CONTROL OF DNA SYNTHESIS IN MAMMALIAN CELLS IN CULTURE

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THE synthesis of DNA is the first step that has been clearly identified in the pathway leading to cell division. In a typical mammalian cell with a doubling time of 24 hr, the initiation of DNA synthesis is preceded by a period of about 12 hr (G_1) during which protein and RNA synthesis takes place. It may be asked what relation exists between the sequence of synthetic steps in G_1 and the beginning of DNA synthesis? Is DNA synthesis triggered by a special mechanism in an otherwise competent cell or is the process initiated by the appearance of enzymes specific for DNA synthesis?

In a previous report on the effects of inhibition of protein synthesis on cell division [18] it was noted that in the cells of an exponential culture the rate of DNA synthesis gradually decreased to zero in a time equal to the duration of the normal DNA synthetic period (S). It has been shown in some bacteria that inhibition of protein synthesis allows DNA replication to be completed in those cells which were making DNA while preventing the initiation of a new round of replication [5]. Since there is a superficial resemblance between this case and the observations on mammalian cells, a study was undertaken to attempt to determine the relation of RNA and protein synthesis to the onset of DNA synthesis.

The present results indicated that DNA synthesis required the synthesis of both RNA and protein. The protein(s) involved have not been identified but in cultures partially synchronized by cyclic additions of FUdeR[2] and thymidine, DNA synthesis did not appear to be limited by deoxynucleotide kinases and DNA polymerase.

MATERIALS AND METHODS

Cell cultures

All experiments were performed on human cells, strain KB [2] grown in suspension cultures with a normal doubling time of 24 hr. Cell counts were made with a Coulter

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[2] Abbreviations used: TdeR, thymidine deoxyriboside; FUdeR, 5′ fluorodeoxyuridine; TMP, TDP, TTP, thymidine mono, di, and triphosphate; deATP, deoxyadenosine triphosphate; deGTP, deoxyguanosine triphosphate; deCTP deoxycytidine triphosphate; PCA, perchloric acid.

counter. The cells were maintained in logarithmic growth at a density of between 10^5 and 2.5×10^5 cells per ml by daily feeding with prewarmed growth medium. Mitotic index measurements were made on aliquots fixed in alcohol–acetic acid (1:1) and stained with methyl-green-pyronin. Radioautographs were made by a dipping method using Kodak NTB-2 emulsion, and were stained through the emulsion with methyl-green-pyronin. The methods outlined above have been described in detail in a previous publication [18].

Determination of DNA, RNA and protein

Total amounts of DNA, RNA and protein per ml of cell culture were determined by standard colorimetric methods. Aliquots of the culture were washed three times by centrifugation in phosphate-saline buffer and then twice extracted for 5 min with ice-cold 0.2 *N* perchloric acid. For the determination of protein the residue was dissolved in normal sodium hydroxide by incubation overnight at 37°C and assayed by the method of Lowry [12] as modified by Oyama and Eagle [15]. Nucleic acids were extracted by heating the residue with 1N perchloric acid for 20 min at 90°C. The DNA content of the extract was determined by Burton's modification [1] of the diphenylamine reaction, and the RNA content by the orcinol method [13].

Synthesis of DNA, RNA, and protein was measured by the incorporation of ^{14}C-thymidine or ^{3}H-thymidine, of ^{14}C-adenine, or of ^{14}C-DL-valine, respectively (all radioactive compounds were obtained from New England Nuclear Corporation, Boston, Mass.). The labelled compound, dissolved in phosphate-buffered saline (140 m*M* NaCl, 27 m*M* KCl, 1.5 m*M* Na_2HPO_4, pH 7.4) was added to the culture flasks. Aliquots were withdrawn and pipetted into centrifuge tubes containing 10 ml ice-cold phosphate-saline. Cells were washed three times by centrifugation in phosphate-saline and filtered onto a millipore filter (pore size 0.45 μ). The material was extracted twice for 10 min with 0.2 *N* PCA and the filter was cemented to a planchet for counting in a Nuclear Chicago Counter.

In some experiments tritium labelled thymidine was used for measurements of DNA synthesis. In this case the cells were washed by centrifugation as before, extracted with cold 0.2 *N* PCA and the residue was dissolved in 1 ml of 10 X Hyamine T (Packard Instruments, Chicago, Illinois) by allowing tubes to incubate overnight at 37°C. The solution was transferred to a counting bottle with 10 ml of diotol [6] and counted in a Packard Tri-Carb Scintillation Counter.

The procedure was slightly modified for the measurements of rates of synthesis. A 3 ml aliquot of the culture was pipetted into a test tube containing the labelled compound dissolved in 2 ml of growth medium and subjected to gentle agitation for 20 min on a rotary shaker. All operations were performed in a warm room (36.8°C). The reaction was stopped by the addition of ice-cold phosphate-saline containing an excess of the unlabelled metabolite and the samples were then treated as described above.

Incorporation of labelled compounds per ml of cell culture increased linearly with time for periods up to 12 hr. For longer periods the rate generally showed a detectable increase due to increase in cell numbers. The 20 min period for rate measurements was selected as a compromise between sufficient incorporation and time resolution.

Enzyme assays

Thymidine kinases.—Phosphorylation of thymidine deoxyriboside to thymidine mono, di and triphosphates was carried out by a procedure adopted from Keilley [8]. Cells were suspended in 0.24 *M* sucrose, 1 m*M* $MgCl_2$, 0.01 *M* tris buffer pH 7.4 and homogenized in the stainless steel-nylon homogenizer described previously [18]. Nuclei were separated by layering the homogenate over 0.34 *M* sucrose, 1 m*M* $MgCl_2$, tris buffer pH 7.4, and spinning for 10 min at 700 *g*. Soluble supernatant was obtained by centrifugation for 1 hr at 100,000 *g*. The incubation mixtures consisted of 0.3 ml ATP solution (0.02 *M* potassium ATP, 0.02 *M* $MgCl_2$, 0.1 *M* tris buffer pH 7.9), 0.1 ml of a solution containing 10 μc of ^{3}H-thymidine and 0.3 ml of the soluble supernatant. The reaction was carried out at 37°C in a water bath, and terminated by heating for 1–2 min in boiling water. Control experiments indicated a negligible hydrolysis of TTP and TDP for this period of heating. Insoluble material was removed by centrifugation and 30 μl aliquots were spotted on thin layer chromatography plates (cellulose) together with 20 μl of a solution 10^{-2} *M* in TdeR, TMP, TDP and TTP. Plates were developed by ascending chromatography in *t*-amylalcohol–formic acid–water (3–2–1). The four thymidine derivatives were obtained as separate spots, although TTP showed some streaking and was overlapped by the ATP present in the assay mixture. Approximate Rf's were 0.35, 0.47, 0.67 and 0.83 for TTP, TDP, TMP and TdeR respectively. The spots were scraped off with a razor blade, eluted with 1 ml water, and aliquots taken for scintillation counting.

Cytoplasmic DNA polymerase.—The assay conditions were essentially those described by Lehman *et al.* [10]. The incubation mixture contained 0.3 ml cell supernatant extract, 0.3 ml ATP solution [see (*a*) above], 0.05 ml heat denatured Salmon DNA (500 γ/ml in 0.05 *M* tris buffer pH 7.5; a gift of Dr. E. Peter Geiduschek), 0.3 ml assay mixture (the assay mixture contained per ml—deATP, deCTP, deGTP, 5×10^{-8} moles), 0.015 ml 2-mercapto-ethanol (0.1 *M*), 0.2 ml $MgCl_2$ (0.1 *M*), 0.2 ml glycine buffer (1 *M* pH 9.2), 0.2 ml tritium labelled TTP solution.

The tritium labelled TTP was prepared from cell extracts as described above. The crude material was stored at −20°C until used. Thin layer chromatographic analysis showed that about 70–80 per cent of the radioactivity was present as TDP and TTP. Incubations were performed at 37°C and the reaction was terminated by pipetting an aliquot into a tube containing 0.2 ml carrier DNA (1 mg/ml) and adding cold *N* PCA to a final concentration of 0.5 *N*. The precipitate was washed by centrifugation in 0.2 *N* PCA and dissolved in Hyamine for counting. The radioactivity in the acid insoluble material was identified with DNA since it was resistant to hydrolysis by 0.3 *N* NaOH at 37°C, and was degraded by DNase.

Nuclear DNA polymerase.—The cells were homogenized in 0.24 *M* sucrose containing 0.5 per cent Brij 58 (Atlas Chemical Industries, Wilmington, Delaware) using a glass Potter homogenizer. Nuclei were washed three times by centrifugation in 0.24 *M* sucrose. Polymerase activity was determined using whole nuclei or a lysate prepared by sonication. The results were similar except that polymerase activity in sonicates was generally about 40 per cent less than in whole nuclei. Omission of denatured primer DNA from the assay reduced the incorporation tenfold.

In all kinase and polymerase experiments the enzyme activity was obtained from the slope of the linear portion of an incorporation versus time curve.

Puromycin and actinomycin were gifts of the American Cyanamid Company, Pearl River, New York and Dr. R. Haselkorn, University of Chicago. Fluorodeoxyuridine was provided by the Cancer Chemotherapy National Service Center of the Natl. Cancer Institute, Bethesda, Maryland.

RESULTS

Effects of Inhibition of Protein and RNA Synthesis in Logarithmically Growing Cultures

In a previous report [18] puromycin was shown to be a relatively specific inhibitor of protein synthesis in KB cells. The rate of synthesis, relative to the control (α), decreased to a constant value in less than 15 min and α values were reproducible to an accuracy of about 10 per cent. The effect was reversible for exposure times of more than 10 hr for values of α greater than 0.2. For concentrations of 5 μM or more of puromycin the culture did not return to logarithmic growth phase after an 8 hr exposure to the inhibitor. In the experiments described below puromycin concentrations were chosen to give α values from 0.5 to 0.2.

For very small values of α the incorporation of label may not be a measure of the relative rate of functional protein synthesis, since the label may be largely in incomplete polypeptide chains. In our previous study [18] the functional response in terms of entry into mitosis was completely blocked for α less than 0.2. Consequently we shall regard α values of less than 0.2 as complete inhibition.

RNA synthesis was inhibited by actinomycin. Preliminary experiments showed that the rate of synthesis as measured by the incorporation of ^{14}C-adenine, was reduced to less than 10 per cent at a concentration of 0.2 γ/ml. (Under the experimental conditions about 10 per cent of the incorporation in the control was resistant to alkaline hydrolysis at 37°C, and presumably represented DNA synthesis.) Inhibition of RNA synthesis was complete in about 15 min while a decrease in the rate of protein synthesis was not detectable until 1.5 to 3 hr after actinomycin addition. The actinomycin effects were not completely reversible after a 5–6 hr exposure to the inhibitor. Cultures resuspended twice in fresh medium regained only about half the control rates of RNA and protein synthesis. This result may be due in part to the difficulty of removing bound actinomycin from the cells.

The rate of DNA synthesis as a function of time at various levels of puromycin was measured by following the time course of incorporation of labelled thymidine and by incubation of aliquots of the culture with labelled thymi-

dine for 20 min. Aliquots were also incubated with ^{3}H-thymidine for 30 min for the preparation of radioautographs. Representative results of the relative rates of DNA synthesis and relative numbers of labelled nuclei are shown in Fig. 1 and Fig. 2. Experiments were performed at least twice for

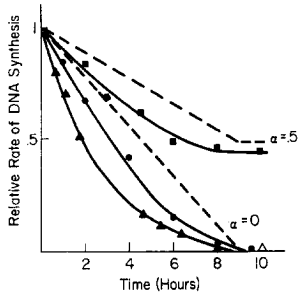

Fig. 1.

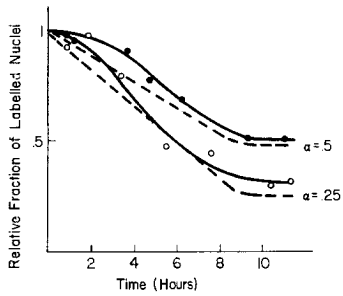

Fig. 2.

Fig. 1.—Rate of incorporation of ^{14}C-thymidine into DNA after addition of puromycin, relative to uninhibited control rate at $t = 0$. ●, ▲, separate experiments for relative rate of protein synthesis (α) of 0.2 (4.2×10^{-6} *M* puromycin), ■, $\alpha = 0.5$ (0.6×10^{-6} *M* puromycin). Dashed curves are theoretical plots for $\alpha = 0$ and 0.5. For details see text.

Fig. 2.—Relative number of labelled nuclei for relative rates of protein synthesis (α) of 0.5 (●) and 0.25 (○) corresponding to puromycin concentrations of 0.6 and 3×10^{-6} *M*. Dotted curves are theoretical plots for $\alpha = 0.25$ and 0.5. For details see text.

relative rates of protein synthesis (α) of 0.5, 0.3, 0.25 and 0.2. Rates of DNA synthesis after inhibition of RNA synthesis with actinomycin are shown in Fig. 3.

Puromycin caused a reduction in the rate of DNA synthesis beginning at approximately zero time. The rate fell to zero in 8 to 9 hr for $\alpha = 0.2$. For larger values of α the relative rate of DNA synthesis tended to reach a constant value in 8 to 9 hr, which was roughly equal to the value of α. In view of the experimental errors involved and the slow further decrease in rate for very long exposures, this correlation can be regarded as only approximate.

The decrease in the number of labelled nuclei was less rapid than the decrease in the rate of DNA synthesis. The number was not significantly reduced for about 2 hr even for low rates of protein synthesis, while the rate of DNA synthesis had fallen to about 0.6 at this time.

The curve for DNA synthesis after actinomycin addition differed from that for puromycin, in that there was a short lag (30 min). Following this period the rate declined more rapidly than for puromycin inhibition ($\alpha = 0.2$).

The marked decrease in DNA synthesis was evident well before any change could be detected in the rate of protein synthesis.

The dotted curves in Figs. 1–3 were calculated on the assumptions that the relative rate of entry of cells into the S period is determined by the relative rate of protein synthesis while cells already synthesizing DNA are unaffected by inhibition of RNA or protein synthesis (see Discussion section). It can be seen that the rate of DNA synthesis declines much faster than required by the model while the decrease in labelled nuclei is probably slower at least at early times.

Inhibition of DNA Synthesis in Synchronized Cultures

To clarify the nature of the rapid reduction in the rate of DNA synthesis, experiments were undertaken with partially synchronized cultures. Partial synchrony was obtained by addition of FUdeR, 10^{-6} *M*, followed by thymidine, 5×10^{-6} *M* at 16–18 hr. This method has been described by a number of investigators [17, 19] and since our results are essentially similar they will not be described in great detail. Total DNA increased during the period of 8 to 9 hr after thymidine addition, followed by a plateau lasting for a few hours. Similar curves were obtained for incorporation of ^{14}C-thymidine. Cell division commenced at 8–10 hr and there was a wave of mitosis lasting 4 to 6 hr. The synchrony was lost in about two generation times so experiments were performed to try to improve synchrony by holding the cells on a cycle

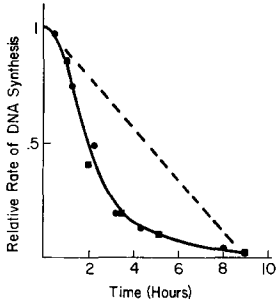

Fig. 3.

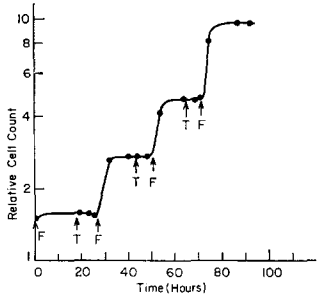

Fig. 4.

Fig. 3.—Rate of incorporation of ^{14}C-thymidine into DNA after addition of actinomycin (0.2 μg/ml) relative to uninhibited control rate at $t = 0$. Dashed curve is a theoretical plot for $\alpha = 0$. For details see text.

Fig. 4.—FUdeR-Thymidine cycle. At times indicated by *T* thymidine (2×10^{-6} *M*) was added and at times indicated by *F* the culture was resuspended in growth medium, containing 10^{-6} *M* FUdeR. Cell counts were made with a Coulter Counter.

consisting of 16 hr in FUdeR medium, 8 hr in the presence of thymidine followed by resuspension in FUdeR medium, etc. The results of such an experiment are shown in Fig. 4. The synchrony was improved at the third division in which a complete doubling was obtained, compared to the value of 80 per cent usually produced in one-step synchrony experiments. The experiment showed that FUdeR inhibition did not affect viability and the interdivision period was still approximately 24 hr.

However measurements of total RNA and protein did not reveal a periodicity. The mitotic index in a one-step experiment reached a peak of 20 per cent and the peak was not raised above 25 per cent in the second or third synchronous division. The experiments described below were performed on cultures undergoing the first or second synchronous division.

Experiments were undertaken to determine the effect of blocking RNA or protein synthesis before or after the addition of thymidine (Figs. 5 and 6). Addition of puromycin 0.5 to 1 hr before thymidine reduced the synthesis of DNA to 20 per cent of the control (average of 5 experiments ranging from 10 to 25 per cent). A similar result was obtained with actinomycin (Table I and Fig. 6). Addition of inhibitor at various times after thymidine had a less pronounced effect in that 40–50 per cent of the total DNA could be synthesized. Synthesis dropped to zero at about the same time in inhibited cultures as in the controls (9 hr).

The shape of the control curve (which was determined more than a dozen times in the course of the experiments) was very reproducible. A small in-

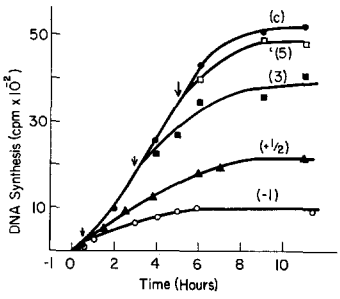

Fig. 5.

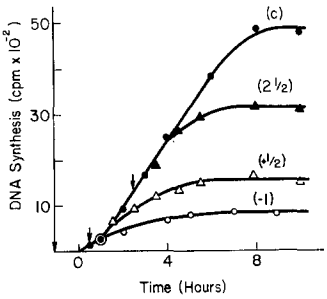

Fig. 6.

Fig. 5.—Incorporation of ^{14}C-thymidine into DNA in cultures partially synchronized by FUdeR treatment. Thymidine was added at $t=0$. Puromycin (4.2×10^{-6} M) was added at -1, $+\frac{1}{2}$, 3, and 5 hr. Curve C refers to the uninhibited control.

Fig. 6.—Incorporation of ^{14}C-thymidine into DNA in cultures partially synchronized by FUdeR treatment. Thymidine was added at $t=0$. Actinomycin (0.2 μg/ml) was added at -1, $+\frac{1}{2}$, and $2\frac{1}{2}$ hr. Curve C refers to the uninhibited control.

crease in rate occurred during the first 1.5 hr in every experiment and the linear portion of the curve extrapolated to about 0.75 hr.

Radioautograph experiments were performed to determine whether the 20 per cent synthesis in blocked cultures was due to 20 per cent of the cells

TABLE I. *Effect of inhibition of protein or RNA synthesis on DNA duplication in partially synchronized cultures.*

At time zero thymidine (10^{-5} *M*) was added to a culture which had been exposed to FUdeR for 16 hr. Results are expressed as per cent DNA synthesized with respect to the total increase in DNA in the uninhibited control. In some cases experiments were performed on cultures in the second FUdeR–thymidine cycle.

Time of addition of inhibitor (hr)		DNA synthesized at time of addition %	Final amount of DNA synthesized %	Increase in DNA %
Puromycin	−1	0	20 ± 6[a]	20 ± 6
(4.2×10^{-6} *M*)	+0.5	3	42	39
	+1	10	66	56
	+2.5	32	71	39
	+3	35	77	32
	+5	68	95	27
Actinomycin	−1	0	17	17
(0.2 μg/ml)	0.5	3	32	29
	2.5	25	65	40

[a] Average of five experiments ± standard deviation.

being capable of complete replication in the absence of protein or RNA synthesis or to the synthesis of 20 per cent of the normal DNA content by any given cell. In the controls 90 to 95 per cent of the nuclei were labelled after 30 min exposure to ^{3}H-thymidine. Puromycin pretreated cultures showed 80–85 per cent labelled nuclei at 1.5 hr. The principal difference was a higher grain count in the cells from controls versus inhibited cultures. Thus, essentially all the cells are capable of some DNA synthesis in the absence of protein or RNA synthesis, although the amount is quite small.

Fluctuations in Protein and RNA Synthesis in Synchronized Cultures

The apparent requirement for RNA and protein synthesis at the beginning of replication prompted us to re-examine their rates of synthesis after thy-

midine addition. Colorimetric determinations of total protein and RNA failed to show any sudden increase during this period but the method is not sensitive enough to detect small increases in rate extending over a brief interval. The rate of synthesis was measured by withdrawing duplicate aliquots at 20 min intervals and incubating for 15 min with ^{14}C-valine or ^{14}C-adenine. In five out of six experiments a small increase in rate of protein synthesis of 10–20 per cent was found, which reached a maximum at 1½ to 2 hr and returned to the base line at 2½ to 4 hr. The average of the data defining the peak differed from the mean control level by two standard deviations, so the results appear to be statistically significant. The peak was eliminated by prior treatment with actinomycin. An increase of similar magnitude in the rate of RNA synthesis occurred during approximately the same time interval in three experiments. However the detection of increased rates extends the experiments to the limits of accuracy and the only conclusion that can safely be drawn is that any increase in protein or RNA synthesis is quite small and the extra synthesis corresponds to less than 0.5 per cent of the total protein or RNA content.

Activities of DNA Polymerase and Thymidine Kinases

A possible explanation of the requirement for protein synthesis is that enzymes of DNA metabolism must be synthesized after thymidine addition. The normal pathway is blocked at the step involving conversion of deUMP to TMP and thymidine addition allows the synthesis of TMP via TdeR kinase. Experiments were performed to determine the levels of DNA polymerase and of the kinases necessary for the formation of TTP.

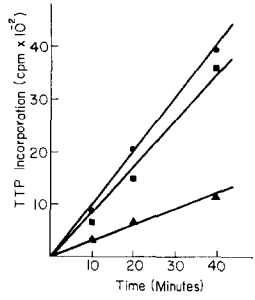

Fig. 7.—Incorporation of ^{3}H-TTP into acid insoluble material by soluble extracts. Extracts were prepared from FUdeR treated culture (●), from FUDR treated culture harvested 2.5 hr after addition of thymidine (■), from FUdeR treated culture to which puromycin (4.2×10^{-6} *M*) was added at $t = -1$ hr, thymidine at $t = 0$, and cells harvested at $t = 2.5$ hr (▲).

Preliminary experiments with cell homogenates showed the presence of a phosphorylase which converted TTP to TMP. This inhibitory activity was largely removed by centrifugation of the homogenate for 1 hr at 15,000 *g*. However nuclei prepared in sucrose could not be freed of particulate material which could apparently inhibit DNA synthesis by hydrolysis of substrate.

TABLE II. *Effects of addition of thymidine, puromycin and actinomycin on the DNA polymerase activity of partially synchronized cultures.*

The FUdeR blocked culture was divided into three flasks. At zero time thymidine 10^{-5} was added to one, puromycin (4.2×10^{-6} *M*) or actinomycin (0.2 μg/ml) was added to the second at −1 hr and thymidine at time zero, and the third received no additions. Cultures were harvested at times indicated in column 2, and polymerase activity determined by the method described in the text (p. 318). Activities are expressed relative to the FUdeR blocked control.

		Relative cytoplasmic DNA polymerase activity		
Experiment	Time after thymidine addition (hr)	Thymidine	Puromycin plus thymidine	Actinomycin plus thymidine
1	2.5	0.80	0.35	
2	2.5	0.78	0.30	
3	2.5	0.83	0.43	
Average		0.80 ± 0.02	0.36 ± 0.05	
4	1	1.0		1.0
4	3	Not determined		0.70
		Relative nuclear DNA polymerase activity		
5	2.5	0.75	0.80	
6	2.5	1.15	1.20	
7	2.5	1.1	1.25	
8	2.5	1.0	1.40	
Average		1.0 ± 0.16	1.16 ± 0.28	

For nuclear polymerase assays the cells were homogenized in Brij 58 which gave much cleaner preparations with high polymerase activity.

Polymerase activity was assayed in cytoplasmic or nuclear extracts prepared (*a*) from the FUdeR blocked control culture (*b*) from the culture 2.5 hr after thymidine addition (*c*) from the culture to which puromycin or actinomycin was added 1 hr before thymidine. The results of a typical experiment are plotted in Fig. 7.

Addition of thymidine did not increase the activity of cytoplasmic polymerase (Table II). In the presence of puromycin the rate was reduced to

30 per cent while somewhat higher values (70 per cent) were obtained with actinomycin. However, the polymerase activity of whole or lysed nuclei (Table II) was not affected by puromycin treatment.

TdeR kinase activity was high in FUdeR blocked cultures and the rate of formation of TMP was too rapid to compare activities under the assay con-

TABLE III. *Effects of addition of thymidine, puromycin and actinomycin on TMP (TDP) kinase activity of partial synchronized cultures.*

Experimental design was the same as for Table II. Puromycin and actinomycin were added 1 hr before thymidine and cultures were harvested 2.5 hr after thymidine additions. Results are expressed relative to the FUdeR blocked control.

	Relative TMP (TDP) kinase activity		
Experiment	Thymidine	Puromycin + thymidine	Actinomycin + thymidine
1	0.80	1.5	
2	1.20	1.6	
3	0.92	1.3	
4	0.93	1.3	
5	0.80		1.6
6	1.30		1.7
Average	0.92 ± 0.2	1.43 ± 0.17	1.65

ditions used. The TMP produced served as a substrate for the assay of TMP (TDP) kinase. The activity of the enzyme or enzymes catalyzing conversion of TMP to TTP was not increased by thymidine addition and the activity was actually higher after puromycin and actinomycin treatment (Table III).

DISCUSSION

The experiments described in the preceding sections demonstrated a requirement for RNA and protein synthesis both immediately prior to and during DNA replication. In logarithmically growing cultures the rate of DNA synthesis decreased to essentially zero in the 9 to 10 hr period following the inhibition of protein synthesis with puromycin or RNA synthesis with actinomycin. The S period in these cells was 8 to 9 hr. A partial inhibition of protein synthesis did not abolish the synthesis of DNA and the relative rate after 9 hr was roughly equal to the relative rate of protein synthesis.

The major difference in the shape of the DNA rate curves after addition of inhibitors was the presence of a 30 min lag for actinomycin inhibition

(Fig. 3 versus Fig. 1). The rate of total protein synthesis did not decrease at this concentration of actinomycin for at least 1 to 2 hr. Thus the results are consistent with a process in which RNA synthesis is necessary for the synthesis of a protein which is in turn required for the synthesis of DNA. The lag with actinomycin may be a measure of the duration of the steps involving RNA. Expressed in current terminology, a new messenger RNA is required and the lag is a measure of the duration of the steps involving synthesis and possibly transfer of messenger to the ribosomes. The much longer lag in the effect of actinomycin on total protein synthesis could arise from the slow decay of existing messenger RNA. However, in the present instance, the theoretical concepts are too far removed from the experimental measurements to be regarded as more than a convenient description.

The relative number of labelled nuclei decreased more slowly than the relative rate of DNA synthesis (Figs. 1 and 2). Since the reproducibility of the ratios of labelled nuclei was about 10 per cent, the shape of the curve during the first 2 hr after addition of inhibitor could not be determined accurately. It can only be concluded that there was a lag or a slowly decreasing rate during this interval in contrast to a more than 50 per cent decrease in the rate of DNA synthesis. It should be noted that nuclei are scored either as labelled or unlabelled and thus changes in the relative number of labelled nuclei with time need not parallel changes in the rate of DNA synthesis.

The experimental data allows a test of a simple model of the response to inhibition, namely: (1) the rate of protein synthesis determines the rate of passage of cells through G_1, the final step being the synthesis of protein necessary for the initiation of DNA synthesis (2) the rate of DNA synthesis for cells in the S period is not dependent on further protein synthesis. Equations giving the relative rate of DNA synthesis and the relative number of labelled nuclei as a function of time are derived in the appendix. The theoretical curves are shown as the broken lines in Figs. 1–3. The rate of DNA synthesis decreased more rapidly than predicted by the model while the curve for labelled nuclei tended to lie above the theoretical curve at early times. The predictions of the model were in agreement with experiment only in respect to the final rate of DNA synthesis, which was reduced in proportion to the relative rate of protein synthesis. This agreement cannot be accepted without reservations since the experimental error is relatively large and the apparent plateau at 8 to 9 hr is partly a result of the time scale. The rate showed a further slow decrease when followed over a 30 hr period.

A simple explanation of the rapid decrease in DNA synthesis is provided by the experiments on synchronized cultures, if it can be assumed that the

synchronization procedure does not affect the mechanisms controlling the initiation of DNA synthesis. Justification for this assumption is provided if the response of DNA synthesis to inhibitors in logarithmically growing cultures can be accurately predicted from the data on synchronized cultures. As discussed below the two sets of data are in qualitative agreement but the accuracy of the measurements is not sufficient to allow an exact comparison.

Addition of inhibitors after the beginning of DNA synthesis allowed the formation of only 30–50 per cent of the total DNA (Table I). Thus in logarithmic growth the rate of DNA synthesis must decrease faster than expected from simply blocking the entry of cells into the S phase. On the other hand inhibition of protein or RNA synthesis before the addition of thymidine permitted only a 20 per cent increase in DNA. Part of this synthesis is due to cells blocked in S by the previous treatment with FUdeR. Since about 30 per cent of the cells are in S before thymidine addition [19], these cells could contribute at least half of the total DNA synthesized, in the inhibited culture. Thus inhibition of protein or RNA synthesis prior to the beginning of S phase reduces the average amount of DNA synthesized per cell to about 10 per cent of the normal amount. Therefore, the cell is much more sensitive to inhibition of protein or RNA synthesis prior to the initiation of DNA synthesis than after the process has begun. In fact, the synthesis of the final 30 per cent of the DNA was unaffected by inhibition of protein or RNA synthesis.

The small amount of DNA synthesis permitted by prior inhibition of protein or RNA synthesis cannot be attributed to an underestimate of the amount of DNA synthesized by the cells blocked in S. Radioautographs of the puromycin or actinomycin inhibited cultures showed that at least 80 per cent of the nuclei were labelled by a 30 min pulse while at most 30 per cent of the cells could have been trapped in S.

The general features of FUdeR synchronized growth described by Till *et al.* [19] for mouse L cells are very similar to the results reported here with the exception of the effect of synchrony on the duration of the S period. These authors found the S period to be reduced from 8 to 4 hr for the first duplication of DNA after release of FUdeR inhibition. In our experiments the duration of DNA synthesis was 8–9 hr in a dozen experiments and was therefore very little different from the normal duration of S. However the time separation between the waves of DNA synthesis and cell division was only 9 to 11 hr compared with the normal duration of S + G_2 + mitosis of 11–12 hr. Thus some phase of the cycle is shortened but in our case the S period was not markedly reduced.

During the course of this work a similar experiment on the effect of puromycin on synchronized HeLa cells was published by Mueller *et al.* [14]. Our results (Fig. 5) are in remarkably close agreement with their findings (Chart 6). These authors also noted an increasing rate of DNA synthesis during the first hour or two after thymidine addition. The same effect was evident in our experiments and the linear portion of the rate curve extrapolated to a lag of 0.75 hr. Since essentially all nuclei were labelled in less than 30 min, this period of increasing rate is due to an increase in rate per cell and not to an increase in the number of cells synthesizing DNA. If protein or RNA synthesis is inhibited before thymidine addition the period of increasing rate is absent (Fig. 5).

In *E. coli* amino acid starvation prevents the initiation of replication, and since this begins at a particular point on the bacterial chromosome, it has been suggested that a specific protein is involved [16]. A mechanism for the genetic control of replication has been proposed by Jacob and Brenner [7]. The unit of replication, or replicon is controlled by an initiator gene which acts on a region located at the beginning of the replicon. The evidence presented in the present work suggests that the requirement for protein and RNA synthesis in replication is not connected with metabolic enzymes and consequently that the process is under genetic control. However, it does not provide support for the replicon hypothesis as opposed to some other genetic scheme.

A large number of enzymes are involved in DNA synthesis and it would be a formidable task to show that no one of these could account for the protein requirement. Assays were limited to DNA polymerase and the enzymes involved in the conversion of added TdeR to TTP, since these enzymes might be expected to be affected under the conditions of the experiments. The addition of thymidine to the FUdeR-blocked cultures did not stimulate the activities of these enzymes (Tables II and III).

Since the enzyme levels may be maintained by a balance between synthesis and degradation, activities were determined after puromycin and actinomycin treatment. Kinase activity was actually stimulated by the inhibitors (Table III) and although the significance of this effect is not clear, the limitation of DNA synthesis by kinases can be eliminated.

DNA polymerase activity was reduced in the cytoplasm while the nuclei may have shown a slight increase (Table II). In three out of four experiments the nuclear activity was higher but if one doubtful experiment (No. 5) is included the increase is not statistically significant.

Littlefield *et al.* [11] and Gold and Helleiner [4] have noted a decrease in

cytoplasmic and an increase in nuclear DNA polymerase in L cells after the addition of thymidine to FUdeR blocked cultures. In the present experiments the enzyme activity was assayed too soon after thymidine addition to obtain the maximum effect reported by these authors and was detected only in the puromycin treated culture. The low cytoplasmic DNA polymerase activity after puromycin treatment may be the result of more efficient enzyme transfer.

The kinetics of DNA synthesis inhibition cannot be readily explained by considering the low cytoplasmic polymerase to be a result of enzyme degradation: (1) Since there is an available pool of cytoplasmic enzyme, loss of enzyme should not affect DNA synthesis until the nuclear level falls below a critical value. Therefore inhibition of DNA synthesis should show a lag while none was observed with puromycin. (2) Actinomycin was an equally good inhibitor of DNA synthesis while loss of cytoplasmic enzyme was much less. (3) A loss of about 50 per cent of the enzyme through degradation in 3 hr would require an unreasonable turnover rate (15–20 per cent per hr versus about 1 per cent for total cell protein [3]).

Inhibition of RNA synthesis did not affect the mitotic index for about 3 hr. This result indicates that RNA synthesis necessary for mitosis is completed about 3 hr before the beginning of prophase (i.e., at the end of S). Previously it was concluded on the basis of models of the kinetics of inhibition of mitosis by puromycin, that the last step in the preparation for mitosis requiring protein synthesis commenced about 3 hr before prophase [18]. The effect of actinomycin is in agreement with this conclusion. Kishimoto and Lieberman [9] have also concluded that the last step requiring RNA synthesis occurs at the end of S.

APPENDIX

Rate of DNA Synthesis or Number of Labelled Nuclei After Inhibition

It is assumed

(1) Rate of arrival of cells at S is proportional to the rate of protein synthesis. Thus if the rate of protein synthesis relative to the control is α this is equivalent to changing the time scale. Time t can be replaced by αt for the entry of cells into the S period.

(2) Rate of DNA synthesis for cells in S at $t = 0$ (the time of inhibition of protein synthesis) is unaffected.

(3) Rate of DNA synthesis per cell is constant during S. This assumption

is only approximately correct (Fig. 5) but the error introduced is too small to affect the results of the analysis.

Let $R_{(t)} = \dfrac{\text{number of cells synthesizing DNA at time } t \text{ in the experiment}}{\text{number of cells synthesizing DNA at time } t \text{ in the control}}$.

At $t = 0$ the cells are in exponential growth, with doubling time T_0,

$$N = N_0 e^{\lambda t} \quad \text{and} \quad \lambda = \log_e 2 / T_0,$$

$$R_{(t)} = \int_{T_1 - \alpha t}^{T_2 - t} dN_T \Big/ \left(e^{\lambda t} \int_{T_1}^{T_2} dN_T \right),$$

dN_T is number of cells of age T at $t = 0$, $0 \leqslant T \leqslant T_0$
$dN_T = 2\lambda\, N_0 e^{-\lambda t}\, dT$ (reference 1)
T_1 and T_2 are the ages of the cell at the beginning and end of S in the exponential culture, $S = T_2 - T_1$

$$R_{(t)} = 2\left[e^{-\lambda T_1} e^{\alpha \lambda t} - e^{-\lambda T_2} e^{\lambda t}\right] / 2\left(e^{-\lambda T_1} - e^{-\lambda T_2}\right) e^{\lambda t}.$$

Assumption 3 means that $R_{(t)}$ has the same form for the relative rate of DNA synthesis and the relative number of labelled nuclei. For simplicity experimental data are expressed in terms of the rate relative to the control rate at $t = 0$, and the factor $e^{\lambda t}$ in the denominator can be dropped.

The dotted curves in Figs. 1 to 3 were calculated for $T_0 = 25$ hr, $T_2 = 21$ hr, $T_1 = 12$ hr. The values of the parameters were obtained from the growth rate, the relative number of labelled nuclei for a ^{3}H-thymidine pulse and the time for the appearance of labelled mitoses. Since the curves are almost linear the results are not sensitive to the values of T_0, T_1 and T_2 but do depend on $T_2 - T_1 = S$ and α.

SUMMARY

Inhibition of protein or RNA synthesis causes an inhibition of DNA synthesis in cultures of human cells (strain KB). In cultures partially synchronized by fluorodeoxyuridine-thymidine treatment, DNA synthesis was reduced to 10–20 per cent of the normal amount by addition of puromycin or actinomycin prior to thymidine. After DNA synthesis has begun the cells are much less sensitive to the inhibitors. Assays of DNA polymerase and thymidine kinases indicated that synthesis of these enzymes after thymidine addition could not account for the requirement for protein synthesis. It is suggested that a particular protein must be made which controls the initiation of replication.

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