Thymidine Kinase Activity in Cardiac Muscle during Embryonic and Postnatal Development

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Cytoplasmic thymidine kinase from cardiac muscle of the rat has been characterized. It has a pH optimum of 9.0 and a K_m value for thymidine of $1.6 \mu\text{M}$. The sedimentation coefficient of this enzyme in sucrose gradients is 4.5S, which represents a molecular weight of approx. 69000. Thymidine kinase prepared from cardiac muscle of foetal, neonatal and adult rats is inhibited by dTTP and dTDP; there is neither inhibition nor stimulation by dTMP, dCTP, dATP, dGTP or cyclic AMP. The activity of thymidine kinase in differentiating cardiac muscle of foetal and neonatal rats declines progressively with development, reaching adult values of almost zero by the fifteenth to seventeenth day of postnatal development. This represents a 70-fold decrease in enzyme activity from 3 days before birth to 17 days after birth. The loss of thymidine kinase activity in differentiating cardiac muscle correlates temporally with the cessation of DNA biosynthesis and the loss of cytoplasmic DNA polymerase activity in this tissue.

Thymidine kinase (ATP–thymidine 5′-phosphotransferase, EC 2.7.1.75) is a key enzyme in DNA replication, providing essential building blocks needed for DNA biosynthesis (Sneider *et al.*, 1969; Machovich & Greengard, 1972; Taylor *et al.*, 1972). Its activity shows a high degree of correlation with cellular proliferation rate and DNA-synthetic activity in many developing and regenerating tissues (Machovich & Greengard, 1972) as well as in tumours and in tissues stimulated to proliferate by viruses

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(McAuslan, 1963; Dulbecco *et al.*, 1965; Hatanaka & Dulbecco, 1966) or by isoproterenol (Pegoraro & Baserga, 1970). Thymidine kinase is the first enzyme in one of two major pathways which lead to the synthesis of deoxythymidine triphosphate (Scheme 1). The pathway of pyrimidine biosynthesis *de novo* enters through UMP. The activity of thymidylate synthetase in this pathway has been found to correlate well with cell growth rates in hepatic tissue undergoing rapid proliferation (Sneider *et al.*, 1969). The ‘salvage’ pathway, which begins with the phosphorylation of thymidine by thymidine kinase, has also been found to have a high activity in rapidly

Scheme 1. Schematic representation of the pathways leading to formation of deoxythymidine triphosphate

The pathway from thymidine (TdR) through thymidine monophosphate is termed the ‘salvage’ pathway. The ‘*de novo*’ pathway, which enters the scheme at UMP, leads to dTTP through thymidylate synthetase.

proliferating tissues (Hopkins *et al.*, 1973; Machovich & Greengard, 1972; Taylor *et al.*, 1972). The exact importance of the two pathways in the production of dTTP has not been fully determined, although both orotic acid ('*de novo*' pathway) and thymidine ('salvage' pathway) incorporation into DNA follow a similar time-course after partial hepatectomy (Hopkins *et al.*, 1973).

DNA replication and mitotic activity are apparently irreversibly repressed in cardiac muscle cells of the adult mammal (Rumyantsev, 1963; Morkin & Ashford, 1968; Grove *et al.*, 1969; Sasaki *et al.*, 1968). It has recently been determined that DNA biosynthesis in cardiac muscle of the rat essentially ceases by the seventeenth day of postnatal development, which correlates temporally with an almost total loss of cytoplasmic but not nuclear DNA polymerase activity (Claycomb, 1973, and unpublished work). To investigate further the control of cell proliferation and DNA replication in cardiac muscle during the terminal stages of differentiation, we have characterized and measured the activity of another enzyme involved in DNA synthesis, thymidine kinase. A preliminary report of this work has been published (Claycomb & Gillette, 1974).

Experimental

Pregnant rats were obtained from Holtzman (Madison, Wis., U.S.A.). Newborn rats were killed by decapitation and their hearts rapidly excised. The great vessels and atria were removed and the ventricles washed in ice-cold 0.9% NaCl. A 20% (w/v) homogenate was prepared in ice-cold 10mM-Tris-HCl (pH 7.5) containing 250mM-sucrose and 2mM- β -mercaptoethanol, with a glass homogenizer fitted with a motor-driven Teflon pestle. The homogenate was centrifuged at 800g for 10min at 4°C in a Sorvall SS34 rotor. The precipitate was discarded and the supernatant fluid centrifuged at 105000g for 60min at 4°C in a Beckman model L preparative ultracentrifuge (type 50 rotor).

Thymidine kinase assay

Thymidine kinase activity was assayed by measuring the initial rate of phosphorylation of deoxy-[methyl-³H]thymidine to deoxythymidine monophosphate by a modification of the method of Breitman (1963). The standard reaction mixture contained the following in a total volume of 0.5ml: 100mM-Tris-HCl (pH 9.0); 5mM-ATP; 2.5mM-MgCl₂; 2 μ M-[methyl-³H]thymidine (sp. radioactivity 5.5Ci/mol; Amersham-Searle, Arlington Heights, Ill., U.S.A.); and 50 μ l of the 105000g supernatant fluid. The incubation was carried out for 10min at 37°C and stopped by pipetting 75 μ l of the reaction mixture on to a circle of DEAE-cellulose chromatography paper (Whatman DE81), which retains only

phosphorylated nucleotides after proper washing. The filter paper was washed once (10min) in ice-cold 0.1mM-ammonium formate, twice (10min) at room temperature (22°C) in 0.1mM-ammonium formate, then twice (5min) in water, and finally in 95% (v/v) ethanol (5min). The chromatography-paper discs were then placed in counting vials and dried in an oven. The amount of thymidine converted into dTMP was determined by counting the radioactivity on the paper discs in a Packard Tri-Carb liquid-scintillation spectrometer in 10ml of scintillation fluid containing 4g of PPO (2,5-diphenyloxazole) and 0.05g of dimethyl POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] per litre of toluene. Corrections for quenching were made by using automatic external standardization.

Since freezing the enzyme extract led to an unpredictable loss of activity, all assays were carried out on the day of preparation. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard. Enzyme activity is expressed as d.p.m./10min per μ g of protein. To determine which of the possible phosphorylated nucleotides was the major product of the reaction, incubation mixtures from several different experiments were separated by ascending chromatography on Whatman DE81 cellulose paper in 2.0M-formic acid-0.1M-ammonium formate by the procedure of Sneider *et al.* (1969). The paper was then cut into 1cm² sections and counted for radioactivity as described above.

Sucrose gradient analysis

The sedimentation coefficient of cardiac muscle cytoplasmic thymidine kinase was estimated by loading 0.25ml of the 105000g supernatant fluid on linear sucrose gradients (5-20%, w/v, in 0.1M-potassium phosphate, pH 7.5, containing 2mM- β -mercaptoethanol) and centrifuging for 17h at 40000rev./min in a Beckman SW 50.1 rotor at 2°C. After centrifugation, the bottom of the centrifuge tube was punctured and seven-drop fractions were collected. Each fraction was assayed for thymidine kinase activity as described above. The sedimentation coefficient was calculated by the procedure of Martin & Ames (1961), by using yeast alcohol dehydrogenase (*s*_{20,w} 7.4S), bovine serum albumin (*s*_{20,w} 4.5S) and ovalbumin (*s*_{20,w} 3.6S) as reference standards.

Results and Discussion

Characterization of the thymidine kinase reaction

The activity of cardiac-muscle thymidine kinase is directly proportional to both time of incubation up to 30min and protein concentration. The only product of the reaction was thymidine monophosphate, as determined by paper chromatography

Fig. 1. DEAE-cellulose chromatography of the products of the thymidine kinase reaction

Migration of nucleotide standards is shown in the upper part of the Figure, and the radiochromatogram of the products of the thymidine kinase reaction at zero time (●) and after 10 min of incubation (○) is shown in the lower part of the Figure. The reaction was carried out under standard assay conditions and was stopped by heating to 100°C for 10 min. A portion of the reaction mixture was chromatographed on DEAE-cellulose paper (DE81) with 2.0M-formic acid–0.1M-ammonium formate as the solvent phase.

Fig. 2. Double-reciprocal plot of velocity against substrate concentration

The reactions were carried out under standard conditions. Thymidine kinase from 3-day-old neonatal rats was used to obtain this plot and is representative of similar plots obtained with enzyme assayed from neonatal rats of several ages and from adults. The K_m value of thymidine kinase for thymidine is 1.6 μM .

(Fig. 1). The K_m value of cardiac muscle thymidine kinase for thymidine was found to be 1.6 μM , by using a double-reciprocal plot (Fig. 2). This value agrees well with that of Klemperer & Haynes (1968), who found the K_m value of hepatic thymidine kinase to be 2.1 μM , and with Bresnick & Thompson (1965),

who reported K_m values for thymidine kinase from transplanted hepatic tumours to range from 2.3 μM to 3.7 μM .

The pH optimum for cardiac thymidine kinase activity was 9.0 in foetal, neonatal and adult hearts (Fig. 3). The pH optimum of hepatic thymidine kinase from neonatal rats was determined to be 8.0 (not shown). This latter value agrees with that found for thymidine kinases from neonatal liver, spleen, thymus and many transplanted Morris and Novikoff hepatomas (Klemperer & Haynes, 1968; Sneider *et al.*, 1969; Taylor *et al.*, 1972; Bresnick & Thompson, 1965). The different pH optimum of cardiac-muscle thymidine kinase distinguishes it from thymidine kinases characterized from other tissues. Thymidine kinase activity was optimum at a Mg^{2+} concentration of 2.5 mM (Fig. 4). In this respect, the cardiac enzyme is similar to thymidine kinases characterized from several other developing tissues (Machovich & Greengard, 1972).

Effect of nucleotides on thymidine kinase activity

Cardiac-muscle thymidine kinase was markedly inhibited by dTTP and dTDP but not by dTMP, dCTP, dGTP, or cyclic AMP (Table 1). The inhibitory effect of dTTP and dTDP was concentration-dependent (Fig. 5). Breitman (1963) suggested that both dTTP and dTDP inhibited thymidine kinase directly. Although he recognized the possibility that the apparent inhibition by dTDP might be due to its conversion into dTTP, he considered that the lability *in vitro* of the enzyme carrying out this reaction (dTDP kinase) made the conversion of dTDP into dTTP unlikely. We found, however, that

Fig. 3. Effect of pH on the activity of cardiac-muscle thymidine kinase

The enzyme source was cardiac muscle from 8-day-old neonatal rats. Assays were carried out under standard conditions as outlined in the text.

Fig. 4. Effect of Mg^{2+} concentration on cardiac-muscle thymidine kinase activity

The Mg^{2+} concentration was varied as shown, while other conditions were kept constant. The enzyme source was 9-day-old neonatal rats.

Table 1. Effect of nucleotides on thymidine activity

Assays were carried out by using enzyme extracts prepared from cardiac muscle of 17-day foetal, 7-day-old neonatal and adult rats exactly as outlined in the text except that the nucleotides were added before the reaction was begun. Concentration of nucleotides was $1\mu M$.

Nucleotide added	Thymidine kinase activity (% of control value)		
	Foetal	Neonatal	Adult
None	100	100	100
dTTP	22	0	12
dTDP	0	0	15
dTMP	95	92	96
dCTP	100	99	100
dGTP	96	95	100
dATP	100	97	100
Cyclic AMP	—	93	—

conversion of dTDP into dTTP is rapid *in vitro*. In experiments in which $[^3H]$ dTDP replaced deoxy $[^3H]$ thymidine and the reaction mixture was separated by chromatography on DEAE-cellulose paper, essentially all of the dTDP was converted into dTTP during the 10-min reaction period (results not shown). Therefore the observed inhibition of thymidine kinase by dTDP may be due to the rapid conversion of the latter into dTTP.

Cardiac thymidine kinase differs from the hepatic enzyme in its response to nucleotide inhibitors. Thymidine kinase prepared from foetal, neonatal

and adult cardiac muscle is equally sensitive to inhibition by dTTP (Table 1) in contrast with the hepatic enzyme, which becomes more resistant to dTTP inhibition with increasing age (Klemperer & Haynes, 1968). Foetal, neonatal and adult cardiac thymidine kinase is not inhibited by dCTP; adult but not foetal human hepatic and splenic thymidine kinases, and adult but not foetal rat hepatic thymidine kinase are, however, inhibited by this nucleotide (Taylor *et al.*, 1972; Bresnick *et al.*, 1964). Our observation that dTMP did not inhibit cardiac thymidine kinase is in contrast with the finding of Taylor *et al.* (1972) that dTMP did inhibit human hepatic and splenic thymidine kinases. Hamazaki (1973) reported that cyclic AMP inhibited one of two forms of thymidine kinase isolated from sarcoma cells, whereas the activity of the other form was enhanced by this cyclic nucleotide. We observed that similar concentrations of cyclic AMP have no effect on the activity of cardiac muscle thymidine kinase when added *in vitro* (Table 1) but did inhibit the activity of this enzyme when administered *in vivo* (W. C. Claycomb & P. C. Gillette, unpublished work).

Estimation of the sedimentation coefficient

The sedimentation coefficient of cardiac-muscle thymidine kinase in soluble extracts prepared from 1-, 2- or 6-day-old and adult rats as estimated by sucrose-density-gradient centrifugation is 4.5S.

Fig. 5. Inhibition of cardiac-muscle thymidine kinase activity by dTTP (●) and dTDP (○)

The enzyme source was cardiac muscle from 4-day-old neonatal rats. Activity is expressed as percentage of control values. Inhibitor concentration is expressed as μM added per assay.

Assuming that thymidine kinase is a globular protein, this sedimentation coefficient would represent a molecular weight of approx. 69000 as calculated by the procedure of Martin & Ames (1961). The sedimentation coefficient of cardiac cytoplasmic thymidine kinase is similar to that found for mitochondrial thymidine kinase in several tumour lines by Kit *et al.* (1973), using glycerol gradients, and only slightly different from the sedimentation values they found for cytoplasmic enzyme. Bresnick & Thompson (1965), using Sephadex chromatography, reported a molecular weight in excess of 670000 for the hepatic enzyme, and Hashimoto *et al.* (1972) found values of 120000 and 70000 for two fractions of tumour thymidine kinase by using gel filtration. Although the activity of cardiac thymidine kinase changes with age, it appears to have the same qualitative characteristics as judged by molecular-weight measurement and other biochemical parameters. This is in contrast with the results of Taylor *et al.* (1972) and Klemperer & Haynes (1968), who reported major differences between the foetal and adult thymidine kinases from human and rat hepatic tissue.

Activity of thymidine kinase during postnatal differentiation

Cardiac-muscle thymidine kinase activity decreases 70-fold from 3 days before birth to 17 days after birth (Fig. 6). The decrease in activity is in two phases. The first decrease, from 3 days before birth to birth, was followed by a plateau lasting until 6 days of

Fig. 6. Cytoplasmic thymidine kinase activity in cardiac muscle during foetal and postnatal development

Assays were carried out under standard conditions as outlined in the text.

neonatal development. The second and larger fall in activity occurred from 6 to 17 days. Aspartate transcarbamylase, the first enzyme in the pathway of pyrimidine biosynthesis *de novo*, has also been found to have high activity in foetal and neonatal cardiac muscle but virtually no activity in the adult (Nordmann *et al.*, 1964). These investigators, however, did not document the exact time during cardiac-muscle differentiation when the activity of this enzyme is lost. The relative importance of the 'salvage' versus the '*de novo*' pathway of dTTP synthesis in proliferating cardiac muscle cannot be determined from the present study, but it is clear that the pathway involving thymidine kinase is very active and correlates well with all other measured indices of cell multiplication in this tissue.

The decrease in cardiac thymidine kinase activity with age does not appear to be due to either a soluble activator in neonatal extracts or to a soluble inhibitor in adult extracts, since mixing experiments *in vitro* were additive (not shown). Addition of NaF (1 mM) as a phosphatase inhibitor to reaction mixtures containing muscle extracts from animals of 17 days and older did not restore thymidine kinase activity (not shown). Thus there is no evidence that increased phosphatase activity in cytoplasmic extracts of older animals was hydrolysing the substrate or product of the thymidine kinase reaction, which might make it appear that enzyme activity was decreased. The inhibition of thymidine kinase by dTTP suggests that it has an important regulatory role *in vivo*. However, on the basis of the results of our mixing experiments, which did not indicate the presence of a soluble inhibitor, dTTP probably is not responsible for the almost complete absence of activity found in cardiac tissue from animals of 17 days and older.

Thymidine kinase activity has been correlated with growth rate in several transplanted tumours (Machovich & Greengard, 1972; Sneider *et al.*, 1969). In mammalian intestine, Salser & Balis (1973) reported a correlation between thymidine kinase activity and cell-division rate. Its activity has also been found to correlate with DNA content of normal developing and dystrophic chick skeletal muscle (Weinstock & Dju, 1967). The fact that thymidine kinase activity has been found to correlate well with cell-division rates and DNA-replication rates in such a large variety of cells suggests that it is an important enzyme in DNA biosynthesis. Our studies again confirm that the activity of this enzyme is very closely related to other indices of cell proliferation and extend this observation to differentiating cardiac muscle.

The decrease in cardiac-muscle thymidine kinase activity during development correlates temporally with the decline and cessation of cardiac-muscle DNA biosynthesis and loss of cytoplasmic DNA polymerase activity (Claycomb, 1973, and unpublished work).

The results presented here and in previous studies thus establish that the cessation of DNA synthesis and cell proliferation in terminally differentiating cardiac muscle is closely correlated with the loss of several enzymes needed for DNA replication. Since it is now generally held that a cell actively synthesizing DNA, with few exceptions, is already committed to continue through the cell cycle, enter mitosis and divide, the loss or inactivation of essential DNA enzymes may be an important causative factor responsible for the cessation of DNA synthesis and hence of cell proliferation in terminally differentiating cardiac muscle.

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References

- Breitman, T. R. (1963) *Biochim. Biophys. Acta* **67**, 153-155
- Bresnick, E. & Thompson, V. B. (1965) *J. Biol. Chem.* **240**, 3967-3974
- Bresnick, E., Thompson, J. B., Morris, H. P. & Liebelt, A. G. (1964) *Biochem. Biophys. Res. Commun.* **16**, 278-283
- Claycomb, W. C. (1973) *Biochem. Biophys. Res. Commun.* **54**, 715-720
- Claycomb, W. C. & Gillette, P. C. (1974) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **33**, 1412
- Dulbecco, R., Hartwell, L. H. & Vogt, M. (1965) *Proc. Nat. Acad. Sci. U.S.* **53**, 403-410
- Grove, D., Zak, R., Nair, K. G. & Aschenbrenner, V. (1969) *Circ. Res.* **25**, 473-485
- Hamazaki, T. (1973) *Gann* **64**, 219-226
- Hashimoto, T., Arima, T., Okuda, H. & Fujii, H. (1972) *Cancer Res.* **32**, 67-72
- Hatanaka, M. & Dulbecco, R. (1966) *Proc. Nat. Acad. Sci. U.S.* **56**, 736-740
- Hopkins, H. A., Campbell, H. A., Barbiroli, B. & Potter, V. A. (1973) *Biochem. J.* **136**, 955-966
- Klemperer, H. G. & Haynes, G. R. (1968) *Biochem. J.* **108**, 541-546
- Kit, S., Leung, W. C. & Trkula, D. (1973) *Arch. Biochem. Biophys.* **158**, 503-513
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Machovich, R. & Greengard, O. (1972) *Biochim. Biophys. Acta* **286**, 375-381
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372-1379
- McAuslan, B. R. (1963) *Virology* **21**, 383-389
- Morkin, E. & Ashford, T. P. (1968) *Amer. J. Physiol.* **215**, 1409-1413
- Nordmann, Y., Hurwitz, R. & Kretchmer, N. (1964) *Nature (London)* **201**, 616-617
- Pegoraro, L. & Baserga, R. (1970) *Lab. Invest.* **22**, 266-271
- Rumyantsev, P. P. (1963) *Folia Histochem. Cytochem.* **1**, 463-471
- Salser, J. S. & Balis, M. E. (1973) *Cancer Res.* **33**, 1889-1897
- Sasaki, R., Watanabe, Y., Morishita, T. & Yamagata, S. (1968) *Tohoku J. Exp. Med.* **95**, 177-184
- Sneider, T. W., Potter, V. R. & Morris, H. P. (1969) *Cancer Res.* **29**, 40-54
- Taylor, A. T., Stafford, M. A. & Jones, O. W. (1972) *J. Biol. Chem.* **247**, 1930-1935
- Weinstock, I. M. & Dju, M. Y. (1967) *Life Sci.* **6**, 797-802