the same subset of cell cycle-dependent genes activated by wild-type virus.

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