JOURNAL OF VIROLOGY, Aug. 1976, p. 291-295

Vol. 19, No. 1
Printed in U.S.A.

Induction of DNA Polymerase in Polyoma Virus-Infected Mouse Cells Requires Transcription and Translation

ERHARD WINTERSBERGER* AND ULRIKE WINTERSBERGER

Physiologisch-Chemisches Institut der Universität Würzburg, D-87 Würzburg, Federal Republic of Germany, Institut für Krebsforschung der Universität Wien, A-1090 Vienna, Austria, and Département de Biologie Moléculaire, Université de Genève, Geneva, Switzerland*

Received for publication 9 February 1976

The induction of DNA polymerase activity and of DNA synthesis in polyoma virus-infected mouse kidney cells is inhibited by actinomycin D and cycloheximide, even in cells that are already T-antigen positive. This indicates that the induction of the DNA-synthesizing apparatus requires transcription and translation. The increase in cellular DNA polymerase activity and in the DNA synthesizing capacity follows the appearance of T-antigen only after a characteristic lag period of several hours.

Polyoma virus infection of mouse cells leads to an induction of DNA polymerase α, DNA ligase, and of a variety of cellular enzymes involved in the synthesis of DNA precursors (2-4, 9, 10, 14, 19). This process is initiated after the appearance of virus-specific T-antigen (15). In the course of studies on the induction of DNA polymerase α in primary mouse kidney cells (19), we became interested in the question of whether this process involves the synthesis of the enzyme or an activation process independent of translation and transcription. Experiments to answer this question have been carried out earlier with other cellular enzymes that show enhanced levels of activity after infection with polyoma virus or with simian virus 40 (3, 5, 10, 14). In these earlier studies, however, the chronological course of events following the infection was not known in any detail. Now it is established that the induction of cellular enzymes as well as of cellular and viral DNA synthesis requires the prior expression of the early viral function, the synthesis of T-antigen (15). Hence, experiments on the mechanism of induction of cellular enzymes are only conclusive if they are designed such that one can distinguish between effects on T-antigen synthesis and on the induction of the enzymes themselves.

If virus-infected cells are incubated at 27 C rather than at 37 C, the time course of infection is slowed down and successive processes can be studied more easily (15). Synthesis of T-antigen in the majority of cells takes place between 24 and 48 h, whereas induction of DNA synthesis and of cellular enzymes is not initiated before 48 h after infection (15). These two processes are therefore much more precisely separated from each other at 27 C than at 37 C. It is thus possible to study enzyme induction under conditions where production of T-antigen is not effected. By using these conditions we have shown that induction of DNA polymerase activity requires RNA and protein synthesis even when T-antigen is already synthesized and therefore is not simply due to activation of preexisting enzyme molecules.

MATERIALS AND METHODS

Cell culture. Primary mouse kidney cells were grown in plastic petri dishes (100 mm in diameter) as described earlier (18). Cells were infected 1 day after reaching confluence with 0.4 ml of a stock (10^9 PFU/ml) of plaque-purified polyoma virus per dish. After adsorption of the virus for 2 h at 37 C, plates were covered with 10 ml of Eagle medium without serum and incubated in a CO_2 incubator at 27 C for 48 h. At this time the majority of cells was T-antigen positive, but DNA synthesis or cellular enzymes were not yet induced. Such cells were used to study the induction of DNA polymerase. To accelerate this process, cells were shifted to 37 C, which resulted in an increase of DNA polymerase activity and initiation of DNA synthesis within several hours. If the effect of inhibitors was studied, cycloheximide (20 μg/ml for exposures up to 5 h, 10 μg/ml for longer exposures) or actinomycin D (4 μg/ml) was added to the cultures at the times indicated in the individual experiments.

Preparation of cell extracts and determination of DNA polymerase activity. Cells were lysed as described previously (19). In most experiments DNA polymerase activity was determined in a total cell lysate without prior separation of nuclei and cytoplasm. The standard assay (pH 7.4) was used (19). Specific activity is expressed as nanomoles of TMP incorporated per milligram of protein in 30 min at 37 C.

Other methods. The DNA synthetic capacity of

cells was determined by the following protocol: a cover slip (22 by 22 mm) was placed into each of the 100-mm-diamter petri dishes before seeding the cells. When the cells of the respective dishes were used for the determination of DNA polymerase activity the cover slips were removed, washed, and placed in small (35-mm-diameter) petri dishes with 1 ml of medium containing 5 μCi of [^{3}H]thymidine. The cells on the cover slip were labeled for 20 min at 37 C, washed twice with phosphate-buffered saline, and fixed with acetone-methanol (7:3) at −20 C. After drying the cover slips were placed in scintillation vials and covered with toluene-based scintillator, and radioactivity was determined. This measures the total incorporation of thymidine into cells in the cover slip. The same cells could be used for autoradiographic estimation of the percentage of labeled nuclei. For this purpose the cover slips were removed from the scintillation vials, washed with ethanol, dried, and processed for autoradiography according to standard procedures (13, 15).

The protein synthetic capacity of cells was determined similarly except that [^{3}H]methionine was used. Cells, which had been washed after the pulse with phosphate-buffered saline, were treated with 5% trichloroacetic acid for 15 min at room temperature prior to acetone-methanol fixation.

Polyoma T-antigen was determined by immunofluorescence as described previously (13, 15).

RESULTS

If polyoma virus-infected primary mouse kidney cells are incubated at 27 C rather than at 37 C the appearance of T-antigen on the one hand and the induction of cellular and viral DNA synthesis and the increase of activity of cellular enzymes on the other hand are well separated in time (15). About 70% of cells are T-antigen positive 48 h after infection, when synthesis of DNA has not yet started to increase. This moment was therefore considered suitable as the starting point for exploring the role of RNA and protein synthesis in the induction of cellular enzymes involved in DNA replication. To speed up the induction of the DNA-synthesizing apparatus, cells, incubated for 48 h at 27 C, were shifted to the higher temperature (37 C), and DNA polymerase activity and DNA synthesis were measured in intervals during a period of 10 to 12 h. The protocol outlined in the experimental section allowed the determination of all the parameters of interest with the same batch of cells. All measurements were carried out in duplicate, and each experiment was repeated at least once. DNA synthesis measured by incorporation of tritiated thymidine into the cells or by autoradiography was initiated after a lag period of approximately 2 h after the shift to the higher temperature (Fig. 1). DNA polymerase activity was induced after a similar lag period. To see whether the synthesis of cellular and

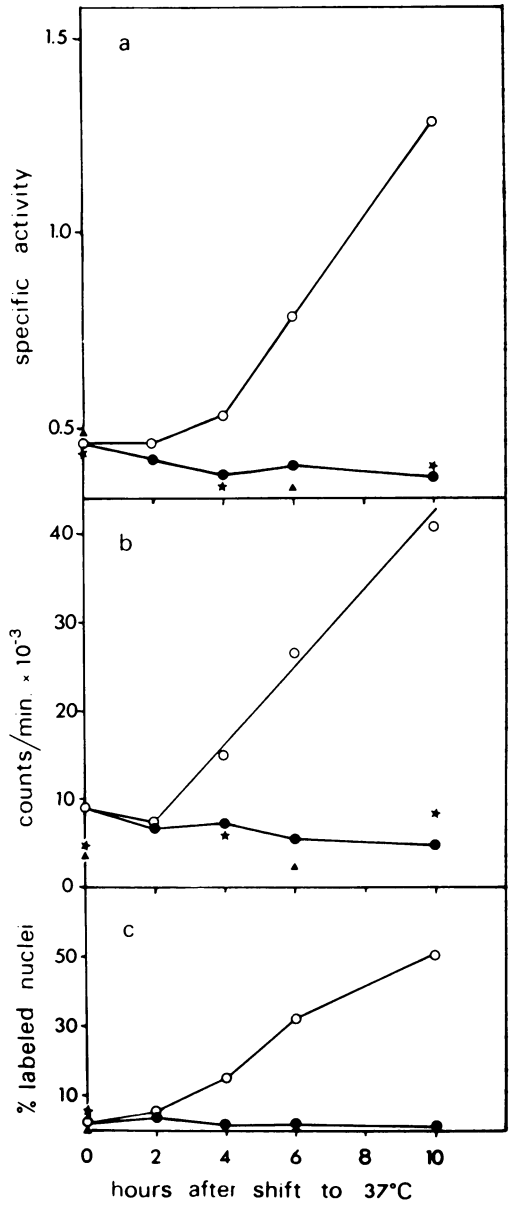

FIG. 1. *Effect of cycloheximide on the increase of DNA polymerase activity and rate of DNA synthesis in polyoma virus-infected mouse kidney cells. Cells, grown and infected as described, were incubated for 48 h at 27 C and then shifted to 37 C (time 0). Determination of DNA polymerase activity (a), estimation of the total incorporation of [^{3}H]thymidine into cells (b), and autoradiography of labeled nuclei (c) were carried out as described. Symbols: ○, infected cells incubated without cycloheximide; ●, cycloheximide (20 μg/ml of medium) added at the time of the shift to 37 C; ▲, cycloheximide (10 μg/ml of medium) added 24 h after infection; ★, mock-infected cells.*

viral DNA followed similar kinetics, cells, pulse labeled for 20 min, were exposed to the selective extraction procedure of Hirt (7), and radioactivity in cellular and viral DNA was measured separately. This experiment revealed a lag period of about 2 h for the initiation of cellular DNA replication. An even longer period of approximately 4 h elapsed before synthesis of viral DNA became measurable (data not shown). The increase of viral DNA in the Hirt supernatant initially followed exponential kinetics. If cycloheximide was added to the cultures at the time of the shift to the higher temperature, neither DNA synthesis nor DNA polymerase activity was induced. The same was true if cycloheximide was added 24 h after infection of the cells. The result of the latter experiment was, of course, expected, as inhibition of T-antigen synthesis, which occurs between 24 and 48 h at 27 C (15), abolishes further effects of virus infection, including the induction of the DNA-synthesizing apparatus. The experiment in which cycloheximide was added to cells that were already T-antigen positive (48 h after infection), however, indicates that protein synthesis is required not only for the synthesis of T-antigen but also for the induction of DNA replication and of DNA polymerase activity. The simplest interpretation would be that DNA polymerase α and other enzymes involved in DNA replication have to be newly synthesized. To analyze this question in more detail, the experiment shown in Fig. 2 was conducted. In this experiment cycloheximide was added to cultures 4 or 6 h after the shift to the higher temperature, when the rise in DNA polymerase activity was already in progress. It was found that cycloheximide effected an immediate stop of any further increase of DNA polymerase activity. This observation substantiates the above conclusion. Although it cannot be completely excluded from these experiments that proteins other than the enzyme itself are being synthesized, which activate DNA polymerase by stoichiometric reaction with preexisting molecules, it seems to us more likely that the increase of DNA polymerase activity is due to synthesis of new enzyme.

Figure 2 also shows the result that was obtained when actinomycin D was added to the culture at the time of the shift to 37 C. Also, actinomycin D completely abolishes the increase of DNA polymerase activity, indicating that, in addition to protein synthesis, transcription is required for the induction process.

Figure 3a shows an experiment in which cycloheximide was present from 24 to 48 h after infection and was removed at the time of the shift to 37 C. Under these conditions no T-antigen can be synthesized up to 48 h, and DNA polymerase activity increases only after a lag period of several hours following removal of the drug. Obviously, during this time T-antigen has to be produced before the induction process can occur. This assumption was tested in a separate experiment (Fig. 3b). Cells grown on cover slips in small petri dishes were cycloheximide treated like those used for the experiment shown in Fig. 3a. After the release from cycloheximide, cells were tested for T-antigen every 2 h. A constant increase of T-antigen-positive nuclei was observed. Only after the percentage of T-antigen-positive nuclei had reached a similarly high value (about 70%) as in cultures without cycloheximide treatment at the beginning of the temperature shift did DNA polymerase activity start to rise. In other words, there is a lag of several hours between the onset of T-antigen synthesis (determined by immunofluorescence) and the induction of DNA polymerase, during which so far unknown processes take place.

In the second experiment depicted in Fig. 3a,

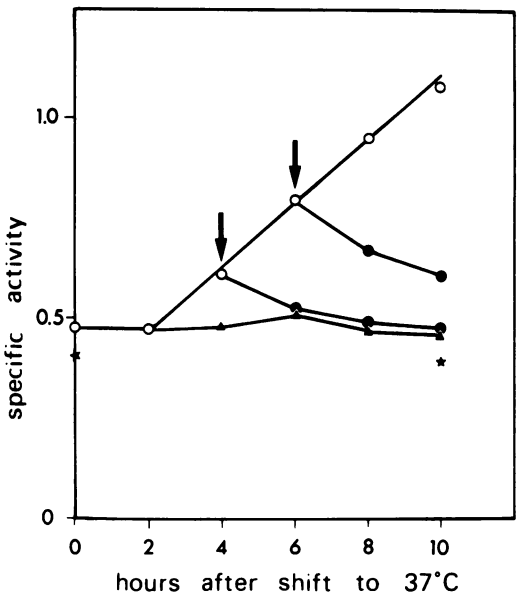

FIG. 2. *Immediate block of the elevation of DNA polymerase activity by the addition of cycloheximide. Polyoma virus-infected mouse kidney cells were incubated at 27 C for 48 h and then shifted to 37 C (time 0). DNA polymerase activity was determined in extracts prepared from cells that had been incubated without or with cycloheximide. Symbols: ○, infected cells incubated without cycloheximide; ●, cultures to which cycloheximide (20 μg/ml of medium) was added at the times indicated by the arrows; ▲, effect of actinomycin D (4 μg/ml of medium) added at the time of the shift to 37 C; ★, mock-infected cells.*

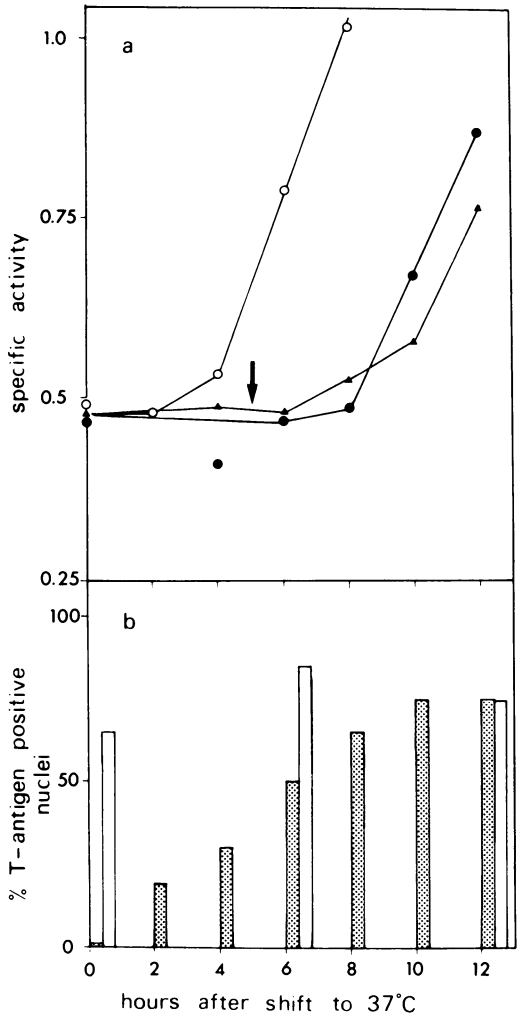

FIG. 3. *DNA polymerase activity (a) and T-antigen-positive nuclei (b) in cells after release from a temporary treatment with cycloheximide. Polyoma virus-infected mouse kidney cells were incubated at 27 C for 48 h and then shifted to 37 C (time 0). (a) DNA polymerase activity in: (○) cells incubated without cycloheximide; (●) cultures to which cycloheximide (10 μg/ml of medium) was added 24 h after infection and removed 48 h after infection at the time of the shift to 37 C; (▲) cultures to which cycloheximide (20 μg/ml of medium) was added 48 h after infection (at the time of the shift to 37 C) and removed 5 h later (indicated by the arrow). (b) Determination of T-antigen by immunofluorescence. Open bars, infected cells incubated without cycloheximide (corresponding to the open circles in [a]); shaded bars, infected cells which received cycloheximide (10 μg/ml of medium) 24 h after infection. The drug was removed 48 h after infection at the time of the shift to 37 C (corresponding to filled circles in [a]).*

cycloheximide was added 48 h after infection, when cells were shifted to the higher temperature. It was left there for 5 h (that is, up to a time at which under our standard conditions DNA polymerase activity is increasing) and then removed from the cultures. Enzyme activity started to rise with a lag period of 2 h after removal of cycloheximide. This lag period is similar to that in the experiment described in Fig. 1, indicating again that some processes that require protein and RNA synthesis have to occur after the synthesis of T-antigen before an increase of DNA polymerase activity and initiation of DNA synthesis can take place. In control experiments we found that recovery of the protein synthetic capacity of cells occurred nearly instantaneously after removal of cycloheximide and therefore cannot be the cause of the observed lag period.

DISCUSSION

Three conclusions can be drawn from the experiments described in this communication. (i) The induction of DNA polymerase activity is most likely due to de novo synthesis of the enzyme. In accord with this conclusion is a recent observation, namely, that the increase of DNA polymerase activity in HeLa cells entering S-phase is also due to enzyme synthesis (1). (ii) The induction of DNA polymerase and the initiation of cellular and viral DNA replication require the prior synthesis of virus-specific T-antigen. (iii) The observation that there is always a lag pariod of several hours between the appearance of T-antigen and the activation of DNA synthesis is most significant. Only about 8 h after T-antigen is demonstrable in approximately 20% of the infected cells, the nearly parallel increase of both DNA polymerase activity and rate of DNA synthesis starts (see Fig. 3). Other studies had indicated that in virus-infected nuclei the initiation of DNA synthesis is preceeded by an activation of transcription, which results in the production of cellular RNA (16). Our results, therefore, are in line with the assumption (15) that T-antigen causes an activation of specific regions in the host genome that are transcribed and translated into protein. These regions of the cellular genome activated by T-antigen could be those that are active in proliferating cells getting ready for the S-phase and which are shutoff in quiescent cells. T-antigen might thus be a substitute for a cellular protein that is active in dividing but inactive in nondividing cells (see also 16). The elevation of the activity of certain cellular enzymes may be a direct or an indirect consequence of this nuclear function of T-anti-

gen. This hypothesis contrasts with the idea (e.g., reference 12) that the activation of cellular and viral DNA synthesis is solely due to a direct action of T-antigen as initiator protein in DNA synthesis. It is well established that inhibition of protein synthesis interferes with DNA replication, particularly with its initiation (4, 6, 8, 11, 17). Our observation (Fig. 1) that cycloheximide inhibits the onset of DNA replication in T-antigen-positive cells indicates a requirement for the synthesis of proteins necessary for this process, which has to occur after T-antigen production. Our results do not of course exclude an additional, direct role of T-antigen on the initiation of DNA synthesis, particularly of viral DNA replication. Further studies on T-antigen-mediated functional modifications of host chromatin and of the transcriptional and translational capacity of infected cells could help to elucidate cellular processes leading to the initiation of chromatin replication.

ACKNOWLEDGMENTS

This work was carried out during a stay in the laboratory of Roger Weil at the Département de Biologie Moléculaire, Université de Genève. We are grateful to Roger Weil and to Hans Türler for many stimulating discussions and helpful suggestions. We also thank Hans Türler for his help with the determination of T-antigen and with the autoradiography and N. Bensemmane for the cell culture.

This work was supported by the Schweizerische Nationalfonds (grant no. 3097.73) and the "Deutsche Forschungsgemeinschaft." U. W. thanks the Austrian Ministery for Science and Research for a grant from the Swiss-Austrian Exchange Programme.

LITERATURE CITED

1. **Chiu, R. W., and E. F. Baril.** 1975. Nuclear DNA polymerases and the HeLa cell cycle. J. Biol. Chem. 250:7951-7957.
2. **Frearson, P. M., S. Kit, and D. R. Dubbs.** 1965. Deoxythymidilate synthetase and deoxythymidine kinase activities of virus-infected animal cells. Cancer Res. 25:737-744.
3. **Frearson, P. M., S. Kit, and D. R. Dubbs.** 1966. Induction of dihydrofolate reductase activity by SV 40 and polyoma virus. Cancer Res. 26:1653-1660.
4. **Hand, R.** 1975. DNA replication in mammalian cells. Altered patterns of initiation during inhibition of protein synthesis. J. Cell Biol. 67:761-773.
5. **Hartwell, L. H., M. Vogt, and R. Dulbecco.** 1965. Induction of cellular DNA synthesis by polyoma. II. Increase in the rate of enzyme synthesis after infection with polyoma virus in mouse embryo cells. Virology 27:262-272.
6. **Highfield, D. P., and W. C. Dewey.** 1972. Inhibition of DNA synthesis in synchronized Chinese hamster cells treated in G-1 or early S phase with cycloheximide or puromycin. Exp. Cell Res. 75:314-320.
7. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365-369.
8. **Hori, T., and K. G. Lark.** 1973. Effect of puromycin on DNA replication in Chinese hamster cells. J. Mol. Biol. 77:391-404.
9. **Kara, J., and R. Weil.** 1967. Specific activation of the DNA synthesizing apparatus in contact-inhibited mouse kidney cells by polyoma virus. Proc. Natl. Acad. Sci. U.S.A. 57:63-70.
10. **Kit, S., D. R. Dubbs, and P. M. Frearson.** 1966. Enzymes of nucleic acid metabolism in cells infected with polyoma virus. Cancer Res. 26:638-646.
11. **Littlefield, J. W., and P. S. Jacobs.** 1965. The relation between DNA and protein synthesis in mouse fibroblasts. Biochim. Biophys. Acta 108:652-658.
12. **Martin, R. G., J. Y. Chou, J. Avila, and R. Saral.** 1974. The semiautonomous replicon: a molecular model for the oncogenicity of SV 40. Cold Spring Harbor Symp. Quant. Biol. 39:17-24.
13. **Peturrson, G., and R. Weil.** 1968. A study on the mechanism of polyoma-induced activation of the cellular DNA-synthesizing apparatus: synchronization by FdU of virus-induced DNA synthesis. Arch. Gesamte Virusforsch. 24:1-29.
14. **Sambrook, J., and A. J. Shatkin.** 1969. Polynucleotide ligase activity in cells infected with simian virus 40, polyoma virus, or vaccina virus. J. Virol. 4:719-726.
15. **Weil, R., and J. Kara.** 1970. Polyoma "tumor antigen": an activator of chromosome replication? Proc. Natl. Acad. Sci. U.S.A. 67:1011-1017.
16. **Weil, R., C. Salomon, E. May, and P. May.** 1974. A simplifying concept in tumor virology: virus-specific "pleiotropic effectors." Cold Spring Harbor Symp. Quant. Biol. 39:381-395.
17. **Weiss, B. G.** 1969. The dependence of DNA synthesis upon protein synthesis in HeLa cells. J. Cell Physiol. 73:85-90.
18. **Winocour, E.** 1963. Purification of polyoma virus. Virology 19:158-168.
19. **Wintersberger, U., and E. Wintersberger.** 1975. DNA polymerases in polyoma virus-infected mouse kidney cells. J. Virol. 16:1095-1100.