[CANCER RESEARCH 32, 107–113, January 1972]

Studies on the Induction of DNA Polymerase during Transformation of Human Lymphocytes[1]

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SUMMARY

The addition of phytohemagglutinin to cultures of human peripheral lymphocytes initiates a series of metabolic alterations leading to DNA replication and cell division. DNA polymerase activity increases immediately before the onset of thymidine incorporation and thereafter both increase proportionally. Addition of actinomycin D in the first 2 hr after phytohemagglutinin is added completely prevents the increase in DNA polymerase activity. However, the earliest increase in DNA polymerase activity is detected only after 18 to 20 hr in culture. This may be an example of translational delay. Actinomycin D added at 6 hr after phytohemagglutinin slows and limits the increase in DNA polymerase activity by only 50%. Subsequently, the increase in DNA polymerase activity becomes increasingly less sensitive to the effect of actinomycin D. Induction of DNA polymerase activity thus appears to be programmed by an actinomycin D-sensitive product made early during lymphocyte transformation. However, there appears to be no delay between the synthesis of polymerase and the appearance of activity, since the addition of either puromycin or cycloheximide (inhibitors of protein synthesis) to cultures of stimulated lymphocytes prevents further increases in DNA polymerase activity and thymidine incorporation.

INTRODUCTION

The induction of DNA replication in populations of nondividing animal cells appears to require the synthesis of the enzymes associated with this replication. For example, human peripheral blood lymphocytes are well-differentiated cells which ordinarily do not divide *in vivo*. Their quiescent behavior is also maintained in culture. However, when they are cultured in the presence of a mitogenic stimulus such as PHA,[4] an extract of the red kidney bean, *Phaseolus vulgaris*, a series of events is initiated (7, 8, 12, 14, 22) that lead to DNA synthesis and cell division (19). These include increases in activities of some of the enzymes in the DNA-synthetic pathway (17, 18). Immediately prior to DNA synthesis, there is a marked stimulation of DNA polymerase activity in these cells. This increase in activity parallels the ability of the cells to incorporate thymidine into DNA (16). We now report on how DNA polymerase activity and thymidine incorporation are affected by interference with RNA and protein synthesis at different times during lymphocyte transformation.

MATERIALS AND METHODS

dTTP-α-^{32}P was purchased from International Chemical and Nuclear Corp., City of Industry, Calif. The purity of this compound was routinely monitored by chromatography and by effectiveness as a substrate for purified sea urchin nuclear DNA polymerase (15). All other radioactive compounds including actinomycin D-^{3}H (3.38 Ci/mM) were obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y. Calf thymus DNA and pancreatic DNase were acquired from Worthington Biochemical Corp., Freehold, N. J. Activated calf thymus DNA was made by digestion of the DNA with minute amounts of pancreatic DNase until maximal priming was achieved (15). Actinomycin D was a gift from Merck, Sharp and Dohme, Rahway, N. J. PHA-M was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

Lymphocytes were isolated from human peripheral blood by the method of Bach and Hirschhorn (1). They were suspended at a concentration of 7.5 X 10^5 cells/ml in Eagle's minimal essential medium (Spinner modification) supplemented with 20% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (1%). All of the constituents of the medium were obtained from Grand Island Biological Co., Grand Island, New York. PHA-M (0.025 ml) was added to each ml of cell suspension. Aliquots of 1 or 2 ml of cell suspension were incubated in 16- x 125-mm disposable plastic culture tubes (Falcon Plastics, Los Angeles, Calif.) at 37° in a humidified air atmosphere containing 5% CO_2. Amounts of actinomycin D and puromycin given in the text refer to the final concentrations (μg/ml) in the lymphocyte cultures.

Incorporation of Radioactive Precursors. We determined the relative rates of protein, RNA, and DNA synthesis by adding 2.5 μCi or L-leucine-^{3}H, uridine-^{3}H, or thymidine-methyl-^{3}H, respectively, to 1-ml cultures 1 hr before harvesting the cells. Incorporation was terminated by adding 10 μmoles of the

[1] This investigation was supported by the NIH (CA-11524 and CA-06551), the National Science Foundation (GB-18419), and by the following grants to this Institute: National Institutes of Health Grants CA-06927 and RR-05539, a grant from the Stanley C. Dordick Foundation, and an appropriation from the Commonwealth of Pennsylvania.

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[4] The abbreviation used is: PHA, phytohemagglutinin.

Received August 16, 1971; accepted September 15, 1971.

corresponding unlabeled precursor and chilling the cultures in an ice water bath. The cells were then collected and washed by centrifugation for 10 min at 2500 × *g* in 2 ml of 0.15 M potassium chloride. The amount of leucine incorporated into acid-insoluble material was determined as previously described (17). In a similar manner, cells labeled with uridine were washed 3 times with ice-cold 0.5 N perchloric acid containing 0.05 M $MgCl_2$ and once with 95% ethanol; then the precipitate was dissolved in 1 ml Hyamine hydroxide (Packard Instrument Co., Downers Grove, Calif.) prior to the determination of radioactivity. Cells labeled with thymidine were suspended in 0.1 ml of a solution containing 20% (w/v) glycerol (Matheson, Coleman and Bell, East Rutherford, N. J.; Spectroquality grade); 0.02 M potassium phosphate buffer (pH 7.4); 0.001 M potassium EDTA; and 0.004 M reduced glutathione. Thereafter the cells were disrupted twice by rapid freezing and thawing. The amount of radioactivity incorporated into acid-insoluble material was determined along with the assay of DNA polymerase activity.

Assay of DNA Polymerase Activity. The assay for DNA polymerase activity measures the incorporation of residues of dTMP-^{32}P into an acid-insoluble product. The reaction mixture in a total volume of 0.3 ml, consisted of the following: 25 μmoles of Tris-maleate buffer, pH 8.0; 3 μmoles of magnesium chloride; 1 μmole of potassium chloride; 0.3 μmole of β-mercaptoethanol; 25 mμmoles each of dATP, dCTP, and dGTP; 10 mμmoles of dTTP-α-^{32}P (about 5 × 10^4 dpm/mμmole); 266 mμmoles of activated calf thymus DNA; and 0.1 ml of the lymphocyte preparation. Incubation was for 1 hr at 37°, and the reaction was stopped by the addition of 0.5 ml of cold 1 M perchloric acid containing 0.01 M sodium pyrophosphate. The acid-insoluble material was collected on glass-fiber filters. Radioactivity was determined by standard, dual-labeling techniques, with liquid scintillation spectroscopy. During the polymerase assay, the thymidine which had been incorporated into DNA was not degraded into acid-soluble nucleotides. After correction for crossover for ^{3}H and ^{32}P in the acid-insoluble precipitate, we were able to determine both thymidine incorporation (^{3}H) and polymerase activity (^{32}P) in the same sample of cells.

RESULTS

Effect of Different Amounts of Actinomycin D. When human peripheral blood lymphocytes are cultured in the presence of PHA, a marked increase in DNA polymerase activity and rate of thymidine incorporation is observed, starting at 18 to 20 hr (16). The increase in polymerase activity precedes by 2 to 3 hr the increase in thymidine incorporation. During the 1st 15 hr, the cells do not incorporate significant amounts of exogenous thymidine, and there is no increase in DNA polymerase activity. In contrast, RNA synthesis, as measured by the incorporation of labeled uridine into acid-insoluble material, is stimulated at a much earlier time, even within the 1st 2 hr after PHA stimulation (7). In initial experiments we studied the effect of adding, simultaneously with PHA, graded amounts of actinomycin D, an inhibitor of RNA synthesis, and harvesting the cells 66 hr later (18). As little as 0.001 μg of actinomycin D per ml was capable of preventing 90% of the PHA stimulation of DNA polymerase activity. Inhibition was complete with 0.01 μg of actinomycin D per ml. Although 90% of uridine incorporation into an acid-insoluble precipitate was inhibited in PHA-stimulated cultures when actinomycin D, 0.001 μg/ml, was added simultaneously with PHA, the cells were still capable of undergoing morphological transformation (18). In cultures treated with actinomycin D, 0.02 μg/ml, more than 56% of the cells were still viable (70% in control cultures with only PHA), as indicated by their ability to exclude the dye, trypan blue. In this report, we have inhibited RNA synthesis by adding actinomycin D at various times during lymphocyte transformation and have measured the effects on the induction of DNA polymerase and DNA replication.

Effects of Actinomycin D on Unstimulated Lymphocytes. Human peripheral lymphocytes seldom divided *in vivo* and have little DNA polymerase activity *in vitro* (17). In the absence of PHA or other stimulatory agents, there is little change in polymerase activity (17, 18), and actinomycin D has little effect on the basal level of this activity (Chart 1). In the presence of actinomycin D (0.02 μg/ml), in the 1st 6 hr after PHA stimulation there was no change in DNA polymerase activity, and in the next 6 hr polymerase activity appeared to decrease slightly. In another experiment, even after 66 hr in the presence of actinomycin D, there was no effect on DNA polymerase activity. Polymerase activity was 21 ± 1 μμmoles of dTMP-^{32}P per ml of culture per hr in the actinomycin D-treated cultures, compared with 16 ± 5 μμmoles in the control cultures. Similarly, DNA polymerase activity in unstimulated cultures is not greatly affected by puromycin. At a concentration of 10 μg/ml of culture, puromycin inhibits by more than 90% the incorporation of algal hydrolysate ^{3}H-labeled into acid-insoluble precipitate, yet did not decrease polymerase activity (Chart 1). The insensitivity of polymerase activity to actinomycin D and puromycin suggests little turnover of the enzyme in unstimulated lymphocytes.

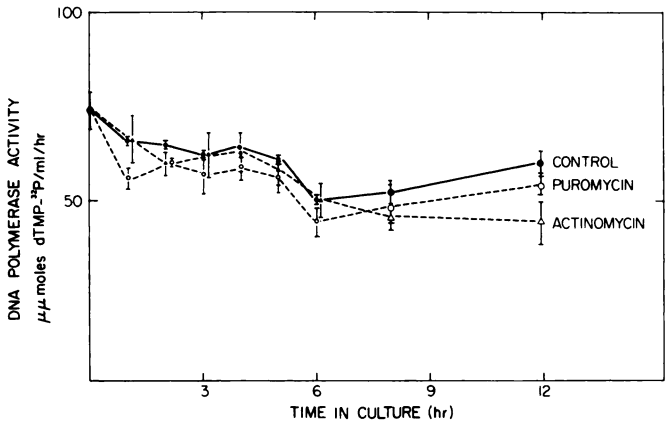

Chart 1. Effect of actinomycin D and puromycin on the amount of DNA polymerase activity in unstimulated lymphocytes. Lymphocytes were cultured in the absence of PHA, and at the indicated times the cells were harvested and assayed for DNA polymerase activity, as described in "Materials and Methods." Results are given as the mean ± S.D. of a set of triplicate cultures. •, control cultures to which 0.05 ml of Eagle's medium was added; △, cultures to which 0.4 μg of actinomycin D in 0.05 ml of Eagle's medium was added; ○, cultures to which 10 μg of puromycin were similarly added.

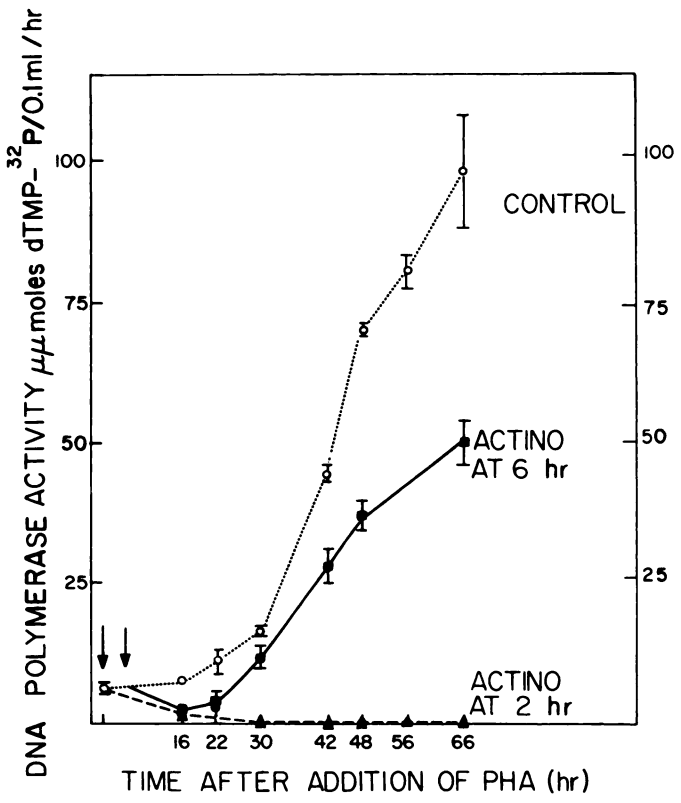

Chart 2. Effect of low doses of actinomycin D (*ACTINO*) on DNA polymerase activity in PHA-stimulated lymphocytes. PHA was added to all cultures in the beginning. Actinomycin D (0.02 μg) was added to each 1-ml culture containing 7.5 X 10^5 cells at times designated by the *arrows*. Cultures were harvested at varying times thereafter. Each *point* represents mean ± S.D. of triplicate cultures.

regression is also very similar (0.0015 mμmole/hr for control

Effect of Actinomycin D on the Kinetics of the Induction of DNA Polymerase in PHA-stimulated Lymphocytes. Actinomycin D (0.02 μg/ml) was added at different times during lymphocyte transformation, and the changes in DNA polymerase activity and thymidine incorporation were determined (Charts 2 and 4 to 6). When actinomycin D was added 2 hr after the addition of PHA, no increase in DNA polymerase activity was observed, even in cultures grown for 66 hr (Chart 2). However, when actinomycin D was added 6 hr after the addition of PHA, there was some increase in DNA polymerase activity. The final activity attained was about 50% of the control cultures (PHA without actinomycin D). These results suggest that sometime between 2 and 6 hr a substantial amount of actinomycin D-sensitive product is synthesized, on which synthesis the subsequent increase in DNA polymerase activity is dependent. The effect of actinomycin D on the rate of thymidine incorporation on the same set of cultures determined simultaneously is shown in Chart 3. Inhibition of polymerase is nearly paralleled by a decrease in thymidine incorporation (compare Chart 2 and Chart 3).

In contrast to the marked sensitivity of the stimulated cells early during lymphocyte transformation, at later times the cells are more resistant. The effect of the addition of actinomycin D at 16, 20, and 24 hr after the addition of PHA on DNA polymerase activity is shown in Chart 4. At these times, actinomycin D has very little effect on the increase in DNA polymerase activity. The maximal activity in cultures with actinomycin D is comparable to that in control cultures, and the rate of increase as calculated from the coefficient of

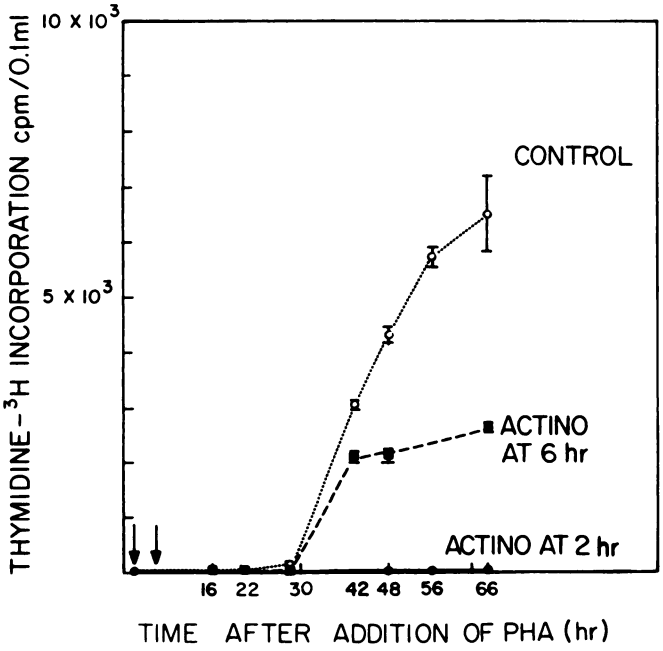

Chart 3. Effect of low doses of actinomycin D (*ACTINO*) on thymidine-^{3}H incorporation in PHA-stimulated lymphocytes. For details, see legend to Chart 2.

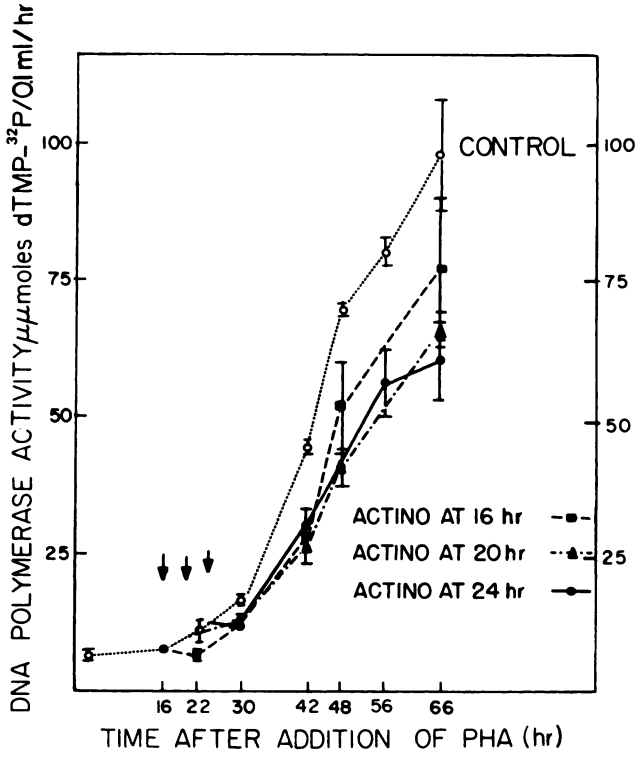

Chart 4. See legend for Chart 2.

Table 1

Effects of different concentrations of actinomycin D during the course of lymphocyte transformation

All cultures were harvested at 44 hr after the addition of PHA. Corresponding values for control cultures without PHA were 15 ± 5 μμmoles of dTMP-^{32}P/hr/ml and 161 ± 28 cpm/hr/ml for DNA polymerase activity and thymidine incorporation, respectively.

In PHA-stimulated cultures without actinomycin D, the values for DNA polymerase activity and thymidine uptake were 177 ± 29 μμmoles of dTMP-^{32}P/hr/ml and 3447 ± 709 cpm/hr/ml, respectively.

Time of addition of actinomycin D (hr after PHA)	DNA polymerase activity (μμmoles of dTMP-^{32}P/hr/ml) at following concentrations of actinomycin D				Rate of thymidine uptake (cpm/hr/ml) at following concentrations of actinomycin D			
	0.01 μg/ml	0.02 μg/ml	0.04 μg/ml	0.08 μg/ml	0.01 μg/ml	0.02 μg/ml	0.04 μg/ml	0.08 μg/ml
0	23 ± 3	21 ± 2			69 ± 20	198 ± 32		
7	30 ± 2	24 ± 4			169 ± 26	208 ± 32		
22	118 ± 18	68 ± 8	49 ± 3		1097 ± 142	1078 ± 120	437 ± 30	
30	114 ± 4	129 ± 15	91 ± 4		3342 ± 303	3069 ± 132	2101 ± 154	
38	173 ± 9	122 ± 15	99 ± 9	108 ± 6	3020 ± 341	2427 ± 303	2362 ± 100	1776 ± 28

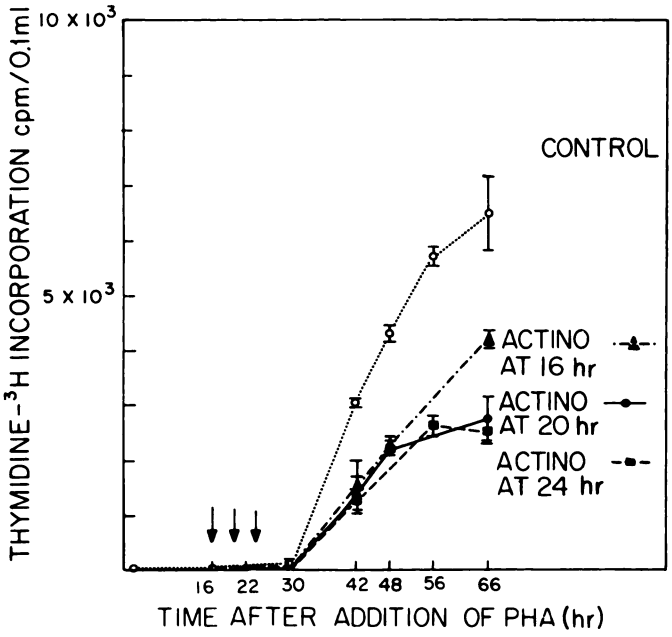

Chart 5. See legend for Chart 3.

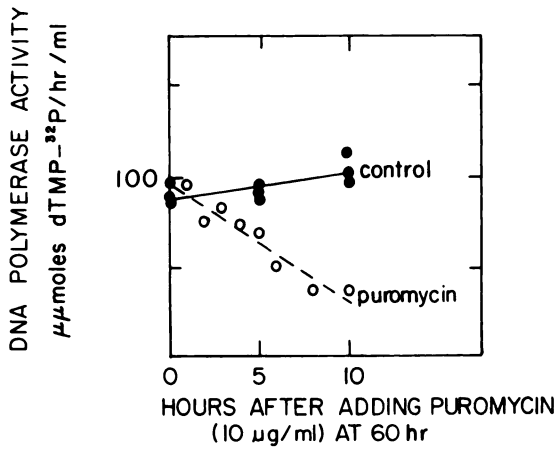

Chart 6. Requirement for protein synthesis. All cultures were stimulated with PHA for 60 hr, and then 0.05 ml of Eagle's medium, alone or with 10 μg of puromycin, was added to each culture. At the indicated times, the cells were harvested and assayed for DNA polymerase activity.

cultures, 0.0015 mμmole/hr for cultures to which actinomycin D was added at 16 hr, 0.0014 mμmole/hr for cultures to which it was added at 20 hr, and 0.0014 mμmole/hr for cultures to which it was added at 24 hr). Corresponding results on the rate of thymidine incorporation are shown in Chart 5. The effect of actinomycin D on RNA synthesis was studied in a separate set of cultures in the above experiment. Uridine incorporation was inhibited 95% when actinomycin D was added at 2 hr and was inhibited 73, 72.8, and 74% when the same concentration was added at 6, 16, and 30 hr, respectively, after the addition of PHA. Part of the labeled uridine could have been incorporated into DNA, but in this group of cultures there was no significant incorporation of labeled thymidine into DNA until a later time, 30 hr after PHA was added to the cultures.

The insensitivity of the lymphocytes at later periods could be due to insufficient amounts of the drug. We measured the effects of the addition of different amounts of actinomycin D at different times during lymphocyte transformation. In Table 1, the stimulated cultures were harvested at 44 hr, and polymerase activity and thymidine incorporation were determined. The pattern is the same: early during lymphocyte transformation, cells are sensitive to actinomycin (0 and 7 hr), and later the cells are more resistant (22, 30, and 38 hr). We have observed some variations in the absolute timing of actinomycin D-sensitive and -resistant periods. This might reflect the lack of synchrony in the stimulated cultures and/or genetic variation in the response of the lymphocytes from different individuals. However, in 6 other experiments with lymphocytes from different donors, we found that the cells were sensitive to actinomycin D in the 1st 20 hr after PHA stimulation and that they then became increasingly refractory to actinomycin D.

Uptake of Actinomycin D-^{3}H. Differences in sensitivity to actinomycin D at different times after PHA stimulation could reflect variations in the permeability of the cells to actinomycin D. We directly measured the rate of uptake of radioactive actinomycin D (5 μCi/culture) by the cells during the course of lymphocyte transformation. The PHA-stimulated cells are permeable to actinomycin D-^{3}H at all times after the addition of PHA. Furthermore, the *rate* of uptake of radioactive actinomycin D increases with time after the

addition of PHA, being 645, 1270, 3909, and 4974 cpm/hr/culture at 4, 20, 42, and 66 hr, respectively.

Requirements for RNA and Protein Synthesis. The above experiments have indicated that a continued increase in DNA polymerase activity does not require continued synthesis of RNA. We therefore studied the stability of the template for DNA polymerase activity. At 60 hr, when cells were engaged in linear growth, separate cultures were treated with either actinomycin D, 0.4 μg/ml, or puromycin, 10 μg/ml. Thereafter, at hourly intervals, the effects of these agents on DNA polymerase activity and thymidine incorporation were determined. In the presence of puromycin, there is complete cessation of any subsequent increase in DNA polymerase activity (Chart 6). The existing enzymatic activity is exponentially degraded in the course of the next 12 hr. This indicates that, even for the maintenance of a particular level of DNA polymerase activity in PHA-stimulated lymphocytes, its continued synthesis is required. Chart 7 shows that the RNA template required for this continued synthesis is very stable. Even when all RNA synthesis was completely blocked by the use of a very high concentration of actinomycin D, there was no decrease in the DNA polymerase activity during the next 12 hr.

As mentioned earlier, there is no measurable increase in DNA polymerase activity during the 1st 20 hr after the addition of PHA to lymphocyte cultures. It is possible that the polymerase is synthesized during this period in an inactive form and is first activated at 20 hr. If so, lymphocytes cultured with PHA for more than 20 hr might have an activator for the polymerase. Table 2 shows the results of the experiment in which graded amounts of homogenates of stimulated lymphocytes (62 hr) were added to homogenates of cells which had been grown with PHA for only 20 hr. There is no evidence for an excess of a substance present in the 62-hr cultures able to activate a putative polymerase which might have been synthesized in the lymphocytes exposed to PHA for only 20 hr.

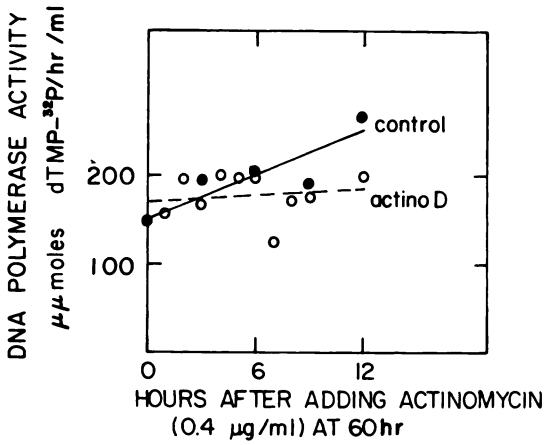

Chart 7. Effect of inhibition of RNA synthesis. The protocol is similar to that in Chart 6 except that, when indicated, the cultures received 0.4 μg of actinomycin D (*actino D*) instead of puromycin.

DISCUSSION

The data presented indicate that the increase in DNA polymerase activity and associated thymidine incorporation during lymphocyte transformation is programmed by an actinomycin D-sensitive product made at a much earlier time. The cells are most sensitive to actinomycin in the 1st 6 hr after the addition of PHA, yet the increase in polymerase activity is not detectable for 16 to 18 hr. When polymerase activity is increasing, the addition of actinomycin to cultures does not inhibit this increase.

Evidence indicates that the inhibition of the induction of DNA polymerase activity by actinomycin D early during the course of PHA stimulation is not mediated by cytotoxicity. Cells grown in the presence of actinomycin D, 0.02 μg/ml, are able to exclude trypan blue. More important, when low doses of actinomycin D block the increase in DNA polymerase activity and DNA synthesis, 60 to 70% of the cells can still undergo morphological transformation into large, blast-like cells. These results are in agreement with the findings of Kay *et al.* (13) and Salzman *et al.* (26) that morphological transformation does not depend upon prior DNA replication.

The principal effect of actinomycin D on macromolecular metabolism is considered to be the inhibition of DNA-dependent RNA synthesis (23). In our studies, this was evidenced by more than 95% inhibition of uridine-^{3}H-incorporation in PHA-stimulated lymphocytes by 0.02 μg of actinomycin D per ml. This concentration of actinomycin could inhibit the synthesis of rRNA (13, 21, 24), preventing the assembly of functioning polyribosomal units

Table 2

Lack of apparent "inactive" enzymes during 1st 20 hr in culture

DNA polymerase activity was assayed in duplicate, as described in "Materials and Methods."

No. of cells per assay (a or b)	DNA polymerase activity (μμmoles dTMP-^{32}P incorporated/hr) PHA exposure for 0 to 20 hr (a)	PHA exposure for 0 to 62 hr (b)	Equal amounts of (a) + (b)
			Experimental
6 × 10^4	<1	6	10
15 × 10^4	3	21	24
30 × 10^4	5	60	50
45 × 10^4	9	100	78

which may be required for the synthesis of DNA polymerase. There is both biochemical and morphological evidence that during PHA stimulation there occurs a marked accumulation of ribosomes in the cell cytoplasm (28). In resting lymphocytes, only 2 to 4% of overall RNA synthesis is directed towards cytoplasmic rRNA synthesis. Following stimulation with PHA, this pattern changes within the 1st few hours. There is a 50- to 100-fold increase in rRNA synthesis by 20 hr after the addition of PHA, and it constitutes up to 20% of the total RNA synthesis (6). Small doses of actinomycin D (0.05 and 0.01 μg/ml) have been shown to inhibit significantly the accumulation of 28 S and 18 S RNA's in the cytoplasm of PHA-stimulated lymphocytes (13). Lack of availability of functional ribosomes could limit protein synthesis. If the effect of actinomycin on the induction of DNA polymerase activity is through the inhibition of the synthesis of ribosomes, sufficient ribosomal precursor RNA's must have been synthesized early after PHA stimulation to allow continued increase in DNA polymerase activity at later times in the presence of actinomycin D. The half-life of rRNA in several systems has been reported to be 5 to 6 days (10), and therefore a continued turnover would not be a restricting problem in a short-term lymphocyte culture of 3 to 4 days.

Equally, the effect of actinomycin D early during PHA stimulation might be to prevent the synthesis of specific informational RNA that programs the synthesis of the polymerase. In fact, the entire spectrum of biochemical events following the addition of PHA to the cultures of lymphocytes is highly suggestive of massive gene activation (3). Although initial attempts to identify unique species of RNA during lymphocyte transformation have not been successful (29), the method used in these studies has obvious limitations (5). New methods of approach appear to be required to prove whether the synthesis of a specific template is necessary for the subsequent increase in DNA polymerase activity.

Since the induction of polymerase is most easily blocked early during PHA stimulation, at a time before increases in polymerase activity are detectable, we speculate that the template for polymerase is made early and translated into active enzyme at a later time. This speculation is based on our inability in simple mixing experiments to detect any enzyme in an "inactive form" during early hours following PHA stimulation. Furthermore, puromycin and cycloheximide, when added at 20 hr following PHA stimulation, can completely prevent any increase in DNA polymerase activity. This indicates that, although most transcription for the polymerase is completed in early hours following PHA stimulation (as determined by sensitivity to actinomycin D), translation into active enzyme does not begin for some time. This could be an example of translational delay. A similar delay was observed by Sussman and Sussman (27), in studies on the slime mold (*Dictyostelium discoideum*), in which UDP-galactose polysaccharide transferase transcription begins as much as 7 hr before the appearance of active enzyme (25) and was observed also by Pegoraro and Baserga (20) in studies on isoproterenol-stimulated salivary glands in which actinomycin D prevents subsequent increase in activities of thymidine and TMP kinases. A more exaggerated delay is implicit in the "masked messenger" concept observed during early development of sea urchin embryos (30) and cotton cotyledons (11). In these systems, the specific mRNA's are presumably formed at a much earlier time and are not able to function until early development.

In the present study, thymidine incorporation and DNA polymerase activity were determined simultaneously on the same sample of cells. Inhibition of the induction of DNA polymerase activity was always accompanied by a corresponding decrease in thymidine incorporation. This was true for both the kinetics and the magnitude of the response. It would seem that the induction of DNA polymerase activity is not only essential but in fact may be a limiting factor for DNA synthesis. If our results can be generalized, the initiation of DNA replication in a population of nondividing cells requires the synthesis of DNA polymerase and perhaps of other enzymatic and structural proteins functioning in DNA replication. For example, polymerase activity is increased in isoproterenol-stimulated mouse salivary glands (2) and after partial hepatectomy in rats (4). In the latter, Guiduice and Novelli (9) found that actinomycin D not only inhibited DNA synthesis but also concomitantly blocked the increase in DNA polymerase activity.

ACKNOWLEDGMENTS

We thank Miss Sylvia S. Bugbee for assistance. We are indebted to Dr. Jack Schultz for counsel and dedicate this paper to his memory.

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