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Biochemical Studies on Adenovirus Multiplication

XVI. Transcription of the Adenovirus Genome During Abortive Infection at Elevated Temperatures

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The synthesis of infectious virus is reduced by 99% in adenovirus type 2-infected KB cells maintained at 42 C in suspension. Studies to delineate the steps in virus biosynthesis blocked at 42 C revealed the following. (i) The inhibition of ribosomal ribonucleic acid (RNA) synthesis and the block in the conversion of ribosomal RNA precursors to ribosomal RNA which normally occur in infected cells at 37 C was accentuated at 42 C. (ii) The same species of early and late viral messenger RNA were synthesized at 37 and 42 C. (iii) The shift from host cell deoxyribonucleic acid (DNA) synthesis to viral DNA replication occurred earlier at 42 C than at 37 C. These findings indicate that the thermosensitive block(s) in virion formation does not occur at the level of viral DNA replication or transcription of viral mRNA but probably involves the synthesis of late viral proteins or the maturation of the virion.

Thermosensitive events occur during the replication of poliovirus (10), vaccinia virus (9), simian-papovavirus SV40 (8), and adenovirus (Ad) types 5 (12), 1, 2, 6, 12, and 14 (8). At certain elevated temperatures, the formation of infectious virus is reduced extensively, but the synthesis of some virus-specific macromolecules may occur. For example, the early synthesis of T antigen in Ad 12-infected KB cells is unimpeded at 41 C (Samaille and Warocquier, *unpublished data*), and studies with metabolic inhibitors, e.g., actinomycin D, puromycin, and 5-fluorophenylalanine, have suggested that the viral genome is transcribed at elevated temperatures (15). In further studies, the effect of incubation at 42 C on the synthesis of the following cell and viral components in Ad 2-infected cells was determined: (i) infectious virus; (ii) total cellular protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA); (iii) ribosomal RNA (rRNA); (iv) early and late viral messenger RNA (mRNA); (v) cell DNA; and (vi) progeny viral DNA.

MATERIALS AND METHODS

Cells, virus, and viral DNA. The methods of Green and Piña (6, 7) were used for cell and virus growth and the purification of viral DNA. Cloning of cells after infection was performed routinely to ensure complete infection (5).

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Labeling of infected cell RNA and DNA. Suspension cultures of KB cells at 2.5×10^5 cells/ml were infected with an input multiplicity of 100 plaque-forming units of Ad 2 cells and maintained at 37 or 42 C. At various times after infection, 200- to 500-ml portions were labeled for 1 hr with 0.5 or 1.0 μc of ^{3}H-uridine (20 c/mmole) per ml or for 3 hr with 0.1 μc of ^{3}H-thymidine (15 c/mmole) per ml. Incubation was continued at the initial temperature of incubation. Isotope incorporation was stopped by pouring the culture over one-half volume of frozen 0.15 M NaCl. Cells were centrifuged, washed with phosphate-buffered saline, and stored at −20 C.

RNA isolation and analysis. RNA was extracted from frozen cell pellets by the hot phenol-sodium dodecyl sulfate (SDS) method (13). Sucrose density gradient sedimentation of RNA was performed as described by Girard et al. (3).

DNA isolation. Cells (0.2 ml pellet) were suspended in 2 ml of 1 N NaCl, 0.001 M ethylenediaminetetraacetate, 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.5), and disrupted by 15 strokes of a Dounce homogenizer. A 40-μliter amount of papain (29.5 mg/ml) and 30 μliters of 1 M cysteine-hydrochloride were added, and the suspension was incubated at 37 C for 3 hr. A 200-μliter amount of 5% SDS was added; the solution was shaken gently with an equal volume of chloroform-isoamyl alcohol (21:1) for 5 min and centrifuged at 5,000 × *g* for 5 min. The aqueous phase was removed with a wide-mouth pipette, and the organic phase was shaken further with 2 ml of buffer. The two aqueous fractions were combined, extracted with chloroform-isoamyl alcohol, and dialyzed against 0.1 × SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate). Recovery

of total DNA as measured by the diphenylamine reaction ranged from 55 to 75%. DNA was further purified by centrifugation in CsCl (ρ = 1.715) in a Spinco SW39 rotor for 48 hr at 100,000 × g at 15 C. Gradients were collected through a needle held 0.5 cm above the bottom to avoid contamination of DNA with the RNA pellet. The pooled DNA fractions were dialyzed against 0.1 × SSC.

Rate of synthesis of RNA, DNA, and protein. At different times after infection, 20-ml portions of uninfected and Ad 2-infected cell cultures were incubated with ^{3}H-uridine (0.5 μc/ml, 20 c/mmole), ^{3}H-thymidine (0.5 μc/ml, 15 c/mmole), or ^{3}H-valine (0.5 μc/ml, 0.34 c/mmole). Nine-milliliter portions were removed at 30 and 60 min and chilled in ice. Unlabeled carrier uridine, thymidine, or valine was added at 100 times the concentration of labeled precursor, and the cells were centrifuged at 300 × g for 10 min at 4 C. Cell pellets were extracted twice with 500 μliters of cold 0.3 M trichloroacetic acid, resuspended in 2 ml of 0.3 M trichloroacetic acid, collected on membrane filters, dried, and counted in a liquid scintillation spectrometer.

DNA-RNA hybridization. Denatured viral DNA, immobilized on nitrocellulose membrane filters, was annealed with labeled RNA isolated from infected cells to measure the amount of virus-specific RNA synthesized at different times after infection at different temperatures. DNA-RNA hybridization was performed by the procedure of Gillespie and Spiegelman (2) with several modifications (1).

A two-step DNA-RNA hybridization-inhibition reaction (Green et al., *in press*) was used to compare the species of virus-specific RNA synthesized at different times after infection at 37 and 42 C. In the first step, viral DNA was annealed for 20 hr at 66 C with increasing amounts of unlabeled RNA isolated from infected cells in 1 ml of 2 × SSC with 0.1% SDS. Filters were rinsed twice with 2 ml of 2 × SSC and annealed in the second step with labeled RNA isolated from infected cells. Annealing conditions were identical to those described for the first reaction. Filters were washed, treated with ribonuclease, and washed again. Bound radioactivity was measured in a scintillation counter (1).

DNA-DNA hybridization. DNA-DNA hybridization was used to measure the relative amount of viral DNA synthesized at different temperatures. The procedure followed was that of Warnaar and Cohen (14) with several modifications (Green et al., *in press*). Filters containing immobilized DNA (1) were annealed for 24 hr at 60 C with labeled DNA in 2 × SSC containing 0.1% SDS. The ratio of immobilized DNA to DNA in solution was at least 10:1. Filters were washed with 3 mM Tris buffer (pH 9.3) and counted in a scintillation spectrometer.

RESULTS

Synthesis of virus and macromolecules in adenovirus-infected cells at different temperatures. The replication of Ad 2 at temperatures from 37 to 42 C is shown in Table 1. At 41.5 and 42 C, the synthesis of virus was reduced drastically. The overall synthesis of DNA, RNA, and protein was determined by the incorporation of ^{3}H-uridine, ^{3}H-thymidine, and ^{3}H-valine. The incorporation of these radioactive precursors appears to be linear for 60 min at 37 and 42 C (Table 2). No block was observed at 42 C. The overall synthesis of RNA, DNA, and protein in uninfected and infected cells was reduced at 42 C as compared to 37 C. However, as shown below, the synthesis of viral DNA and viral mRNA is increased at 42 C.

TABLE 1. *Virus synthesis in AD 2-infected KB cells at various temperatures*

Incubation temp	Per cent of maximum yield[a]
C	
37	100
41	30
41.5	2
42	0.1–0.4

[a] Cultures were incubated for 36 hr at the indicated temperature.

Synthesis of RNA and RNA precursors in Ad 2-infected cells at 37 and 42 C. Infected cells maintained at 37 and 42 C were incubated for 1 hr with ^{3}H-uridine at 6, 18, and 28 hr after infection. The RNA species synthesized at 37 and 42 C were analyzed by sucrose gradient sedimentation (Fig. 1). The synthesis of all RNA species at 6 and 18 hr after infection, as revealed by specific radioactivity (counts per minute per optical density unit), was lower in cells incubated at 42 C than at 37 C. RNA synthesis was markedly reduced at 28 hr after infection of cells both at 37 and 42 C.

At 6 hr after infection at 37 C, the sucrose gradient patterns of RNA species (Fig. 1) were identical to that of uninfected cells (not included in Fig. 1). At 6 hr after infection, cells synthesized 45S and 32S rRNA precursors at both 37 and 42 C, but the cleavage of the 45S to 18S RNA was blocked at 42 C (Fig. 1). The synthesis of 18S rRNA is blocked in uninfected cells maintained at 42 C (Warocquier and Scherrer, *in preparation*). Appreciable amounts of 45S and 32S components were not formed at 18 hr after infection at 37 and 42 C; most labeled RNA had a sedimentation coefficient of about 41S. It is not known whether 41S RNA represents degraded rRNA precursor or mRNA species. Previous studies (Raskas, Thomas, and Green, *unpublished data*) have shown that rRNA synthesis was reduced markedly at 37 C in Ad 2-infected KB cells at 18 hr after infection. The data presented here suggest that this inhibition may be caused by a block in the conversion of rRNA precursors to rRNA.

Synthesis of virus-specific RNA at 37 and 42 C. KB cells maintained at 37 and 42 C were labeled

with ^{3}H-uridine for 1 hr at 6, 18, and 28 hr after infection with Ad 2. Labeled RNA was isolated and annealed with viral DNA to determine the amount of virus-specific RNA synthesized at these times. Over 1% of 37 C RNA synthesized at 6 hr after infection was virus-specific, but twice as much 42 C RNA was virus-specific at this time (Table 3). At 18 and 28 hr after infection, larger

TABLE 2. *Incorporation of ^{3}H-uridine, ^{3}H-thymidine, and ^{3}H-valine into uninfected and AD 2-infected KB cells at 37 and 42 C*

Time after infection, and incubation temp	Incorporation of ^{3}H-uridine[b]		Incorporation of ^{3}H-thymidine[b]		Incorporation of ^{3}H-valine[b]	
	30 min	60 min	30 min	60 min	30 min	60 min
Infected cells						
6 hr-37 C	5,600	10,160	5,970	11,440	3,615	5,350
18 hr-37 C	4,910	13,660	12,390	18,460	3,309	6,670
28 hr-37 C	7,190	11,860	5,322	14,340	4,475	8,680
6 hr-42 C	3,875	5,770	1,424	2,808	2,673	4,435
18 hr-42 C	3,010	5,390	2,159	5,650	954	2,213
28 hr-42 C	600	1,610	620	1,412	347	725
Uninfected cells						
18 hr-37 C	7,000	13,680	9,185	20,150	3,030	4,440
18 hr-42 C	5,420	6,700	1,560	1,340	3,020	3,680

[a] KB cells infected with Ad 2 at an input multiplicity of 100 plaque-forming units/cell were maintained at 37 and 42 C. At 6, 18, and 28 hr after infection, 20-ml samples were labeled with 0.5 μc of ^{3}H-uridine (10^{-5} M), ^{3}H-thymidine (5×10^{-6} M), or ^{3}H-valine (1.7×10^{-7} M) per ml. Uninfected cultures were labeled under the same conditions after 18 hr of incubation at 37 or 42 C. These experiments were repeated once with similar results (±30%).

[b] Label was incorporated into trichloroacetic acid-precipitable material. Results are expressed as counts per minute per 10^6 cells.

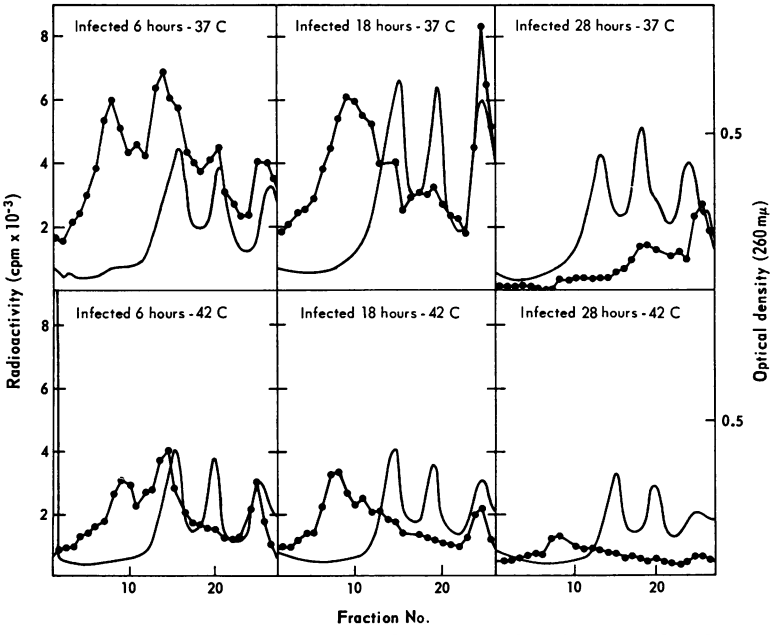

FIG. 1. *Sedimentation analysis of pulse-labeled RNA from Ad 2-infected cells incubated at 37 and 42 C. Cells were labeled for 60 min with ^{3}H-uridine (0.5 μc/ml, 20 c/mmole) at 6, 18, and 28 hr after infection. Total cellular RNA was extracted by the hot phenol-SDS method and centrifuged in an SW25.3 (Spinco) rotor at 75,000 × g for 16 hr at 15 C with a 15 to 30 (w/w) sucrose-SDS buffer gradient. Acid-precipitable radioactivity profile (●) and optical density profile of 28S and 18S ribosomal RNA and 4S transfer RNA components (solid line) are plotted against fraction number.*

TABLE 3. *Hybridization of viral DNA with pulse-labeled RNA from AD 2-infected cells maintained at 37 C and 42 C*[a]

Source of RNA[b]	Input RNA	Adenovirus DNA	Bound RNA	Bound
	counts/min	*μg/filter*	*counts/min*	*%*
6 hr-37 C	3,360	3.0	41	1.22
	3,360	3.0	39	1.16
	3,360	None	0	0
18 hr-37 C	2,320	3.0	434	18.9
	2,320	3.0	414	17.9
	2,320	None	3	0.13
28 hr-37 C	3,140	3.0	87	3.03
	3,140	3.0	87	3.03
	3,140	None	8	0.25
6 hr-42 C	2,100	3.0	49	2.52
	2,100	3.0	48	2.52
	2,100	None	4	0.19
18 hr-42 C	2,660	3.0	543	20.4
	2,660	3.0	536	20.2
	2,660	None	1	0.03
28 hr-42 C	2,260	3.0	147	7.0
	2,260	3.0	140	6.7
	2,260	None	11	0.19

[a] Portions (200 ml) of Ad 2-infected KB cells maintained at 37 or 42 C were labeled for 1 hr with ^{3}H-uridine (1 μc/ml; 20 c/mmole). RNA were extracted and annealed with viral DNA as described. These experiments were repeated twice with similar results.

[b] From infected cells (time after infection—temperature of incubation).

proportions of virus-specific RNA appear to be synthesized at 42 C than at 37 C. Maximal amounts of virus-specific RNA were synthesized at 18 hr after infection at both 37 and 42 C.

Species of virus-specific RNA synthesized at 37 and 42 C. The synthesis of Ad 2-DNA begins at about 7 hr after infection at 37 C (4, 10). "Early" mRNA sequences are those formed prior to the initiation of viral DNA synthesis, and "late" mRNA sequences are those made after viral DNA synthesis has begun. The species of virus-specific RNA synthesized at 6 (early) and 18 (late) hr after infection of cells maintained at 37 and 42 C were compared by hybridization-inhibition measurements.

First, the amount of early and late 37 and 42 C virus-specific RNA required to saturate a fixed amount of viral DNA was determined (Fig. 2). Low levels of late 37 and 42 C RNA-saturated viral DNA, but high levels of early 37 and 42 C RNA did not saturate viral DNA completely. The saturation curves indicate that larger amounts of Ad 2-mRNA were synthesized late than early after infection at 37 and 42 C.

The hybridization of early 37 and 42 C RNA (6 hr) with viral DNA was blocked 80 to 90% by

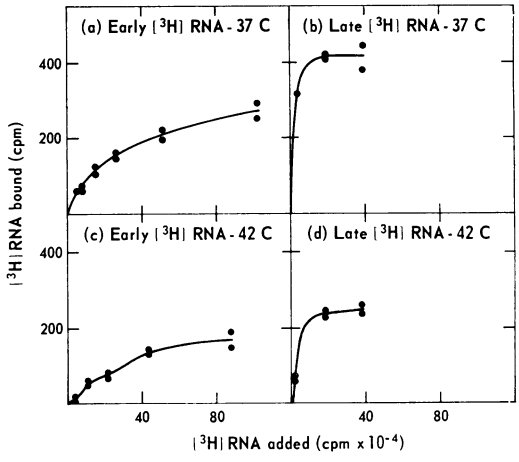

FIG. 2. *Saturation of Ad 2-DNA with early and late Ad 2 virus-specific ^{3}H-RNA synthesized at 37 and 42 C. Filters containing (a) 0.50 μg, (b) 0.10 μg, (c) 0.25 μg, and (d) 0.10 μg of alkali-denatured Ad 2 DNA were annealed with increasing amounts of (a) early 37 C ^{3}H-RNA (3,255 counts per min per μg), (b) late 37 C ^{3}H-RNA (1,716 counts per min per μg), (c) early 42 C ^{3}H-RNA (2,440 counts per min per μg), and (d) late 42 C ^{3}H-RNA (582 counts per min per μg) for 24 hr at 66 C. Radioactivity bound was plotted against the amount of ^{3}H-RNA added.*

both unlabeled early and late 37 and 42 C RNA (Fig. 3). Thus the same sequences of virus-specific RNA were synthesized at 6 hr after infection of cells maintained at both 37 and 42 C.

The binding of labeled late 37 and 42 C RNA to viral DNA was inhibited 90 to 95% by both unlabeled late 37 C RNA and 42 C RNA (Fig. 4). The same fraction of late RNA sequences were present early after infection (6 hr) at 37 and 42 C, as shown by the similar inhibition of labeled late virus-specific RNA by unlabeled early 37 and 42 C RNA. [Uninfected cell RNA at levels of 2 to 3 mg per filter inhibited nonspecifically the binding of virus-specific RNA to viral DNA by 20% (Fig. 4b). These amounts are necessary often for competition with early RNA species, which are present at low levels.] We conclude that the same species of virus-specific RNA are synthesized late after infection of cells at 42 and 37 C.

Synthesis of cellular and viral DNA at 37 and 42 C. KB cells incubated at 37 and 42 C were labeled with ^{3}H-thymidine for 3 hr at 6 and 18 hr after infection. Labeled DNA was isolated and annealed with cellular DNA and with Ad 2-DNA to determine the relative amounts of viral and cellular DNA synthesized at 37 and at 42 C. The synthesis of Ad 2-DNA was detected between 6 and 9 hr after infection (Table 4), confirming the results of inhibitor (4) and isotope (11) studies. Only 4 to 5% of DNA labeled from 6 to 9 hr at

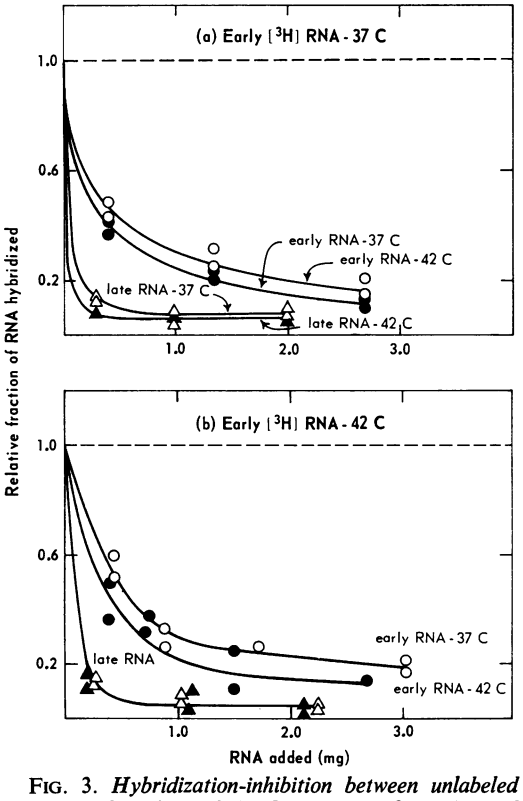

FIG. 3. *Hybridization-inhibition between unlabeled early and late 37 and 42 C virus-specific RNA and labeled early 37 and 42 C RNA. Filters containing 0.25 μg (42 C RNA) or 0.50 μg (37 C RNA) of alkali-denatured Ad 2-DNA were annealed first with unlabeled early or late 37 C or 42 C RNA and then with labeled early 37 C and 42 C RNA.*

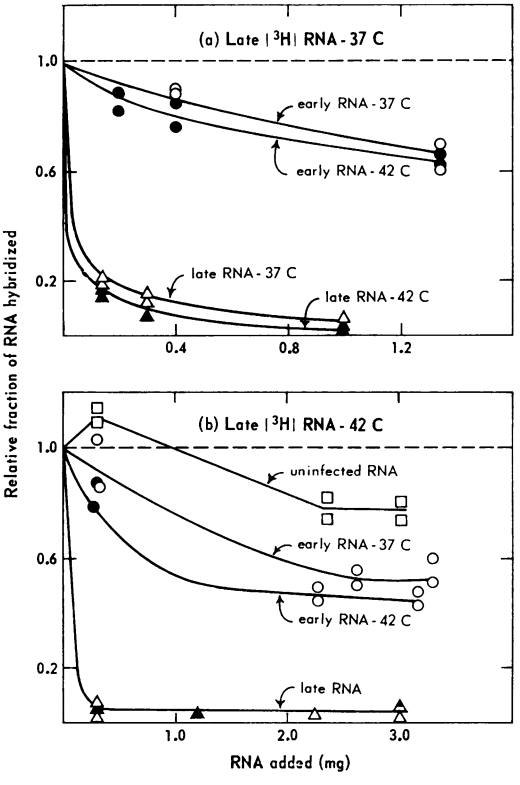

FIG. 4. *Hybridization-inhibition between unlabeled early and late 37 and 42 C virus-specific RNA and labeled late 37 and 42 C RNA. The conditions described in the legend to Fig. 3 were used. Filters contained 0.1 μg of Ad 2-DNA.*

TABLE 4. *Hybridization of cellular DNA and viral DNA with labeled DNA from AD 2-infected cells maintained at 37 and 42 C*[a]

Source of labeled DNA[b]	Input DNA	Cell DNA	Bound DNA	Bound[c]	Ad 2-DNA	Bound DNA	Bound[c]
	counts/min	*μg/filter*	*counts/min*	%	*μg/filter*	*counts/min*	%
6 to 9 hr-37 C	2,802	10.1	1,347	48.0	3.0	128	4.57
	2,802	10.1	1,420	50.4	3.0	126	4.50
	2,802	None	13	0.46	None	16	0.57
18 to 21 hr-37 C	3,500	10.0	236	6.74	3.0	2,910	83.0
	3,500	10.0	238	6.80	3.0	2,970	85.0
	3,500	None	16	0.46	None	10	0.28
6 to 9 hr-42 C	3,147	10.0	865	27.4	3.0	629	20.0
	3,147	10.0	745	23.6	3.0	623	19.7
	3,147	None	11	0.35	None	8	0.25
18 to 21 hr-42 C	3,112	10.0	108	3.5	3.0	2,995	96.3
	3,112	10.0	86	2.8	3.0	3,017	96.4
	3,112	None	10	0.32	None	3	0.10

[a] Portions (200 ml) of Ad 2-infected KB cells maintained at 37 and 42 C were labeled for 3 hr with ^{3}H-thymidine (0.1 μc/ml, 15 c/mmole). DNA was extracted and annealed with cellular and viral DNA as described. The hybridization reaction was repeated four times with similar results.

[b] From infected cells (time after infection—temperature of incubation).

[c] The sum of the percentage of labeled DNA that anneals with cellular and viral DNA does not equal 100%, because the efficiency of DNA-RNA hybrid formation is 80% between viral DNA molecules and 30 to 50% between cellular DNA molecules (Green et al, *in press*).

37 C hybridized with Ad 2-DNA, but at 42 C this value increased to 20%. Large amounts of cellular DNA were synthesized at 6 hr after infection at 37 C, in agreement with previous data (Piña and Green, *Virology, in press*), but the amount of cellular DNA synthesized at 42 C was reduced by 50%. At 18 hr after infection, 84 and 95% of synthesized DNA at 37 and 42 C, respectively, hybridized with viral DNA, whereas only 7 and 3% hybridized with cellular DNA. These results show that the synthesis of viral DNA was stimulated at 42 C as compared to 37 C, and that the shut-off of cellular DNA synthesis was enhanced at 42 C.

DISCUSSION

Incubation of Ad 2-infected KB cells at 42 C produced an "abortive" infection in which less than 0.5% of the normal yield of virus was produced. Analysis of RNA metabolism showed that: (i) the inhibition of rRNA synthesis which occurred at 37 C in infected cells was accentuated at 42 C; (ii) the synthesis of early and late viral mRNA was increased at 42 C; (iii) the same species of viral mRNA molecules were synthesized at 6 and 18 hr after infection at 37 and 42 C; and (iv) the changeover from host cell DNA synthesis to viral DNA replication was accelerated at 42 C. Thus, the critical thermosensitive step in virus synthesis does not appear to be at the level of transcription or viral DNA synthesis. Most likely some step in the translation of late viral mRNA or in the assembly of the virion is blocked at high temperature. Possible defective viral proteins are synthesized which are not assembled, or miscoding in translation occurs at high temperatures. The synthesis of Ad 2 at 42 C may provide a good system for the study of the intermediates in virus assembly.

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