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Biochemical Aspects of Cardiac Muscle Differentiation

DETERMINATION OF DEOXYRIBONUCLEIC ACID TEMPLATE AVAILABILITY AND 3′-HYDROXYL TERMINI IN NUCLEI AND CHROMATIN BY USING EXOGENOUS DEOXYRIBONUCLEIC ACID POLYMERASES*

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Experiments were designed to determine whether DNA synthesis ceases in terminally differentiating cardiac muscle of the rat because the activity of the putative replicative DNA polymerase (DNA polymerase α) is lost or whether the activity of this enzyme is lost because DNA synthesis ceases. DNA-template availability and 3′-hydroxyl termini in nuclei and chromatin, isolated from cardiac muscle at various times during the developmental period in which DNA synthesis and the activity of DNA polymerase α are decreasing, were measured by using *Escherichia coli* DNA polymerase I, *Micrococcus luteus* DNA polymerase and DNA polymerase α under optimal conditions. Density-shift experiments with bromodeoxyuridine triphosphate and isopycnic analysis indicate that DNA chains being replicated semi-conservatively *in vivo* continue to be elongated in isolated nuclei by exogenous DNA polymerases. DNA template and 3′-hydroxyl termini available to exogenously added DNA polymerases do not change as cardiac muscle differentiates and the rate of DNA synthesis decreases and ceases *in vivo*. Template availability and 3′-hydroxyl termini are also not changed in nuclei isolated from cardiac muscle in which DNA synthesis had been inhibited by administration of isoproterenol and theophylline to newborn rats. DNA-template availability and 3′-hydroxyl termini, however, were substantially increased in nuclei and chromatin from cardiac muscle of adult rats. This increase is not due to elevated deoxyribonuclease activity in nuclei and chromatin of the adult. Electron microscopy indicates that this increase is also not due to dispersal of the chromatin or disruption of nuclear morphology. Density-shift experiments and isopycnic analysis of DNA from cardiac muscle of the adult show that it is more fragmented than DNA from cardiac-muscle cells that are, or have recently ceased, dividing. These studies indicate that DNA synthesis ceases in terminally differentiating cardiac muscle because the activity of a replicative DNA polymerase is lost, rather than the activity of this enzyme being lost because DNA synthesis ceases.

Semi-conservative DNA replication is restricted progressively in terminally differentiating ventricular cardiac muscle of the rat and essentially ceases by week 3 of postnatal development (Claycomb, 1975, 1976*a*). Accompanying this loss of DNA synthetic activity is an almost complete loss in activity of the putative replicative DNA polymerase, DNA polymerase α (Claycomb, 1975). A logical question therefore is: does DNA synthesis cease in this tissue because the activity of this enzyme is lost, or is the activity of this enzyme lost because DNA synthesis ceases? Which is cause and which is effect? A further question that can be asked is: do the number of 3′-hydroxyl primer termini decrease in DNA as DNA synthesis is restricted? In an attempt to answer these questions DNA-template availability and 3′-hydroxyl termini in nuclei and chromatin, isolated from cardiac muscle at various times during the developmental period in which DNA synthesis is being and has been restricted, were measured by using exogenous DNA polymerases. Preliminary accounts of some of these observations have been reported (Claycomb, 1976*b*, 1977*a*).

Abbreviations used: BrdUTP, 5-bromo-2′-deoxyuridine 5′-triphosphate; DNAase, deoxyribonuclease; RNAase, ribonuclease.

* This is paper VII in a series on Biochemical Aspects of Cardiac-Muscle Cell Proliferation and Cell Differentiation. Claycomb (1977*b*,*c*) are papers V and VI in this series.

Experimental

Isotopes and chemicals

[^{3}H]dTTP (sp. radioactivity 30 Ci/mmol), [^{14}C]-dATP (sp. radioactivity 524 mCi/mmol) and

[*methyl*-^{3}H]thymidine (sp. radioactivity 18.5Ci/mmol) were from Amersham/Searle, Arlington Heights, IL, U.S.A.; [^{3}H]dCTP (sp. radioactivity 15.9Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.; [^{3}H]dATP (sp. radioactivity 12Ci/mmol) was from Schwartz/Mann, Orangeburg, NY, U.S.A.; ^{3}H-labelled linear duplex and closed circular duplex SV-40-viral DNA (sp. radioactivity 50000d.p.m./μg of DNA) were from Bethesda Research Laboratories, Rockville, MD, U.S.A.; BrdUTP was from P–L Biochemicals, Milwaukee, WI, U.S.A. The source of all other chemicals and materials was as previously described (Claycomb, 1975, 1976*a*,*c*).

Enzymes

Micrococcus luteus DNA polymerase (EC 2.7.7.7) prepared by the procedure of Harwood *et al.* (1970) was from Miles Laboratories, Elkhart, IN, U.S.A. One enzyme unit is defined as the amount of enzyme required to convert 10nmol of dNTP into acid-insoluble material in 30min in the presence of poly[d(A-T)] at 37°C. *Escherichia coli* DNA polymerase I (EC 2.7.7.7) (fraction VII), prepared and assayed by the procedure of Richardson *et al.* (1964*a*) was obtained from GIBCO, Grand Island, NY, U.S.A. One enzyme unit is defined as the amount of enzyme required to convert 10nmol of dNTP into acid-insoluble material in 30min in the presence of poly[d(A-T)] at 37°C. Calf thymus DNA polymerase α (EC 2.7.7.7) prepared by the procedure of Bollum (1968) was obtained from GIBCO. One enzyme unit is defined as the amount of enzyme required to convert 1nmol of dNTP into acid-insoluble material in 60min in the presence of denatured calf thymus DNA at 37°C. Endonuclease-free *E. coli* exonuclease III (EC 3.1.4.27) prepared by the procedure of Richardson *et al.* (1964*b*,*c*) was obtained from Miles Laboratories. One enzyme unit produces 1nmol of acid-soluble radioactivity in 30min in the presence of sonicated duplex [^{3}H]DNA at 37°C.

Animals

Timed pregnant rats were obtained from Holtzman (Madison, WI, U.S.A.) on day 14 of gestation. They were housed in individual cages and maintained on water and standard laboratory chow *ad libitum*. Neonatal rats were raised in litters of ten.

Isolation of nuclei and preparation of chromatin

Nuclei were isolated from ventricular cardiac muscle and washed with 0.5% Triton X-100 exactly as described previously (Claycomb, 1976*c*, 1977*b*). The purified nuclei were suspended in 0.01M-Tris/HCl, pH7.5, containing 0.3M-sucrose and 1mM-β-mercaptoethanol at a final DNA concentration of 200–400μg/ml. To prepare chromatin the nuclei were suspended in 0.15M-NaCl/0.01M-Tris/HCl (pH8.0)/1mM-EDTA and washed twice by resuspending and centrifuging at 10000*g* for 15min at 4°C. The nuclei were suspended in ice-cold water and left to lyse in an ice bath for 30min. The chromatin was centrifuged at 10000*g* for 10min at 4°C and resuspended in 0.1M-Tris/HCl, pH8.0, containing 1mM-β-mercaptoethanol at a concentration of approx. 400μg of DNA/ml. The chromatin was resuspended by hand with a glass/Teflon homogenizer just before use. The protein/DNA ratio of the chromatin preparations ranged between 2.10 and 2.59 and the average A_{260}/A_{280} was 0.66. The pH of all buffers was determined at 23°C.

Assay of template availability and determination of 3′-hydroxyl termini

DNA-template availability in nuclei and chromatin towards exogenous DNA polymerase was determined by using a reaction mixture (final volume 0.5ml) containing 0.1M-Tris/HCl (at the optimum pH for each enzyme), 6mM-$MgCl_2$, 4mM-ATP, 0.1mM-dCTP, -dGTP and -dATP, 165pmol of [^{3}H]dTTP and the indicated DNA polymerase. Template availability is defined here as the DNA in nuclei and chromatin that is accessible for DNA polymerase binding and that can be used as a template to catalyse incorporation of deoxyribonucleotides into DNA. This assay provides the polymerase the potential to catalyse dNTP incorporation into DNA by several different processes. These include incorporation due to elongation of DNA chains that were growing *in vivo* and DNA-repair synthesis such as gap filling (Kornberg, 1974; Bollum, 1975; Weissbach 1975; Lehman & Uyemura, 1976). The 3′-hydroxyl termini in the DNA are measured by omitting the three non-radioactive dNTP species from the reaction mixture. At saturating concentrations of DNA polymerase the number of 3′-hydroxyl primer termini in DNA can be determined by measuring the incorporation of a single radioactive dNTP (Adler *et al.*, 1958; Sedwick *et al.*, 1972; Kornberg, 1974; McClure & Jovin, 1975). In these reactions the amount of [^{3}H]dTTP added was increased to 330pmol. Reactions for both types of determinations were initiated by addition of approx. 5μg of either nuclear or chromatin DNA and were left to proceed for 30min at 37°C. The reactions were terminated and incorporated radioactivity was determined as previously described (Claycomb, 1975). Each assay was done in triplicate and the results were corrected for zero-time controls, which were less than 500d.p.m. above background. Any modifications to these conditions are given in the individual experiments.

To maintain the nuclei and chromatin as closely as possible to the physiological state that they were in

in vivo, and to be able to compare preparations made on different days from animals of different ages, assays were carried out on the day the preparations were made. It was noted that the activity of freshly isolated nuclei or chromatin and preparations that had been frozen once and stored at −20°C for 1 day to 3 weeks were not appreciably different. Hence this allowed comparisons also to be made on the same day, in a single experiment, of preparations made on different days from different aged animals. This further ensured that comparisons were valid and that any differences were not caused by variations in the individual preparations or individual assays. It was observed that DNA polymerase-catalysed incorporation of radioactivity in either the presence of absence of three non-radioactive dNTP species was substantially increased in nuclear and chromatin preparations that had been repeatedly frozen and thawed. This may possibly be due to disruption of the chromatin, dispersal of chromosomal proteins or activation of latent DNAase activity.

Assay of DNAase activity

Exonuclease activity in the nuclear or chromatin preparations or in the different DNA polymerase preparations was determined by incubating approx. 10 μg of either nuclear or chromatin DNA or 4 units of DNA polymerase with 4 μg of linear duplex SV-40-viral [^{3}H]DNA. The assay was carried out in a reaction mixture (0.15 ml) containing 10 mM-Tris/HCl, pH 7.0, 6 mM-$MgCl_2$ and 1 mM-β-mercaptoethanol. The reaction was for 30 min at 37°C and was terminated by addition of 50 μg of native calf thymus DNA followed by 2.0 ml of ice-cold 10% (w/v) trichloroacetic acid. The amount of hydrolysed DNA was determined by counting for radioactivity portions of the acid-soluble fraction (Claycomb, 1975).

The assay for endonuclease activity is based on an assay described by Gates & Linn (1977) for *E. coli* endonuclease V. It measures the transformation of closed circular SV-40-viral [^{3}H]DNA to a form that is susceptible to hydrolysis by *E. coli* exonuclease III. Approx. 10 μg of either nuclear or chromatin DNA or 4 units of DNA polymerase was incubated with 4 μg of closed circular duplex SV-40-viral [^{3}H]DNA and 2 units of *E. coli* exonuclease III in a reaction mixture (0.15 ml) containing 10 mM-Tris/HCl, pH 7.0, 6 mM-$MgCl_2$ and 1 mM-β-mercaptoethanol. The reaction was carried out at 37°C for 30 min, terminated and the amount of hydrolysed DNA determined as described above.

DNA extraction and isopycnic analysis in CsCl equilibrium density gradients

To obtain sufficient DNA to analyse in CsCl gradients the contents of the template availability reaction mixture were increased by a factor of 5 and the reaction was carried out in a total volume of 2.5 ml with approx. 50 μg of nuclear DNA. BrdUTP (1 mM) was used as a density label and replaced [^{3}H]dTTP in the reaction mixture; as indicated, either [^{14}C]dATP or [^{3}H]dATP was used as the radioactive label. Scaled-down (total vol. 0.5 ml) control experiments demonstrated that substitution of BrdUTP for [^{3}H]dTTP had no anomalous effect on DNA synthesis with any of the enzymes used, in that radioactive dATP was incorporated into the acid-insoluble product of the reaction at the same rate and to the same extent as [^{3}H]dTTP. The incubation time was 30 or 45 min at 37°C and was carried out with freshly isolated nuclei. The reaction was terminated by the addition of 4 ml of ethanol/diethyl ether (3:1 v/v) and chilling on ice for 30 min. A pellet obtained by centrifuging the mixture at 25000*g* for 20 min was dried under N_2 and suspended with a glass stirring rod in 1.5 ml of 0.01 M-Tris/HCl, pH 7.0, containing 1% (w/v) sodium dodecyl sulphate, 0.1 M-EDTA, 0.15 M-NaCl and 15 mM-sodium citrate. It was incubated for 60 min at 37°C with 100 μg of RNAase (previously heated at 90°C for 10 min to inactivate DNAase)/ml followed by a further 60 min incubation with 1 mg of Pronase (predigested for 60 min at 37°C)/ml. The DNA sample was then dialysed overnight against two changes of 0.15 M-NaCl containing 15 mM-sodium citrate, chilled on ice and centrifuged in a clinical centrifuge to remove any remaining sodium dodecyl sulphate. DNA was analysed in alkaline CsCl equilibrium density gradients as previously described (Claycomb, 1976*a*). DNA, where indicated, was sheared by passing samples through a 27-gauge needle five times. Exposure of bromodeoxyuridine-substituted DNA to light was minimized.

Electron microscopy

Nuclei were prepared for electron microscopy exactly as described before (Claycomb, 1977*b*) and were photographed in a Phillips EM-300 electron microscope at 60 kV.

Chemical assays

DNA was determined by the Burton (1956) modification of the diphenylamine reaction, with calf thymus DNA as a standard. Protein was determined by the procedure of Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard.

Results

Characterization of deoxyribonucleotide incorporation catalysed by exogenous DNA polymerases

To ensure that all available template and 3'-hydroxyl termini were being measured assay con-

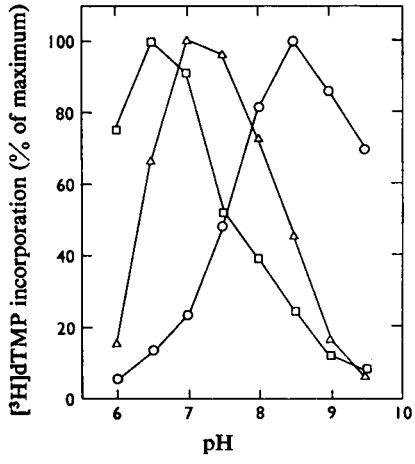


Fig. 1. *Effect of pH on DNA polymerase-catalysed incorporation of [3H]dTMP into DNA of isolated nuclei*

Nuclei were isolated from differentiating cardiac muscle of 2-day-old rats and incubated in a reaction mixture designed to measure template availability as detailed in the text. Nuclei (4.9μg of DNA) were incubated for 30min at 37°C with 4 units of either *E. coli* DNA polymerase I (○), *M. luteus* DNA polymerase (△) or DNA polymerase α (□). The buffer used was 0.1 M-Tris/HCl, at the indicated pH. Incorporation of [3H]dTMP in the absence of exogenous DNA polymerase was determined at each pH. Values for this endogenous incorporation were subtracted when expressing the results.

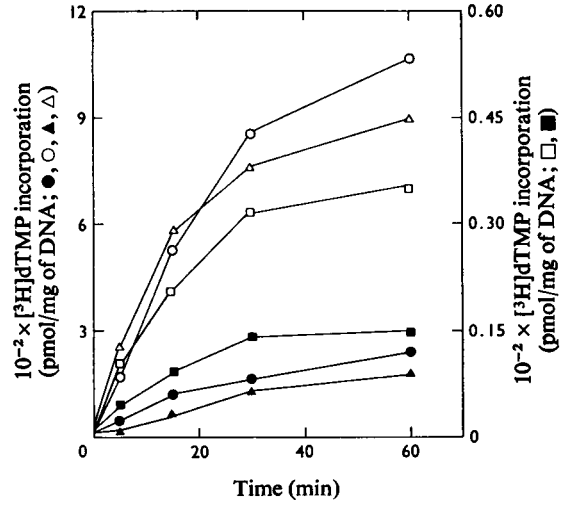


Fig. 2. *Kinetics of [3H]dTMP incorporation into DNA of isolated nuclei*

Nuclei were isolated from differentiating cardiac muscle of 4-day-old rats and incubated in a reaction mixture designed to measure template availability (open symbols) or 3′-hydroxyl termini (closed symbols). Nuclei (4.4μg of DNA) were incubated with *E. coli* DNA polymerase I (4 units) at pH8.5 (○, ●), *M. luteus* DNA polymerase (4 units) at pH7.0 (△, ▲) or DNA polymerase α (4 units) at pH6.5 (□, ■). Further details are given in the text.

ditions were optimized. pH optima were observed at 6.5, 7.0 and 8.5 for DNA polymerase α, *M. luteus* DNA polymerase and *E. coli* polymerase I respectively (Fig. 1). These determinations were made in an assay system designed to measure template availability in isolated nuclei. Identical pH optima were observed for these enzymes with nuclei incubated in the absence of the three non-radioactive dNTP species or with chromatin incubated either in the presence or absence of these precursors. Optimum Mg^{2+} and ATP concentrations with all of these DNA polymerases both in the presence and absence of the three dNTP species, with either nuclei or chromatin, were determined to be 6mM and 4mM respectively. Kinetics of exogenous DNA polymerase-catalysed incorporation of [3H]dTMP into DNA of isolated nuclei are shown in Fig. 2. Incorporation catalysed by *E. coli* DNA polymerase I, *M. luteus* DNA polymerase and DNA polymerase α is linear for approx. 30min both in the presence and absence of the three non-radioactive precursors. To measure all available template and 3′-hydroxyl termini the reactions must be carried out at saturating concentrations of enzyme. These experiments are summarized in Fig. 3. For convenience of presentation enzyme saturation of template in isolated nuclei and saturation of 3′-hydroxyl termini in chromatin are plotted in this Figure. Identical results were obtained in reciprocal experiments, i.e. enzyme saturation of 3′-hydroxyl termini in nuclei and saturation of template in chromatin. Both template and 3′-hydroxyl termini are saturated with 4units of each DNA polymerase in a reaction mixture with approx. 5μg of either nuclear or chromatin DNA. This ratio of DNA/enzyme concentration was used in all subsequent experiments.

In the presence of the three non-radioactive precursors incorporation of [3H]dTMP into DNA of isolated nuclei is increased by the addition of *E. coli* DNA polymerase I, *M. luteus* DNA polymerase and DNA polymerase α approx. 200-, 180- and 7-fold respectively, when compared with endogenous incorporation (Table 1); in their absence these increases are 207-, 205- and 19-fold. Both *E. coli* DNA polymerase I and *M. luteus* DNA polymerase at saturating concentrations under optimal assay conditions are approx. 30 times more active in catalysing the incorporation of [3H]dTMP in the presence of the three non-radioactive dNTP species compared with DNA polymerase α and approx. 10 times more active in their absence (Table 1). When the

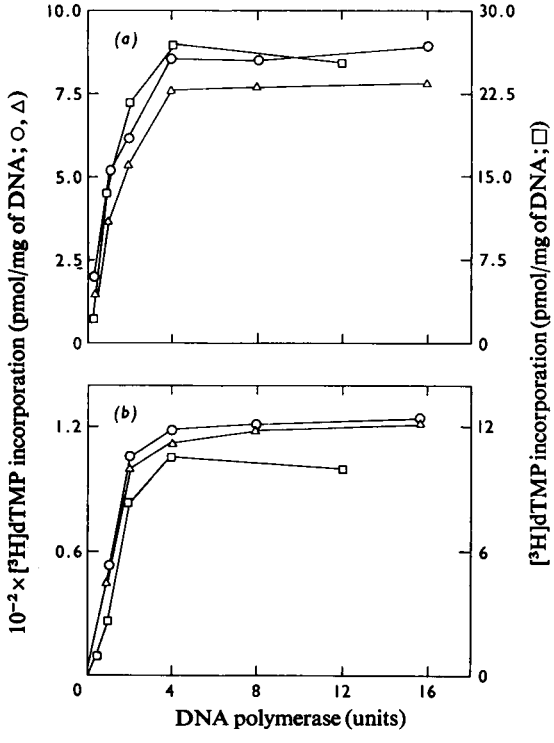


Fig. 3. *Saturation of DNA template and 3'-hydroxyl termini in nuclei and chromatin with exogenous DNA polymerases*

(*a*) Nuclei were isolated from differentiating cardiac muscle of 4-day-old rats and added to a reaction mixture designed to measure template availability. Nuclei (5.2 μg of DNA) were incubated for 30 min at 37°C. (*b*) Chromatin was isolated from differentiating cardiac muscle of 4-day-old rats and incubated in a reaction mixture designed to measure 3'-hydroxyl termini. Chromatin (4.9 μg of DNA) was incubated for 30 min at 37°C. Reactions containing *E. coli* DNA polymerase I (○), *M. luteus* DNA polymerase (△) or DNA polymerase α (□) were carried out at pH 8.5, 7.0 and 6.5 respectively.

number of 3'-hydroxyl termini is increased by addition of DNAase-nicked DNA to the reaction mixture the incorporation is greatly increased (Table 1). This indicates further that these enzymes are at saturating concentrations in these assays and thus are measuring all available template and 3'-hydroxyl termini in nuclei and chromatin.

In reaction mixtures containing the three non-radioactive dNTP species, addition of activated DNA to nuclei or to nuclei incubated with *E. coli* DNA polymerase I or *M. luteus* DNA polymerase, increases the incorporation of [^{3}H]dTMP approx. 5-fold; the increase with DNA polymerase α is 65-fold (Table 1). In the absence of the three non-radioactive dNTP species the increases on addition of activated DNA are 2-, 5-, 7.5- and 35-fold for nuclei alone and for nuclei plus *E. coli* DNA polymerase I, *M. luteus* DNA polymerase or DNA polymerase α respectively.

Isopycnic analysis of DNA synthesized in isolated nuclei

[^{3}H]Thymidine incorporation into DNA of differentiating cardiac muscle *in vivo* is into DNA that is replicating semi-conservatively (Claycomb, 1976*a*). The following series of experiments were done to examine the nature of the DNA polymerase-catalysed incorporation of dNTP into DNA of isolated nuclei. Newborn rats were injected with [^{3}H]thymidine to label DNA of cardiac muscle that was being synthesized *in vivo*. Cardiac-muscle nuclei were then isolated from these animals and incubated with BrdUTP as a density label and [^{14}C]dATP was used as the radioactive label in a reaction mixture containing either *E. coli* DNA polymerase I or *M. luteus* DNA polymerase. The DNA was then isolated, sheared to emphasize a density shift and analysed in alkaline CsCl equilibrium density gradients. Most of the fragmented DNA containing both ^{3}H and ^{14}C was

Table 1. *DNA polymerase-catalysed incorporation of [^{3}H]dTMP into DNA*

Nuclei were isolated from differentiating cardiac muscle of 6-day-old rats and added either to a reaction mixture designed to measure template availability or 3'-hydroxyl termini as detailed in the text. Nuclei (5.1 μg of DNA) were incubated with 4 units of the indicated DNA polymerase and where indicated 10 μg of activated calf thymus DNA was added. Nuclei in the absence of DNA polymerase were incubated at pH 8.5; incubations containing *E. coli* DNA polymerase I, *M. luteus* DNA polymerase or DNA polymerase α were carried out at pH 8.5, 7.0 and 6.5 respectively.

	[^{3}H]dTMP incorporation (pmol/mg of DNA)			
	+dCTP, dGTP, dATP		−dCTP, dGTP, dATP	
Reaction condition		+Activated DNA		+Activated DNA
Nuclei	3.9	15.4	0.6	1.2
Nuclei + *E. coli* DNA polymerase I	739	3780	118	607
Nuclei + *M. luteus* DNA polymerase	716	3755	117	877
Nuclei + DNA polymerase α	28	1815	11	394

shifted towards a heavier density away from the bulk DNA (absorbance) with both *M. luteus* DNA polymerase (Fig. 4*a*) and *E. coli* DNA polymerase I (Fig. 4*b*). A similar result was obtained with calf thymus DNA polymerase α. These data are consistent with these exogenous DNA polymerases catalysing the incorporation of BrdUTP and [^{14}C]dAMP into DNA of isolated nuclei relatively close if not in tandem to the region where [^{3}H]thymidine was incorporated *in vivo* (Magnusson *et al.*, 1972). This indicates that DNA chains initiated and growing by semi-conservative replication *in vivo* continue to be elongated in isolated nuclei by exogenous DNA polymerases.

Template availability and 3′-hydroxyl termini during postnatal differentiation

Template and 3′-hydroxyl termini available to exogenous DNA polymerases in nuclei isolated from cardiac muscle at different periods during postnatal differentiation and from the adult are shown in Fig. 5. This is a representative experiment from a large number of similar experiments and identical

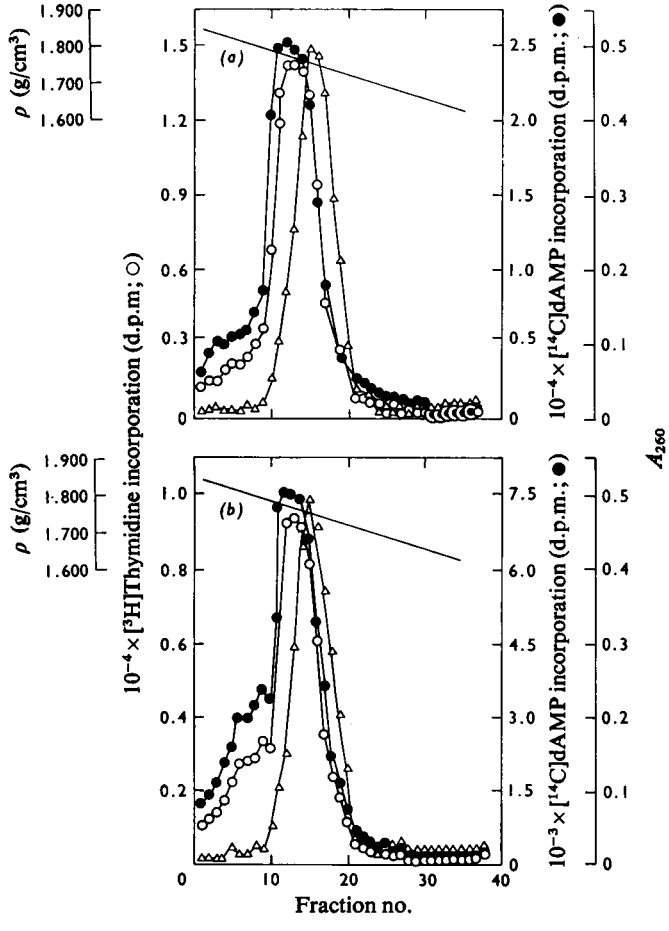


Fig. 4. *Elongation in isolated nuclei of DNA chains growing in vivo by exogenous DNA polymerases*
[^{3}H]Thymidine (10 μCi/g body wt.) was injected into 60 2-day-old rats. Then 1.5 h later they were killed and nuclei were isolated from ventricular cardiac muscle. Nuclei (approx. 50 μg of DNA) were incubated in a reaction mixture designed to measure template availability as detailed in the text. Contents of the standard reaction mixture were increased 5-fold. BrdUTP was used as a density label and replaced [^{3}H]dTTP; [^{14}C]dATP (5 μCi) served as the radioactive label. The reaction was carried out for 30 min at 37°C. (*a*) Nuclei incubated with 40 units of *M. luteus* DNA polymerase at pH 7.0; (*b*) nuclei incubated with 40 units of *E. coli* DNA polymerase I at pH 8.5. DNA was extracted from the nuclei, sheared by passing through a 27-gauge needle and analysed in alkaline CsCl equilibrium density gradients (——). ○, [^{3}H]-Thymidine incorporated into DNA *in vivo*; ●, DNA polymerase-catalysed incorporation of [^{14}C]dAMP into DNA *in vitro*; △, DNA (A_{260}). Further details are given in the text.

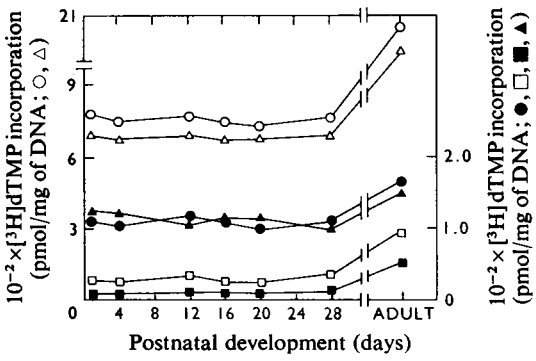


Fig. 5. *Determination of DNA template availability and 3'-hydroxyl termini in nuclei isolated from cardiac muscle during postnatal differentiation and from the adult*

Nuclei were isolated from animals of the indicated age and incubated in a reaction mixture designed to measure template availability (open symbols) or 3'-hydroxyl termini (closed symbols). Nuclei (approx. 5 μg of DNA) were incubated for 30 min at 37°C with 4 units of *E. coli* DNA polymerase I at pH 8.5 (○, ●), 4 units of *M. luteus* DNA polymerase at pH 7.0 (△, ▲) or 4 units of DNA polymerase α at pH 6.5 (□, ■).

qualitative results were obtained whether nuclei or chromatin were used or whether nuclei were prepared in the presence or in the absence of Triton X-100 (Claycomb, 1977*b*). Template available to exogenous DNA polymerases and the number of 3'-hydroxyl termini do not change in isolated nuclei as DNA synthesis is restricted *in vivo* during terminal differentiation. However, available template and 3'-hydroxyl termini were observed to be substantially increased in nuclei and chromatin isolated from cardiac muscle of the adult. This same pattern was also observed when either [^{3}H]dATP or [^{3}H]dCTP was used in reaction mixtures as the radioactive dNTP.

Previous studies have shown that the rate of DNA synthesis in cardiac muscle is inhibited approx. 80% 16h after administration of isoproterenol and theophylline to neonatal rats (Claycomb, 1976*a*). Therefore I decided to inhibit DNA synthesis by this procedure and then examine whether this inhibition resulted in a change in the accessibility of the DNA template to DNA polymerase or in a decrease in the number of 3'-hydroxyl termini. This experiment is summarized in Table 2. Inhibition of DNA synthesis by isoproterenol had no effect on the ability of either *E. coli* DNA polymerase I or DNA polymerase α to use the DNA in these nuclei as template or to catalyse the incorporation of [^{3}H]dTMP into 3'-hydroxyl termini.

Experiments were next carried out to determine whether the lack of change in template or 3'-hydroxyl termini during postnatal differentiation was possibly due to nicking of the DNA during the isolation of nuclei. A failure to observe differences could occur if DNAase activity in nuclear preparations was sufficient to nick the DNA at all available sites. If this occurred it would obscure or obliterate any changes in the DNA that may have taken place before the nuclei were isolated. This does not appear to be the case since DNAase activity in isolated nuclei or chromatin was low and was similar in preparations made from different aged animals including the adult (Table 3). Further, it was observed that DNA extracted from nuclei isolated from cardiac muscle of neonatal rats and analysed in alkaline CsCl gradients was not fragmented (see Fig. 6 for experimental details). This indicates that the DNA in these nuclei had not been nicked to any great extent during isolation.

A further possibility that should be considered is that DNAase activity in the various DNA polymerase

Table 2. *Template availability and 3'-hydroxyl termini in DNA of nuclei isolated from cardiac muscle in which DNA synthesis had been inhibited by isoproterenol*

Three groups of 20 4-day-old rats were injected subcutaneously with isoproterenol (25 mg/kg) together with theophylline (50 mg/kg) (Claycomb, 1976*a*). Three groups of 20 littermates were injected with an equal volume of 0.15 M-NaCl and served as controls. All injections were made in 0.1 ml at 15:00h. Then 18h later the animals were killed and nuclei were isolated from ventricular cardiac muscle and assayed for DNA template availability and 3'-hydroxyl termini with the indicated DNA polymerase (4 units). Nuclei (approx. 5 μg of DNA) were incubated for 30 min at 37°C. Reaction mixtures containing *E. coli* DNA polymerase I were incubated at pH 8.5; those containing DNA polymerase α were incubated at pH 6.5.

Treatment	[^{3}H]dTMP incorporation (pmol/mg of DNA)			
	+dCTP, dGTP, dATP		−dCTP, dGTP, dATP	
	+*E. coli* DNA polymerase I	+DNA polymerase α	+*E. coli* DNA polymerase I	+DNA polymerase α
Control	808	29	112	10
Isoproterenol + theophylline	814	32	109	12

Table 3. *Deoxyribonuclease activity in nuclei and chromatin and in the DNA polymerase preparations*

Approx. 10μg of nuclear or chromatin DNA from animals of the indicated age or 4 units of the specified DNA polymerase preparation was incubated with 4μg of either linear duplex SV-40 viral [^{3}H]DNA (to measure exonuclease activity) or closed circular duplex SV-40-viral [^{3}H]DNA and 2 units of *E. coli* exonuclease III (to measure endonuclease activity). The specific radioactivity of the DNA was 50000 d.p.m./μg of DNA. Zero-time acid-soluble radioactivity was subtracted when expressing the results.

	Acid-soluble radioactivity in reaction mixture (d.p.m.)	
Preparation tested	Exonuclease activity	Endonuclease activity
Nuclei (1 day)	824	1121
Chromatin (1 day)	902	904
Nuclei (10 day)	729	1251
Chromatin (10 day)	862	1048
Nuclei (20 day)	749	1127
Chromatin (20 day)	801	1274
Nuclei (adult)	876	1087
Chromatin (adult)	899	1154
E. coli DNA polymerase I	698	1486
M. luteus DNA polymerase	206	421
DNA polymerase α	129	452

preparations that were used was sufficient enough to substantially nick the DNA in isolated nuclei or chromatin. DNAase activity in these polymerase preparations was either low or non-measurable (Table 3). Failure to detect nuclease activity in these preparations most likely means that none is present, however, it could also mean that nicking of the DNA by nucleases may take place concurrently with DNA synthesis or repair.

Template availability and 3′-hydroxyl termini in cardiac muscle of the adult

Experiments were carried out to investigate why template and 3′-hydroxyl-termini availability were higher in cardiac muscle of the adult compared with the neonatal animal. The possibility existed that nuclei were more disrupted or that the chromatin was more dispersed in nuclei isolated from the adult and thus the DNA in these nuclei would be more accessible to exogenous DNA polymerase. This does not appear to be true, however, since nuclei isolated from the adult had better morphology and the chromatin was more condensed than nuclei isolated from cardiac muscle of neonatal animals (Plate 1). Poor morphology of nuclei isolated from neonatal animals may be due to the rapid cell and nuclear division occurring during this period of development.

Experiments were next carried out to examine the physical state of cardiac-muscle DNA. Nuclei, isolated from 1-day-old rats and from adult rats, were incubated with BrdUTP (to emphasize any density heterogeneity) and *E. coli* DNA polymerase I; the DNA was then extracted without shearing and analysed in alkaline CsCl equilibrium density grad-

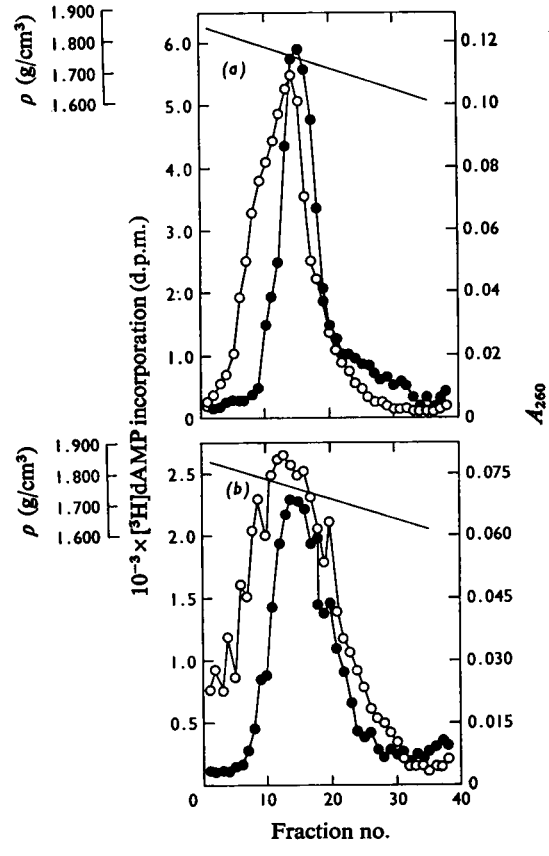
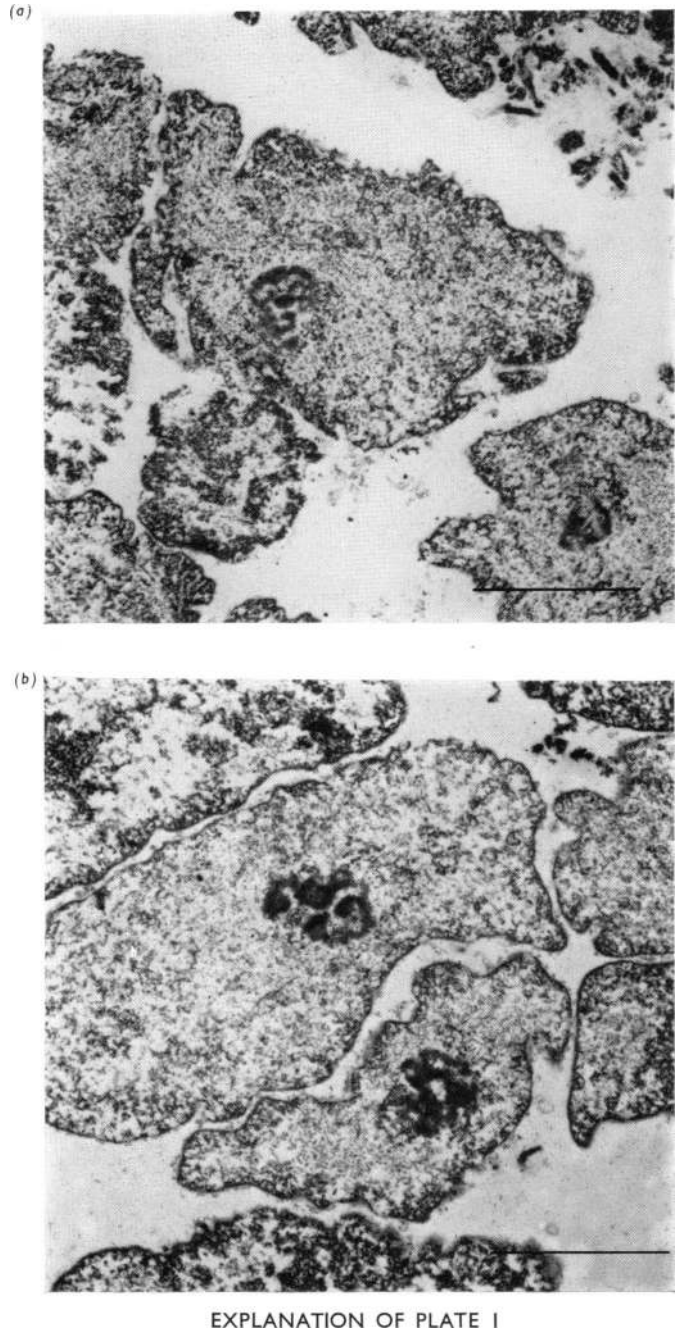


Fig. 6. *Isopycnic analysis of DNA from cardiac muscle of 1-day-old and adult rats*

Nuclei were isolated from cardiac muscle of 1-day-old and adult rats. Adults were females that weighed approx. 250g. Nuclei (approx. 50μg of DNA) were incubated in a reaction mixture designed to measure template availability except that BrdUTP replaced [^{3}H]dTTP and [^{3}H]dATP (10μCi/ml) served as the radioactive label. The reaction was carried out for 45min at 37°C. (*a*) Nuclei isolated from 1-day-old rats incubated with 20 units of *E. coli* DNA polymerase I at pH8.5. (*b*) Nuclei isolated from adult rats incubated with 20 units of *E. coli* DNA polymerase I at pH8.5. The extracted DNA was analysed in alkaline CsCl equilibrium density gradients (——). ○, DNA polymerase-catalysed incorporation of [^{3}H]dAMP into DNA of isolated nuclei; ●, DNA (A_{260}). Further details are given in the legend to Fig. 4 and in the text.



EXPLANATION OF PLATE 1

Electron micrographs of nuclei isolated from ventricular cardiac muscle of the rat

(*a*) Nuclei isolated from 4-day-old rats. Magnification ×11250. The bar represents 2 μm. (*b*) Nuclei isolated from adult rats. Magnification ×11800. The bar represents 2 μm.

ients. DNA of the adult was observed to have a greater density heterogeneity than DNA from neonatal animals (Fig. 6). Results identical with Fig. 6(*a*) were obtained with nuclei isolated from 22-day-old rats. These observations suggest that the increased number of 3'hydroxyl termini seen in DNA of the adult (Fig. 5) is due to fragmentation of the DNA.

Discussion

Experiments reported in the present paper show that accessibility of the DNA template towards exogenous DNA polymerase and the number of accessible 3'-hydroxyl termini in DNA of nuclei or chromatin isolated from cardiac muscle does not change as the cells stop synthesizing DNA and withdraw from the cell cycle. Thus it would appear that 3'-hydroxyl termini are available in repressed cells, but are not being utilized for DNA synthesis. These experiments indicate that DNA synthesis ceases in cardiac muscle because the activity of a DNA-polymerizing enzyme, which apparently functions in replicative DNA synthesis, is no longer available. This conclusion is substantiated by the experiments in which DNA synthesis was inhibited *in vivo* with isoproterenol and accessible template and 3'-hydroxyl termini were found not to be changed in isolated nuclei (Table 2). Inhibition of DNA synthesis in cardiac muscle by isoproterenol has been shown to be accompanied by an inhibition in the activity of DNA polymerase α (Claycomb, 1976*c*).

It should be pointed out that accumulated evidence indicates that approx. 50% of the DNA in chromatin is not accessible to exogenous enzymes such as DNAase or to certain dyes or polylysine (Clark & Felsenfeld, 1971, 1972, 1974; Itzhaki, 1971*a*,*b*; Itzhaki & Saffhill, 1973; Umiel & Plaut, 1972, 1973; Simpson & Polacow, 1973; Brodie *et al.*, 1975). Further, it is not known whether replicon origins (3'-hydroxyl sites for initiation of DNA synthesis) occur in the DNA contained in the nucleosomes or in the DNA between nucleosomes. Failure to observe differences in the number of 3'hydroxyl termini in DNA of nuclei isolated from cells actively synthesizing DNA compared with cells that have ceased DNA synthesis could mean that replicon origins and growing DNA-replication forks lie within inaccessible regions. It has been reported that, during replication, addition of histones to newly synthesized DNA, follows soon after synthesis of DNA and is complete within 15min (Seale, 1975). If this is the case, then exogenous DNA polymerases would not be able to detect differences in the number of free 3'-hydroxyl termini in these inaccessible regions. This possibility could be tested by isolating and removing all chromatin proteins from cardiac-muscle DNA of different aged animals and then measuring 3'-hydroxyl termini with exogenous DNA polymerase. Care would have to be taken in such experiments, however, to ensure that the DNA was not sheared during isolation or nicked by DNAase.

The DNA polymerases used in these experiments are complex enzymes with still incompletely understood functions in the living cell (Kornberg, 1974; Bollum, 1975; Weissbach, 1975; Lehman & Uyemura, 1976); all, however, require a free 3'-hydroxyl primer terminus in the DNA for the enzymic addition and polymerization of dNTP. Because various DNA polymerases have different specificities and ancillary enzyme activities (Kornberg, 1974) I decided to use enzymes from several different organisms and do a comparative study. Identical qualitative results were obtained with all of these DNA polymerases (Fig. 5). In preliminary studies with calf thymus terminal deoxynucleotidyl transferase and a single dNTP ([^{3}H]dATP) in the reaction mixture, results identical with those of Fig. 5 were obtained, i.e. no difference in the number of 3'-hydroxyl termini in nuclei isolated from cardiac muscle of rats between the ages of 1 and 24 days was observed, but there was an increase in the number of 3'-hydroxyl termini in nuclei prepared from cardiac muscle of the adult. This enzyme requires as a primer a DNA chain containing a minimum of three deoxyribonucleotide residues with a free 3'-hydroxyl group (Bollum, 1970). These results further support the conclusion that the number of accessible 3'-hydroxyl termini in DNA does not change as DNA synthesis is restricted in differentiating cardiac muscle.

The observation that the number of 3'-hydroxyl termini were increased approx. 3-fold in nuclei and chromatin from adult cardiac muscle was unexpected. Price *et al.* (1971) have shown in radioautographic studies, in which they added DNA polymerase to fixed tissue sections, that DNA-template activity of several adult mouse tissues, including cardiac muscle, was increased as compared with the activity in tissues from younger animals. They suggested that this was due to an accumulation of DNA strand breaks with aging. Data in the present paper indicate that the increased number of 3'-hydroxyl termini in cardiac muscle of the adult is due to fragmentation of the DNA. The most likely reason for this fragmented DNA is a decrease in the ability of the aged cardiac-muscle cell to repair DNA. This could be the result of a decrease or loss in activity of the DNA repair enzyme(s). The function of DNA polymerase β in the eukaryotic cell is not known, but it is not unreasonable to think that it may function in some capacity in the repair of DNA. Previous studies have shown, however, that the activity of DNA polymerase β is the same in dividing, non-dividing and adult cardiac muscle cells (Claycomb, 1975). Fragmented DNA in aged cells could result from a decrease in the fidelity of this enzyme rather than to a decrease in

grossly detectable enzyme activity. A decrease in the fidelity of a DNA polymerase from aged human fibroblasts has been observed (Linn *et al.*, 1976).

Although the definitive function of DNA polymerase α in eukaryotic cells has not been delineated, it seems logical to consider that it could function in some capacity in DNA-repair synthesis. If this were the case then repair synthesis of DNA would be decreased in cardiac muscle cells when they lose the activity of DNA polymerase α. The period during development when DNA strand breaks first begin to appear in cardiac-muscle DNA has not been determined. They may begin to accumulate when active DNA replication ceases during terminal differentiation. Previous studies by Stockdale and O'Neill and others (Stockdale, 1971; Stockdale & O'Neill, 1972; Hahn *et al.*, 1971; Chan *et al.*, 1976) have shown that, in skeletal muscle, when the cells fuse, DNA synthesis ceases and the ability of nuclei in the myotube to repair DNA is decreased by approx 50%.

It should be noted that failure to repair fragmented DNA in cardiac muscle during aging has important implications for the physiological function of this tissue. If strand breaks become extensive in the adult, the DNA would no longer accurately code for metabolic enzymes and cellular metabolism would be affected. It would be a particularly serious problem if these breaks were to occur in regions of the DNA that code for the contractile proteins.

Finally, the question remains whether loss of DNA polymerase α activity in cardiac muscle is due to an inhibition in the synthesis or to an inhibition in the catalytic activity of existing enzyme protein. Loss of this and possibly other enzymes and proteins that are required for DNA replication may be due to a selective and programmed repression in the genes coding for them. Adrenergic innervation of cardiac muscle may ultimately be responsible for this developmental program with noradrenaline and cyclic AMP serving as chemical mediators (Claycomb, 1976*a*).

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