Vol. 118, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 13, 1984 Pages 310-316

DNA-MEDIATED GENE TRANSFER IS MORE EFFICIENT DURING S-PHASE OF THE CELL CYCLE

Elena Giulotto[1] and Nicole Israel[2]

Department of Genetics, Stanford University School of Medicine, Stanford, California 94305, U.S.A.

Received October 23, 1983

We transformed synchronized mouse L-thymidine kinase deficient cells with a cloned thymidine kinase gene and carrier DNA at different times in the cell cycle. The frequency of resistant colonies was determined after growth in hypoxanthine-aminopterin-thymidine medium for two weeks (late expression) and the frequency of thymidine kinase positive cells was determined 72 hours after transformation (early expression) by incorporation of ^{3}H-thymidine and autoradiography. The frequency of late expression was several-fold higher when cells were exposed to DNA during S-phase, whereas the frequency of early expression did not depend on the phase of the cell cycle.

The frequency of tk^+ colonies in HAT medium after transformation of mouse Ltk cells with the tk gene of herpes simplex virus reaches a plateau at approximately 10^{-4} with increasing DNA concentrations (1). The ability to integrate and express foreign DNA is not a stably heritable trait of a few cells in the population, but it seems to be a transient property of all the cells (2). Four stages can be identified in the transformation process. First, the Ca-phosphate-DNA precipitate enters the cytoplasm. Loyter et al. (3) showed that all mouse Ltk recipient cells can take up DNA initially, but only 1-5% of the cells show the presence of DNA complexes in the nucleus. Thus, the entry of the donor DNA complex from the cytoplasm to the nucleus is a discrete second step and probably limits the overall process.

[1]Present address - Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, England.

[2]Present address - Groupement de Genie Genetique, Institut Pasteur, Rue du Dr. Roux, 75015, Paris, France.

ABBREVIATIONS: tk - thymidine kinase; HAT - hypoxanthine-aminopterin-thymidine; HSV - herpes simplex virus.

0006-291X/84 $1.50

Furthermore, Linsley and Siminovitch (4) showed that the HSV tk gene, added exogenously as Ca-precipitate, is expressed at early times at a frequency 10- to 100-fold higher than the frequency of HAT-resistant colonies measured two weeks later. It is not yet clear whether all the cells that transfer DNA into the nucleus are able to express the heterologous genes. When HSV Tk DNA is injected directly into the nucleus, 50 to 100% of the cells express the tk activity, whereas only 1 out of 500 to 1,000 cells gives rise to a colony in HAT medium (5). From transformation experiments performed in different cell lines and under different conditions, Shen et al. (6) also concludes that the barrier to the early expression of the exogenous tk gene is the nuclear membrane. We can thus surmise two more stages in the process: expression of the gene, probably occurring with a high frequency after its entry into the nucleus, and conversion of the donor DNA into larger molecules which might replicate and eventually integrate into the recipient genome (7).

Since only a small proportion of cells undergo stable transformation and since prolonged incubation with the DNA precipitate is needed to achieve maximum transformation frequency (8), we wondered whether these limitations were due to the dependence of some of the steps in the transformation process on a particular phase of the cell cycle.

MATERIALS AND METHODS

Cell culture. The recipient cells, from the murine cell line Ltk^- (kindly given by Dr. Richard Axel), were maintained in DMEM, supplemented with 10% newborn calf serum (Flow Laboratories) and 30µg/ml BUdR.

Cell synchronization by treatment with colcemid. 3×10^6 cells/100 mm dish were plated in medium without BUdR. After 24 hours Colcemid (demecolcine, Sigma) was added at a final concentration of 0.02 µg/ml. After 13 hours the cells were incubated for 4 hours in the presence of DNA precipitate and colcemid, then the mitotic cells were selectively detached (9) and plated to determine the frequency of HAT-resistant colonies and the plating efficiency in non-selective medium (see below). The mitotic index in this sample was between 87% and 92% in different experiments. Cells in G_2 and S phase: after detachment of cells in mitosis, the residual monolayer contains cells mostly in S and G_2 pnases (10). The cell sample

enriched in S and G_2 phases were detached with trypsin and plated for HAT selection and determination of plating efficiency. Cells in G_1 phase: from parallel plates, treated with colcemid for 13 hours and not exposed to DNA, mitotic cells were also detached and plated in the absence of colcemid to allow progression through the cell cycle. After 6 hours of incubation at 37°C, when the cells were attached to the plate and had hence reached G_1, the DNA precipitate was added for 4 hours. The cells were then detached with trypsin and plated for HAT selection and determination of plating efficiency. Asynchronous control cells were exposed to the DNA precipitate, detached and plated.

Cell synchronization by serum starvation. Cells were plated at a density of 2.5×10^5/60 mm dish. After 24 hours the cells were incubated in medium without serum for 96 hours. After this period of starvation, cells were refed with medium containing serum. Transformations were performed at various times, together with tests to check synchronization and to determine the phase of the cell cycle.

DNA content. Cells were trypsinized and the pellets resuspended in 70% ethanol, then stained with the DNA-specific dye chromomycin A3.(11). Fractions of cells in the G_1, S and G_2M phases of the cell cycle were estimated as described by Dean and Jett (12) using a Flow cytometer for DNA distribution analysis.

Transformation. The purified HSV thymidine kinase gene is contained in the recombinant plasmid pB12 (13). Transformation was performed using the calcium-phosphate method described by Graham and Van der Eb (1), with 0.7 µg of pB12 and 20 µg of salmon sperm carrier DNA per plate. After 3 hours, the DNA precipitate was removed, the cells were washed with PBS, treated with trypsin and replated in three sets of plates to determine the frequency of HAT-resistant cells, the percentage of viable cells and the frequency of early expression of the gene. All experiments were performed in duplicate.

Cloning efficiency and frequency of HAT-resistant colonies. 500 cells previously exposed to DNA were plated in 60 mm dishes. Colonies were scored after one week to test the cloning efficiency. 10^6 cells previously exposed to DNA were plated in 100 mm dishes; 20 hours later this medium was replaced by HAT medium (15 µg/ml hypoxanthine, 0.2 µg/ml aminopterin, 5.0 µg/ml thymidine and 0.22 µg/ml glycine). HAT-resistant clones were scored after 2 weeks. The number of transformants was corrected for cell viability.

Early expression of the thymidine kinase gene. 2×10^5 cells previously exposed to DNA were plated on coverslips by medium containing 2 µCi/ml of (^{3}H) thymidine (Amersham, 76.6 Ci/mmole) for 24 hours. The coverslips were then washed, fixed with methanol-acetic acid (3:1 vol/vol) and stained with 1% acetic orcein. The coverslips were then mounted on microscope slides, dipped in Kodak NTB2 emulsion. After exposure for 3-10 days at 4°C, the photographic emulsion was developed (Kodak LX24) and fixed (Kodak AL4). Clusters of cells were counted instead of individual cells because cells exposed to DNA at different phases of the cell cycle had different probabilities to divide once or twice during the 48 hours before labelling. Another reason is to avoid counting cells labelled because of possible "metabolic cooperation" between cells (14).

RESULTS AND DISCUSSION

Table I shows the results of a transformation experiment performed on cells synchronized by treatment with colcemid. The transformation

TABLE 1. Late expression in cells synchronized with colcemid.

Phases represented in cell population	No. colonies in HAT per 10^6 cells	No. colonies in HAT per 10^6 cells/No. colonies in normal medium per 10^6 cells[a]
	(Mean ± SD)	(Mean ± SD)
G_1, G_2, S, M (asynchronous)	88.5 ± 1.5	20.0 ± 0.7
G_2, S	130.3 ± 5.8	49.7 ± 3.1
M	2.3 ± 1.3	5.9 ± 3.4
G_1	2.5 ± 0.5	7.7 ± 2.5

[a] After incubation of cells with DNA, the cells were detached and replated in HAT medium (10^6 cells/plate) and in normal medium (10^3 cells/plate). The number of colonies in HAT was scored after 2 weeks and corrected by the number of colonies in non-selective medium (cloning efficiency).

efficiency of the cell sample enriched in G_2 and S is significantly higher than that of the other samples, including the asynchronous population.

Serum starvation (15) was then used as synchronization regimen since it does not require the use of drugs like colcemid that could have toxic effects on cellular processes. Mouse Ltk^- cells synchronized with this method maintain the same cloning efficiency as asynchronous cells. We determined at different times of the cell cycle, the frequency of HAT resistant colonies (late expression) and the frequency of cells expressing the tk gene at early times (early expression). (Fig. I and Table II). Parallel cell samples, which had been put through the same synchronization regimen, were analyzed for DNA content by flow cytometry at the indicated times following serum addition. From these data, the proportion of cells in S phase was calculated for each sample. In different experiments the maximum proportion of cells in S phase was obtained at different times. For example, in the experiment presented in Fig. I, the maximum percentage of cells in S phase was between 15 and 18 hours, while it was between 20 and 22 hours in other experiments. In spite of the observed variability in the cell cycle progression, in all the experiments the efficiency of late expression

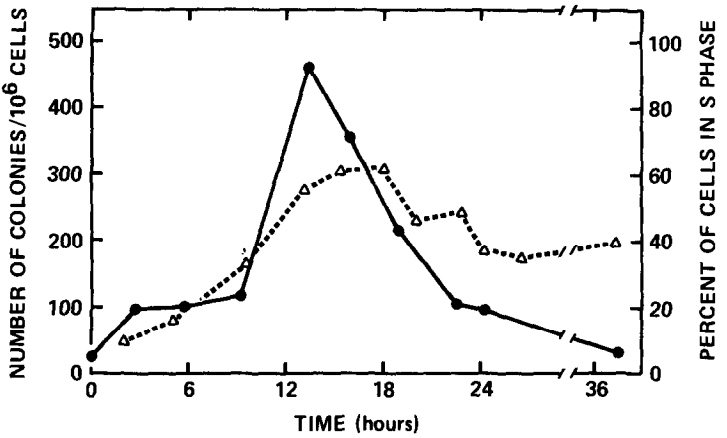

FIGURE 1. Late expression in cells synchronized by serum starvation. Mouse Ltk cells were incubated for 96 hours in medium without serum and refed with serum (time zero). At the indicated times the DNA precipitate was added for 3 hours. The precipitate was removed and the cells were replated to determine the number of colonies in HAT medium (●——●). A parallel set of samples, synchronized by the same protocol but not exposed to DNA, were analyzed for DNA content by fluorocytometry, and the percentage of cells in S phase was determined (▲--------▲).

showed a clear maximum, correlated with the maximum number of cells in S phase. Thus, these experiments confirm the observations reported in Table I and point to the S phase rather than G_2 as the phase during which a critical step of the transformation process occurs.

TABLE 2. Early expression in cells synchronized by serum starvation.

Hours after re-feeding with serum[a]	No. clusters of labelled cells per 10^3 cells		No. colonies in HAT medium per 10^6 cells	% cells in S phase[c]
	3 day exp.	10 day exp.[b]		
0	3	2	134	ND
3	2	10	ND	ND
6	5	4	ND	ND
9	4	4	203	14
16	8	5	ND	ND
20	7	5	325	53
23	7	3	513	ND
25	4	3	248	32

[a] After synchronization by serum starvation, the cells were refed with serum (time zero). At different times after time zero, transformation was performed.

[b] Two sets of samples were exposed to the photographic emulsion for 3 and 10 days; 2,000 cells per sample were scored.

[c] The percentage of cells in S phase was calculated in independent samples not exposed to DNA precipitate.

ND - not determined

The sharpness of the transformation peak with respect to relative broadness of the curve expressing the percentage of cells in S phase (see Fig. I) focuses toward a specific fraction of the S phase as being more crucial in this context. In particular, the data indicated early S phase (or the G_1-S border) as the period of highest 'competence' for transformation (if not the only period of competence, given the lack of perfect synchrony and the fact that the cells were exposed to DNA for 3 hours). Furthermore, as data of Table II show, the early expression of HSV tk gene does not follow the same kinetics as that of the late expression. The frequency of early expression, which is between 10 to 100 times higher than the frequency of late expression, is not related to the cell cycle. In all probability, the late expression occurs in a small proportion of the cells expressing the gene at early times and the early expression is not dependent on the integration of DNA into high molecular weight structures but only on its entry in the nucleus (5,6) . Hence, the factor(s) limiting the transport of the DNA precipitate from the cytoplasm to the nucleus may not be related to the cell cycle.

While the entry of DNA into the nucleus, which occurs soon after its incubation with the cells (3), is probably sufficient for its expression at early times, the incoming DNA may have to be processed to be maintained and expressed in a more stable way. Our data indicate that at least some of the steps in this processing occur preferentially or only in the early S phase. Nevertheless, the S phase dependent factor(s) may not be sufficient to assure the late expression of the genes since only a fraction of the cells expressing the gene at early times can originate colonies resistant to HAT medium even when they have been exposed to DNA during the most appropriate phase.

It is known that the activity of DNA polymerase -α is induced during S phase in synchronized HeLa cells (16, 17) and in human lymphocytes during proliferation along with the activity of other

enzymes involved in DNA metabolism (18). The ability to perform unscheduled DNA synthesis is also higher during S phase in HeLa cells (10). At the beginning of S phase, enzymatic activities necessary for DNA metabolism become available in the cell to accomplish DNA replication and more efficient DNA repair; it seems likely that some of these enzymes would be necessary for processing foreign DNA and integrate it in high molecular weight structures.

ACKNOWLEDGEMENTS

We wish to thank Dr. A.T. Ganesan, John Yoder and Alain Israel for discussions and Joe Gray of Lawrence Livermore National Laboratory for performing the flow cytometry experiments and his critical comments. Elena Giulotto was the recipient of a long-term fellowship from the European Molecular Biology Organization. Nicole Israel was supported by the French Ministère des Affaires Etrangères, the Phillipe Foundation and the Fondation pur la Recherche Medicale Francaise. This research was supported by the grant from the National Institutes of Health GM 14108 to Dr. A.T. Ganesan. The technical help of JoAnn Katheiser is gratefully acknowledged.

REFERENCES

1. Graham, F.L., and van der Eb, A.J. (1973) Virology 52, 456-467.
2. Wigler, M., Sweet, R., Keesim, G., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S., and Axel, R. (1979) Cell 16, 777-785.
3. Loyter, A., Scangos, G.A., and Ruddle, F.H. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 422-426.
4. Linsley, P.S., and Siminovitch, L. (1982) Mol. Cell. Biol. 2, 593-597.
5. Capecchi, M.R. (1980) Cell 22, 479-488.
6. Shen, Y., Hirschhorn, R.R., Mercer, W.E., Surmacz, E., Tsutsui, Y., Soprano, K.J., and Baserga, R. (1982) Mol. Cell. Biol. 2, 1145-1154.
7. Pellicer, A., Wigler, M., Axel, R., and Silverstein, S. (1978) Cell 14, 133-141.
8. Graham, F.L., Bacchetti, S., and McKinnon, R. (1980) Transformation of mammalian cells, p. 3-25. In R. Baserga, C. Croce, and G. Rovera (ed.), the calcium technique. Alan R. Liss, Inc., New York.
9. Terasima, T., and Tolmach, L.J. (1963) Exp. Cell. Res. 30, 344-351.
10. Giulotto, E., Mottura, A., DeCarli, L., and Nuzzo, F. (1978) Exp. Cell Res. 113, 415-420.
11. Gray, J W , and Coffino, P. (1979) Cell cycle analysis by flow cytometry, p. 233-248. In W. Jacoby and I. Pastan (ed.), Methods in Enzymology, vol. 58. Academic Press, Inc., New York.
12. Dean, P.N., and Jett, J.T. (1974) J. Cell Biol. 40, 523-527.
13. Enquist, L.W., Wold, G.F., Wagner, M., Smiley, J.R. and Summers, W.C. (1979) Gene 7, 335-342.
14. Gilula, N.B., Reeves, O.R., and Steinbach, A. (1972) Nature 235, 262-265.
15. Scheffler, I.E., and Buttin, G. (1973) J. Cell. Physiol. 81, 199-216.
16. Spadari, S., and Weissbach, A.J. (1974) J. Mol. Biol. 86, 11-14.
17. Chiu, R.W., amd Baril, E.F. (1975) J. Biol. Chem. 250, 7951-7954.
18. Bertazzoni, U., Stefanini, M., Pedrali-Noy, G., Giulotto, E., Nuzzo, F., Falaschi, A., and Spadari, S. (1976) Proc. Natl. Acad. Sci., U.S.A. 73, 785-789.