JOURNAL OF VIROLOGY, Sept. 1977, p. 455-460
Vol. 23, No. 3

Printed in U.S.A.

Cell-Free Synthesis of Herpes Simplex Virus-Coded Pyrimidine Deoxyribonucleoside Kinase Enzyme

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Received for publication 9 March 1977

The incubation of a cell-free protein-synthesizing system prepared from rabbit reticulocytes with cytoplasmic RNA from herpes simplex virus (HSV)-infected cells resulted in increased thymidine kinase activity. This enzyme activity was specifically inhibited by anti-HSV antiserum and was relatively unaffected by TTP, an inhibitor of cellular thymidine kinases. Induction of the new activity was prevented by addition of inhibitors of eucaryotic protein synthesis, and no new activity was detected when RNA from cells infected with pyrimidine deoxyribonucleoside kinase-deficient mutants, instead of wild-type HSV, was added. An increased deoxycytidine kinase activity with similar properties to the HSV-specified enzyme activity was also present in cell-free systems incubated with RNA from HSV-infected cells. Phosphorylation of thymidine and deoxycytidine at 30°C continued for longer than 11 h. The findings are consistent with the accurate synthesis in vitro of enzymically active HSV-specified pyrimidine deoxyribonucleoside kinase.

Translation of mRNA in bacterial cell-free systems to active enzymes has been achieved with bacterial mRNA (25) and bacteriophage mRNA (6, 18, 22, 23). Such experiments have proved very useful in investigations of the regulation of mRNA synthesis and inactivation, since a single product of known identity can be studied. The synthesis of active bacteriophage enzymes in eucaryotic systems has also been demonstrated (1, 2).

Attempts to study specific mRNA's from eucaryotic organisms and their viruses have generally relied on identification of the in vitro-synthesized product by immunoprecipitation (3, 4, 10, 15, 20, 24), but this approach can be most usefully applied only if the protein of interest can be obtained in a purified form. Analysis of the biological activity of in vitro-synthesized polypeptides overcomes this restriction, but at present the only eucaryotic system of this nature with potential for quantitative work is the cell-free synthesis of interferon (21).

We have previously described the synthesis of herpesvirus-induced polypeptides in a cell-free system derived from reticulocytes (16). Since many of the products appeared to represent authentic virus-induced polypeptides, it seemed worthwhile to investigate the possibility that herpesvirus-specified enzymes were also made. Pyrimidine deoxyribonucleoside kinase (dPyK), an enzyme that phosphorylates many deoxypyrimidine nucleosides including thymidine and deoxycytidine (5), was chosen for three reasons. First, it has biochemical properties that are well defined and that differ from those of cellular enzymes in many respects (9, 11, 12). Second, mutants that lack detectable dPyK activity are available and provide useful controls (8). Third, the molecular weight of the enzyme is reported to be 44,000 (7), a size that is translated efficiently by the cell-free system.

MATERIALS AND METHODS

Isotopes. [*Methyl*-^{3}H]thymidine (50 Ci/mmol) and deoxy[5-^{3}H]cytidine (24 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England.

Cells and virus stocks. The cells used were BHK C13 cells (14). The virus was herpes simplex virus (HSV) type 1, strain 17. Two mutants of the same strain lacking detectable pyrimidine deoxyribonucleoside kinase activity, $dPyK_1^{-1}$ and $dPyK_1^{-7}$, were supplied by A. T. Jamieson. Anti-HSV antiserum (against HSV-infected rabbit kidney cells) and preimmune rabbit serum were gifts from J. Hay.

Preparation of cell extracts. Extracts from uninfected cells or cells infected with HSV-1 (20 PFU/cell) at 7 h postinfection were prepared as described by Jamieson and Subak-Sharpe (9).

Extraction of RNA. BHK cell monolayers were infected with HSV-1 or mutant viruses at 20 PFU/cell and harvested at 5 h after infection at 31°C. Total cytoplasmic RNA was extracted as described previously (16).

Cell-free protein synthesis. The conditions for cell-free protein synthesis (16) were modified slightly. Assay mixtures (20 μl) contained the following: 50 mM *N*-2-hydroxyethyl piperazine-*N'*-2-

ethanesulfonic acid buffer, pH 7.6; 0.5 mM dithiothreitol; 0.5 mM spermidine; 0.15 mM magnesium acetate; 0.6 mM CTP; 1 mM ATP; 0.1 mM GTP; 4 mg of creatine phosphate per ml; 0.2 mg of creatine phosphokinase per ml; 0.05 mM of each of the 20 common amino acids; 2 μl of a rabbit reticulocyte pH 5 fraction; 7 μl of rabbit reticulocyte S100 fraction; and 1.2 μl of a BHK cell ribosomal salt-wash fraction.

Cytoplasmic RNA from uninfected or infected cells, or *Escherichia coli* rRNA, was added to give a final concentration of 200 μg per ml.

Incubation was continued for 90 min at 30°C, after which thymidine kinase or deoxycytidine kinase activity was assayed.

Thymidine kinase assays. Thymidine kinase was measured by a modification of previously described methods (9). Assay mixtures (80 μl) contained the following components: 100 mM sodium phosphate buffer, pH 6.0; 10 mM ATP; 10 mM magnesium acetate; and 100 μCi of [^{3}H]thymidine per ml. TTP, where present, was added at 0.05 mM.

For the analysis of thymidine kinase synthesis in vitro, a concentrated solution of these ingredients was added to the cell-free system.

The activity of extracts of uninfected or infected cells was measured under the same conditions, but the amount of extract added was adjusted to give an activity approximately equal to that of the cell-free systems.

Reticulocyte thymidine kinase was measured by the addition of 20 μl of reticulocyte S100 to the assay mixture.

Incubation of the assay mixtures was at 30°C for 5 h, after which 20 μl of 1 mM thymidine was added. The mixtures were then heated at 100°C for 2 min and centrifuged at 2,000 × *g* for 10 min, and a 60-μl sample of the supernatant was spotted onto a Whatman DE81 paper disk. The disks were washed three times at 37°C in 4 mM ammonium formate containing 10 μM thymidine, once in water, and twice in ethanol and dried before estimating the retained radioactivity by scintillation counting.

Thin-layer chromatography on PEI-cellulose sheets confirmed that the reaction products were thymidine nucleotides.

Antisera, where present, were diluted in phosphate-buffered saline and constituted 25% of the total reaction mixture.

Deoxycytidine kinase assays. Assay conditions for deoxycytidine kinase were the same as for thymidine kinase, except that sodium phosphate buffer was present at a concentration of 50 mM (pH 7.0) and 200 μCi of [^{3}H]deoxycytidine per ml was added instead of [^{3}H]thymidine.

Protein estimations. Protein was estimated by the method of Lowry et al. (13).

RESULTS

Thymidine kinase activity of the fractionated reticulocyte cell-free system. Investigation by the method of Jamieson and Subak-Sharpe (9) of thymidine kinase activity in the fractionated reticulocyte cell-free system revealed no detectable phosphorylation of thymidine. Furthermore, no increase in thymidine kinase could be observed after incubation with cytoplasmic RNA from HSV-infected cells, even though it has been shown that HSV-induced polypeptides can be synthesized in the cell-free system (16). Increasing the sensitivity of the thymidine kinase assay, however, has allowed detection of the in vitro synthesis of the virus-specified enzyme. The important changes are the use of high-specific-activity [^{3}H]thymidine and incubation at 30°C for extended reaction times.

Under these modified conditions, the activity of the cell-free system components could be detected (Table 1), although the value for the reticulocyte S100 fraction was about 2,000 times lower than that found in BHK cells. It should be noted that, owing to the lower concentration of thymidine present, the activities of infected and uninfected BHK cells are lower than previously reported (9). Incubation of the cell-free system with cytoplasmic RNA from HSV-infected cells results in a two- to threefold increase in thymidine kinase activity, whereas no such effect was observed in the presence of cytoplasmic RNA from uninfected cells. This result suggests that in vitro synthesis of the HSV-specified enzyme occurs, and the remainder of this communication concerns the characterization of this induced activity.

Effects of TTP on thymidine kinases. It has been shown previously that cellular thymidine kinase is inhibited by TTP at concentrations as low as 50 μM, although the HSV-induced enzyme is relatively unaffected by this compound (9, 12). Figure 1 shows that the activity of the

TABLE 1. *Thymidine kinase activities of cell-free system components*

Component[a]	Thymidine kinase activity[b] (pmol phosphorylated/h per mg of protein)
Reticulocyte S100	6.2
Reticulocyte pH 5 fraction	9.1
BHK ribosomal salt-wash fraction	126
Total cell-free system	8.4
After incubation with BHK cell RNA	7.6
After incubation with infected-cell RNA	23.2
BHK cell extract	4,600
Infected BHK cell extract	60,000

[a] Thymidine kinase assays were performed on the complete cell-free translation system or on the individual components.

[b] Results are the means of at least three determinations.

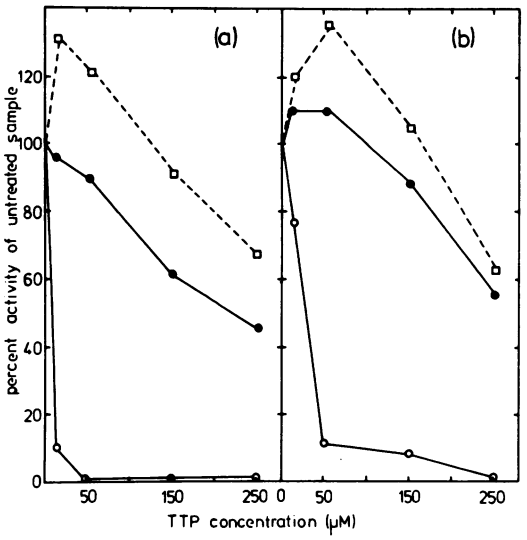

FIG. 1. *Effects of TTP on thymidine kinase activities. (a) Activity in cell-free systems after incubation with cytoplasmic RNA from infected cells (●) or no RNA (○) was assayed. (b) Activity of extracts from infected (●) or uninfected (○) BHK cells was measured. The activity due to the HSV-induced enzyme (□) was calculated by subtraction of the background activity of the cell-free system (a) or BHK cells (b). Note that this calculation depends on the assumption that the activities of reticulocyte and BHK cell enzymes are unaffected by the presence of virus-induced thymidine kinase.*

cell-free system was totally inhibited by the addition of 50 μM TTP, whereas after incubation with cytoplasmic RNA from infected cells, an activity that was very much less affected by TTP was present. Comparison with the behavior of extracts from infected and uninfected BHK cells shows that the response of the enzyme produced in vitro was very similar to that of the virus-specified enzyme induced in vivo. This strongly suggests that in vitro synthesis of active HSV-specified thymidine kinase occurs in the cell-free system, and furthermore demonstrates that the addition of 50 μM TTP allows the specific assay of this enzyme.

Induction of thymidine kinase in vitro requires translation of mRNA. To establish whether induction of TTP-resistant thymidine kinase was due to protein synthesis, the effects of two inhibitors were tested (Table 2). The inclusion of cycloheximide or aurin tricarboxylic acid in the cell-free system before incubation with infected-cell RNA completely abolished production of the enzyme. The possibility that this effect was due to a direct action of the compounds on the enzyme was tested by adding them after in vitro protein synthesis. Cycloheximide then had no effect on the thymidine kinase activity, and although aurin tricarboxylic acid gave a reduction of about 40%, it was clear that the major mode of action of these compounds was through inhibition of protein synthesis.

The appearance of TTP-resistant thymidine kinase was also prevented by incubation of the protein-synthesizing system at 4°C instead of at 30°C.

Further evidence that the in vitro-synthesized enzyme was the HSV-specified thymidine kinase was obtained by comparing various cytoplasmic RNA preparations. RNA from cells infected with dPyK-deficient mutants or from uninfected cells failed to stimulate thymidine kinase synthesis (Table 3), even though many polypeptides were synthesized (16).

These results demonstrate that translation of added RNA was necessary for the production of TTP-resistant thymidine kinase and exclude the possibility that addition of RNA altered the properties of reticulocyte thymidine kinase or stimulated the synthesis of a reticulocyte or BHK cell enzyme.

Immunological characterization of in vitro-synthesized thymidine kinase. The effects of anti-HSV antiserum and pre-immune rabbit serum on thymidine kinase activity are shown

TABLE 2. *Requirements for in vitro synthesis of thymidine kinase*

Treatment to cell-free system[a]	Thymidine kinase activity (cpm × 10^{-3})
No additions	33.8
Cycloheximide (200 μg/ml) added at:	
0 min	0.8
90 min	35.6
Aurin tricarboxylic acid (200 μM) added at:	
0 min	0.1
90 min	19.7
Incubation at 4°C instead of 30°C	3.4

[a] Cell-free systems contained cytoplasmic RNA from infected cells.

TABLE 3. *In vitro synthesis of thymidine kinase directed by various RNA preparations*

Source of RNA	Thymidine kinase activity[a] (cpm × 10^{-3})
HSV-infected cells	34.2
HSV ($dPyK_1^{-1}$)-infected cells	1.2
HSV($dPyK_1^{-7}$)-infected cells	1.0
Uninfected BHK cells	1.4
E. coli rRNA	1.2
No added RNA	1.4

[a] TTP, 50 μM, was present in all incubations.

in Fig. 2. The anti-HSV antiserum totally inhibited the HSV-specified enzyme from infected cells and the in vitro-synthesized thymidine kinase, but had little effect on reticulocyte or BHK cell activities. Separate experiments (not shown) established that the enzyme from infected cells was inhibited to the same extent when added to reaction mixtures containing no added RNA. Pre-immune rabbit serum did not significantly inhibit any of the activities tested. The enzyme synthesized in vitro was, therefore, indistinguishable from HSV-specified thymidine kinase by this criterion.

Time course of thymidine phosphorylation. The sensitivity of the thymidine kinase assay has been considerably increased by the use of an extended period of incubation. A time of 5 h was used in these studies, since this gave adequate detection of in vitro-synthesized thymidine kinase. An examination of the reaction in the presence of 50 μM TTP (Fig. 3) shows that formation of thymidine nucleotides proceeds linearly with time for at least 11 h, and therefore even greater sensitivity of detection of thymidine kinase is possible. A similar time course has been found for the reaction with enzyme synthesized in infected cells (19).

Synthesis of deoxycytidine kinase in vitro. The deoxycytidine kinase activity of cell-free protein-synthesizing systems is shown in Fig. 4. Two main findings emerge. First, the cell-free system incubated with cytoplasmic RNA from HSV-infected cells showed a twofold increase in

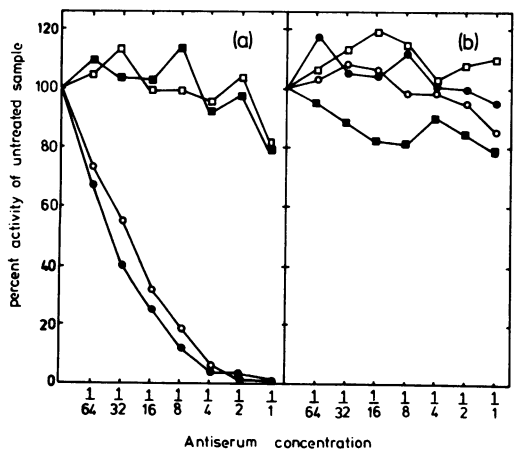

FIG. 2. *Effect of antisera on thymidine kinase activities. Anti-HSV antiserum (a) or pre-immune rabbit serum (b) was added to thymidine kinase assays. Symbols: (●) in vitro-synthesized HSV enzyme (assayed with added TTP); (■) reticulocyte enzyme (assayed without TTP); (○) infected-cell virus-specified enzyme (assayed with added TTP); (□) BHK cell enzyme (assayed without TTP).*

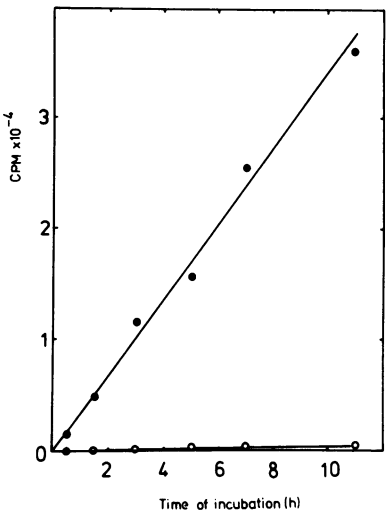

FIG. 3. *Time course of thymidine phosphorylation. Assay mixtures contained 50 μM TTP and samples from cell-free systems after incubation with HSV-infected-cell RNA (●) or no added RNA (○). Addition of cytoplasmic RNA from cells infected with HSV ($dPyK_1^{-7}$) or uninfected cells gave results undistinguishable from those found with no added RNA.*

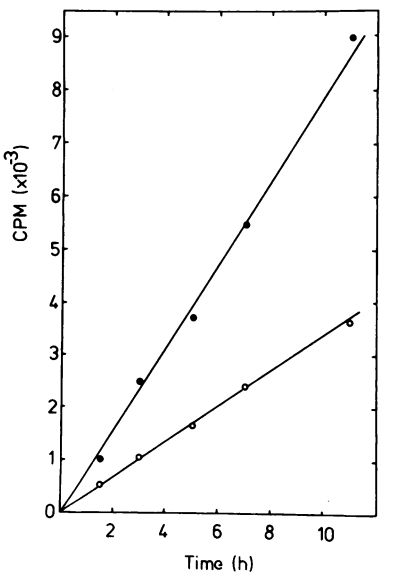

FIG. 4. *Time course of deoxycytidine phosphorylation. Assays contained samples from cell-free systems after incubation with HSV-infected-cell RNA (●) or no added RNA (○). Addition of cytoplasmic RNA from cells infected with HSV ($dPyK_1^{-7}$) or uninfected cells gave results indistinguishable from those found with no added RNA.*

activity compared with cell-free systems incubated with RNA from HSV-1($dPyK_1^{-7}$)-infected cells, BHK cells, or no RNA. This level of

enhancement is similar to that found when thymidine kinase was assayed.

Second, the overall deoxycytidine kinase activity in the presence or absence of infected-cell RNA was substantially lower than the thymidine kinase activity. This was not due to any inhibitory effects of reticulocyte components, since the activity of infected-cell extracts was unaffected by the addition of cell-free translation mixtures (results not shown).

A preliminary characterization of the induced deoxycytidine kinase activity has been carried out (Table 4). This shows that the activity induced by incubation of the cell-free system with cytoplasmic RNA from HSV-infected cells was significantly more sensitive to inhibition by anti-HSV antiserum than the activity of the cell-free system without added RNA, whereas pre-immune rabbit serum had little effect. Furthermore, thymidine, a competitive inhibitor of deoxycytidine phosphorylation by the virus-specified enzyme (9), gave greater inhibition of the induced enzyme than that of background activity.

These findings are consistent with the conclusion that the in vitro-synthesized enzyme can use both thymidine and deoxycytidine as substrates.

DISCUSSION

The experiments reported here demonstrate that cytoplasmic RNA from infected cells can direct the in vitro synthesis of enzymically active HSV-specified dPyK. The newly synthesized enzyme is indistinguishable from HSV dPyK induced in vivo by biochemical and immunological criteria, thus providing compelling evidence for the accuracy of the cell-free translation system. The requirement for an intact viral dPyK gene indicates that the in vitro-synthesized enzyme is HSV coded. As far as we know, this is the first report of translation of mRNA extracted from infected (or uninfected) eucaryotic cells into an enzymically active product.

A major reason for the successful detection of in vitro-synthesized dPyK is the use of a cell-free system derived mainly from reticulocytes. These cells have undergone differentiation to the extent that the nucleus has been lost, and this presumably has been accompanied by decay of the enzymes associated with DNA synthesis. It may therefore be possible to detect in vitro synthesis of other enzymes concerned with DNA synthesis. We have also detected synthesis of dPyK in unfractionated reticulocyte lysates, although the yield of enzyme was lower than reported here.

No in vitro synthesis of BHK-specified dPyK enzymes was found in the experiments described here. This may be due to the presence of only low amounts of functional mRNA for these enzymes, or to a lack of sensitivity of the assay system, which has been optimized for detection of virus-specified dPyK.

It has been shown previously that the cell-free system used in these studies synthesizes many HSV-induced polypeptides that have no in vivo counterparts with identical molecular weights (16). This is thought to be due to the failure of reticulocyte-derived systems to carry out "normal" processing of in vitro-synthesized polypeptides, especially glycosylation (17). The results presented here, therefore, suggest that extensive glycosylation is not essential for the production of active HSV dPyK.

The system described here should be particularly useful in studies on the structure and metabolism of HSV dPyK mRNA.

We have recently learned that in vitro synthesis of the HSV dPyK polypeptide has been reported by others (3a).

TABLE 4. *Partial characterization of in vitro-synthesized deoxycytidine kinase*

Treatment to cell-free system	Deoxycytidine kinase activity (cpm × 10^{-3})	
	+HSV-infected RNA	+BHK cell RNA
No additions	2.80	1.15
+PBS[a]	2.94	1.08
+Pre-immune rabbit serum[a]	3.25	1.16
+Anti-HSV antiserum[a]	1.06	1.25
+25 μM thymidine	1.33	1.26
+50 μM thymidine	1.25	1.13

[a] Phosphate-buffered saline and antisera constituted 25% of the reaction mixtures. Antisera were the undiluted samples shown in Fig. 2.

ACKNOWLEDGMENTS

I am grateful to J. H. Subak-Sharpe and D. McGeoch for encouragement and helpful suggestions, and to M. Dunlop for skilled technical assistance.

LITERATURE CITED

1. Anderson, C. W., J. F. Atkins, and J. J. Dunn. 1976. Bacteriophage T3 and T7 early RNAs are translated by eukaryotic 80S ribosomes: active phage T3 coded S-adenosylmethionine cleaving enzyme is synthesized. Proc. Natl. Acad. Sci. U.S.A. 73:2752-2756.
2. Beck, O. E., and H. G. Gassen. 1977. Translation of bacteriophage T4 mRNA into active lysozyme by a wheat germ cell-free system. Biochem. Biophys. Res. Commun. 74:16-24.
3. Chan, S. J., P. Keim, and D. F. Steiner. 1976. Cell-free synthesis of rat proinsulin: characterization and par-

tial amino acid sequence determination. Proc. Natl. Acad. Sci. U.S.A. 73:1964–1968.

3a. **Cremer, K. J., W. C. Summers, and R. F. Gesteland.** 1977. Cell-free synthesis of herpes simplex virus proteins. J. Virol. 22:750–757.

4. **Duguid, J. R., D. F. Steiner, and W. L. Chick.** 1976. Partial purification and characterization of the mRNA for rat preproinsulin. Proc. Natl. Acad. Sci. U.S.A. 73:3539–3543.

5. **Hay, J., P. A. J. Perera, J. M. Morrison, G. A. Gentry, and J. H. Subak-Sharpe.** 1971. Herpes virus-specified proteins, p. 355–372. *In* G. E. W. Wolstenholme and M. O'Connor (ed.), Strategy of the viral genome. Churchill Livingstone, Edinburgh and London.

6. **Herrlich, P., and M. Schweiger.** 1970. T3 and T7 bacteriophage deoxyribonucleic acid-directed enzyme synthesis in vitro. J. Virol. 6:750–753.

7. **Honess, R. W., and D. H. Watson.** 1974. Herpes simplex virus-specific polypeptides studied by polyacrylamide gel electrophoresis of immune precipitates. J. Gen. Virol. 22:171–185.

8. **Jamieson, A. T., G. A. Gentry, and J. H. Subak-Sharpe.** 1974. Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J. Gen. Virol. 24:465–480.

9. **Jamieson, A. T., and J. H. Subak-Sharpe.** 1974. Biochemical studies on the herpes simplex virus-specified deoxypyrimidine kinase activity. J. Gen. Virol. 24:481–492.

10. **Kellems, R. E., F. W. Alt, and R. T. Schimke.** 1976. Regulation of folate reductase synthesis in sensitive and methotrexate-resistant sarcoma 180 cells. J. Biol. Chem. 251:6987–6993.

11. **Kit, S., and D. R. Dubbs.** 1965. Properties of deoxythymidine kinase partially purified from noninfected and virus-infected mouse fibroblast cells. Virology 26:16–27.

12. **Klemperer, H. G., G. R. Haynes, W. I. H. Sheddon, and D. H. Watson.** 1967. A virus-specific thymidine kinase in BHK 21 cells infected with herpes simplex virus. Virology 31:120–128.

13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

14. **MacPherson, I., and M. Stoker.** 1962. Polyoma transformation of hamster clones—an investigation of genetic factors affecting cell competence. Virology 16:147–151.

15. **Palmiter, R. D.** 1973. Ovalbumin messenger ribonucleic acid translation. J. Biol. Chem. 248:2095–2106.

16. **Preston, C. M.** 1977. The cell-free synthesis of herpesvirus-induced polypeptides. Virology 78:349–353.

17. **Preston, C. M., and J. F. Szilágyi.** 1977. Cell-free translation of RNA synthesized in vitro by a transcribing nucleoprotein complex prepared from purified vesicular stomatitis virus. J. Virol. 21:1002–1009.

18. **Sakiyama, S., and J. M. Buchanan.** 1971. In vitro synthesis of deoxyribonucleotide kinase programmed by bacteriophage T4-RNA. Proc. Natl. Acad. Sci. U.S.A. 68:1376–1380.

19. **Summers, W. P., M. Wagner, and W. C. Summers.** 1975. Possible peptide chain termination mutants in thymidine kinase gene of a mammalian virus, herpes simplex virus. Proc. Natl. Acad. Sci. U.S.A. 72:4081–4084.

20. **Taylor, J. M., and T. P. H. Tse.** 1976. Isolation of rat liver albumin messenger RNA. J. Biol. Chem. 251:7461–7467.

21. **Thang, M. N., D. C. Thang, E. DeMayer, and L. Montagnier.** 1975. Biosynthesis of mouse interferon by translation of its messenger RNA in a cell-free system. Proc. Natl. Acad. Sci. U.S.A. 72:3975–3977.

22. **Walker, A. C., M. L. Walsh, D. Pennica, P. S. Cohen, and H. L. Ennis.** 1976. Transcription-translation and translation-messenger RNA decay coupling: separate mechanisms for different messengers. Proc. Natl. Acad. Sci. U.S.A. 73:1126–1130.

23. **Young, E. T.** 1970. Control of functional T4 messenger synthesis. Cold Spring Harbor Symp. Quant. Biol. 35:189–195.

24. **Zähringer, J., B. S. Baliga, and H. N. Munor.** 1976. Novel mechanism for translational control in regulation of ferritin synthesis by iron. Proc. Natl. Acad. Sci. U.S.A. 73:857–861.

25. **Zubay, G., and D. A. Chambers.** 1969. A DNA-directed cell-free system for β-galactosidase synthesis: characterization of the de novo synthesized enzyme and some aspects of the regulation of synthesis. Cold Spring Harbor Symp. Quant. Biol. 34:753–761.