The Synthesis of Polydeoxyribonucleotide by Cell-Free Extracts of Embryonic Mouse Tissue

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The conditions under which soluble extracts prepared from mouse embryos incorporate [^3H]thymidine 5'-triphosphate into polydeoxyribonucleotide have been studied. In common with similar preparations from other mammalian tissues, mouse-embryo DNA nucleotidyltransferase requires the four complementary deoxyribonucleoside 5'-triphosphates, primer DNA and a bivalent cation for activity. Unlike other mammalian DNA nucleotidyltransferases, the rate and extent of the incorporation of [^3H]thymidine 5'-triphosphate is much greater with Mn^{2+} than with Mg^{2+} and, with either Mg^{2+} or Mn^{2+} , maximum activity occurs at pH 6.4. The difference between Mg^{2+} and Mn^{2+} varies markedly with pH, reaching a maximum of six- to eight-fold at pH 6.4.

Extracts of a variety of mammalian tissues, including rat liver (Bollum & Potter, 1958; Mantsavinos & Canellakis, 1959*b*) and mouse tumours (Mantsavinos & Canellakis, 1959*a*; Smellie, Keir & Davidson, 1959; Keir, Binnie & Smellie, 1962), and of cultured mammalian cells (Magee, 1962; Keir & Gold, 1963) have been shown to contain active DNA nucleotidyltransferase (deoxy-nucleoside triphosphate-DNA nucleotidyltransferase, EC 2.7.7.7). The enzyme has been purified, to various extents, from sources such as regenerating rat liver (Mantsavinos, 1964), calf thymus (Bollum, 1960) and tumour tissue (Keir, 1962; Furlong, 1965; Shepherd & Keir, 1966). The properties of many of these DNA-nucleotidyltransferase preparations have been described (Bollum & Potter, 1958; Mantsavinos & Canellakis, 1959*a,b*; Smellie *et al.* 1959; Bollum, 1960; Magee, 1962; Mantsavinos, 1964; Furlong, 1965; Shepherd & Keir, 1966) and they have been shown to require primer DNA, the four complementary deoxyribonucleoside 5'-triphosphates and a bivalent cation for activity.

The DNA nucleotidyltransferase of embryonic tissues has been little studied, although Smellie *et al.* (1959) showed that chick-embryo extracts actively incorporate [^3H]thymidine into polydeoxyribonucleotide. Reichard, Canellakis & Canellakis (1961) and Miller, Valanju & Balis (1965) have studied the properties of an extract of chick embryos that incorporates ribonucleotides into polydeoxyribonucleotide. The work described in the present paper was undertaken when, during some studies on polyoma-virus-infected mouse-

embryo cells (Birnie & Fox, 1965), it was found that, in contrast with previously described mammalian DNA nucleotidyltransferases, the mouse-embryo enzyme is most active in the presence of Mn^{2+} at pH 6.4.

EXPERIMENTAL

Materials. [^3H]dTTP* (specific activity 1 mc/ μmole) was purchased from Schwarz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). dATP, dGTP, dCTP, ATP and barium 3-phosphoglycerate were purchased from Sigma (London) Chemical Co. Ltd. (London, S.W. 6). Sodium 3-phosphoglycerate was prepared from the barium salt by titration with Na_2SO_4 . Highly polymerized salmon-sperm DNA (Sigma) dissolved in 50 mM-KCl containing 1 mM- MgCl_2 was used as primer DNA. Stock solutions containing 1 mg. of DNA/ml. were denatured by heating at 100° for 10 min. and then cooling rapidly in ice. [$2\text{-}^{14}\text{C}$]Thymidine was purchased from The Radiochemical Centre (Amersham, Bucks.). 5'-Phosphodiesterase (EC 3.1.4.1) from *Crotalus adamanteus* venom, tris (specially purified for enzyme work) and all other materials (AnalaR quality) were obtained from British Drug Houses Ltd. (Poole, Dorset). Actinomycin D was a gift from Dr D. C. Remy of Merck, Sharpe and Dohme Inc. (West Point, Pa., U.S.A.).

Preparation of embryo extracts. Extracts were prepared from 13- or 14-day-old mouse embryos (strain TO). The embryos were removed and immediately chilled in ice-cold phosphate-buffered saline (0.145 M-NaCl-10 mM-sodium phosphate, pH 7.2). All further operations were carried out at 0-4°. The embryos were minced by squeezing them

* Abbreviations: dATP, dGTP, dCTP and dTTP, the 5'-triphosphate esters of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine respectively.

through a fine stainless-steel gauze (22 × 22 mesh). The minced material was washed twice by suspending it in 10 vol. of phosphate-buffered saline, allowing it to settle for 5 min. and then removing the supernatant fluid. The minced tissue was finally collected by centrifugation at 1000g for 5 min. The pellet was homogenized in 2 vol. of 1 mM-tris-HCl buffer, pH 7.5, with an all-glass homogenizer and subjected to ultrasonic vibrations (Dawe Soniprobe, maximum power, $\frac{1}{8}$ in. micro-tip) for three periods of 1 min. The homogenate was centrifuged at 77000g for 90 min. (MSE Superspeed 40 with no. 30 rotor) and the clear supernatant fluid, which formed the standard enzyme preparation, was removed and stored at -20° until used. As a routine these extracts were used within 1 week of preparation although they could be stored at -20° for a month without significant loss of DNA-nucleotidyltransferase activity.

The concentration of protein in the extracts was measured by a quantitative biuret method (Gornall, Bardawill & David, 1949), with crystalline bovine serum albumin as a standard. The embryo extracts contained 6–10 mg. of protein/ml.

Assay of DNA-nucleotidyltransferase activity. Standard reaction mixtures contained tris (100 μ moles/ml.), KCl (100 μ moles/ml.), dATP, dGTP and dCTP (0.1 μ mole of each/ml.), ATP (18 μ moles/ml.), sodium 3-phosphoglycerate (15 μ moles/ml.), heat-denatured DNA (200 μ g./ml.), 2-mercaptoethanol (0.2 μ mole/ml.), [3 H]dTTP [1 m μ mole (1 μ Ci)/ml.] and MgSO₄ (18 μ moles/ml.) or MnSO₄ (18 μ moles/ml.). The pH of the mixtures was adjusted to 6.5 with N-HCl and 0.5 ml. portions were introduced into 10 ml. conical centrifuge tubes in an ice-water bath. The reaction was started by adding embryo extract (0.5 ml.) and incubating at 37°. After 30 min. the tubes were chilled in ice and the reaction was stopped by adding 2 ml. of ice-cold 2N-HClO₄ containing 40 mM-Na₄P₂O₇ to each tube, followed by 1 ml. of carrier calf-thymus DNA (1 mg./ml.; British Drug Houses Ltd.) to ensure complete precipitation of DNA. The mixtures were kept at 0° for 15 min. before being centrifuged at 1500g for 15 min. The nucleoprotein precipitates were washed twice by resuspension in 2 ml. portions of ice-cold 0.2N-HClO₄ containing 4 mM-Na₄P₂O₇ followed by centrifugation. Each washed pellet was drained briefly and dissolved in 2 ml. of 0.5N-KOH by heating at 50° for 30 min. The tubes were chilled in ice and 2 ml. of ice-cold 25% (w/v) trichloroacetic acid containing 40 mM-Na₄P₂O₇ was added to each. After 1 hr. at 0°, the nucleoprotein precipitates were resuspended and each suspension was filtered through a glass-fibre pad (Whatman G/FC, diam. 2.5 cm.). The pads were washed twice with 2 ml. portions of ice-cold 5% (w/v) trichloroacetic acid and twice with 2 ml. portions of ether. The pads were dried at room temperature and mounted on aluminium planchets with a trace of silicone grease.

The amount of radioactivity on each pad was determined with a Nuclear-Chicago gas-flow counter (counting efficiency, 3.5%). Since in any one experiment the same amount of embryo extract was added to each tube and therefore the amount of nucleoprotein on each filter pad was the same, the results have not been corrected for self-absorption losses.

Each reaction mixture was assayed in duplicate, with reproducibility within 10%. Complete reaction mixtures to which HClO₄ was added immediately after the enzyme extract served as controls for non-specific absorption of labelled precursor.

Other enzyme assays. Thymidine-triphosphatase activity was assayed under the conditions for assay of DNA nucleotidyltransferase except that ATP, 3-phosphoglycerate and DNA were omitted. After incubation for 30 min. the reaction was stopped by heating at 100° for 5 min., protein was removed by centrifugation and the reaction products were separated by chromatography on DEAE-cellulose paper (Whatman DE81) (Ives, Morse & Potter, 1963). Radioactive products were located and measured with a Nuclear-Chicago 4 π Actigraph (counting efficiency, 0.8%) equipped with an automatic integrator and print-out (George Kent Ltd., Luton), with reproducibility within 10%.

Deoxyribonuclease activity was assayed under the conditions for assay of DNA nucleotidyltransferase except that [14 C]polydeoxyribonucleotide [150 μ g. (500 counts/min.)/ml.] replaced [3 H]dTTP in the reaction mixture. After incubation for 1 hr. undegraded [14 C]polynucleotide was isolated and measured as described for the DNA-nucleotidyltransferase assay.

Preparation of [14 C]polydeoxyribonucleotide. Standard DNA-nucleotidyltransferase assay mixtures, scaled up sevenfold, in which [2- 14 C]thymidine [20 m μ moles (0.5 μ Ci)/ml.] replaced [3 H]dTTP and which contained either MgSO₄ or MnSO₄, were incubated for 1 hr. After addition of carrier denatured salmon-sperm DNA (2 mg.) the [14 C]nucleoprotein was isolated as described for the DNA-nucleotidyltransferase assay except that the product was further purified by three additional cycles of solution in KOH and precipitation with HClO₄. The [14 C]nucleoprotein was dissolved in 7 ml. of 0.05N-KOH and the solution was adjusted to pH 8 with HCl. Insoluble material was removed by centrifugation at 1500g for 15 min.

RESULTS

Incorporation of [3 H]dTTP into polynucleotide. Extracts of mouse embryos rapidly incorporate tritium from [3 H]dTTP into polydeoxyribonucleotide material. The incorporation of the label is stimulated by the presence of dATP, dGTP and dCTP, heat-denatured DNA, ATP, K⁺ and Mg²⁺, and is inhibited by actinomycin D. The radioactive product of the reaction is stable to cold dilute acid and hot dilute alkali, but is degraded by hot dilute acid and 5'-phosphodiesterase.

Replacement of Mg²⁺ in the reaction mixture by Mn²⁺ results in a striking increase in the rate of incorporation of [3 H]dTTP into polynucleotide (Fig. 1). With either Mg²⁺ or Mn²⁺, incorporation approaches a maximum after the same time-interval. The response of the reaction to various concentrations of Mg²⁺ and Mn²⁺, either alone or in combination, is shown in Fig. 2. At all concentrations studied, the activity of the extracts is several times greater with Mn²⁺ than with Mg²⁺. Various combinations of Mg²⁺ and Mn²⁺ have a marked synergistic effect on the reaction. However, no combination of Mg²⁺ and Mn²⁺ stimulates the incorporation of [3 H]dTTP to as great an extent as a concentration of Mn²⁺ equal to the total concentra-

tion of the combined ions. Total ionic concentrations in excess of 15 mM are inhibitory.

The effect of pH on the activity of embryo extracts was determined over the range pH 6–8. There is no significant change in the optimum pH

when Mn^{2+} replaces Mg^{2+} , maximum incorporation of $[^3\text{H}]\text{dTTP}$ occurring at pH 6.7 or pH 6.4 in the presence of Mg^{2+} or Mn^{2+} respectively (Fig. 3). However, the response of the reaction to changes in pH is much greater with Mn^{2+} than with Mg^{2+} . At all pH values examined, Mn^{2+} stimulates more than Mg^{2+} , the maximum difference between the two ions occurring at pH 6.4. The difference is much less marked at pH 7.5.

Although heat-denatured DNA stimulates $[^3\text{H}]\text{dTTP}$ incorporation to a greater extent with Mn^{2+} than with Mg^{2+} , with either ion the enzyme activity becomes saturated with respect to primer at the same concentration of denatured DNA. When denatured DNA is replaced with unheated DNA, the incorporation of $[^3\text{H}]\text{dTTP}$ is decreased to a greater extent in the Mn^{2+} -stimulated reaction (70%) than in the Mg^{2+} -stimulated reaction (45%) (Table 1). Despite this, when unheated DNA is used as primer, the rate of incorporation with Mn^{2+} is still four times that with Mg^{2+} .

Contaminating enzyme activities. Since no attempt was made to purify the DNA nucleotidyl-transferase of the embryo extracts, the activities of contaminating enzymes that would influence the rate of incorporation of $[^3\text{H}]\text{dTTP}$ into polynucleotide were measured with particular reference to the relative effects of Mg^{2+} and Mn^{2+} . Though mouse-embryo extracts are capable of rapidly degrading

Fig. 1. Time-course of the incorporation of $[^3\text{H}]\text{dTTP}$ into polydeoxyribonucleotide at pH 6.5, in the presence of either 9 mM- MgSO_4 (O) or 9 mM- MnSO_4 (●). The embryo-extract protein concentration was 4.4 mg./ml.

Fig. 2. Effect of Mg^{2+} and Mn^{2+} on the incorporation of $[^3\text{H}]\text{dTTP}$ into polydeoxyribonucleotide at pH 6.5: O, Mg^{2+} ; Δ, $\text{Mg}^{2+} + 2.5 \text{ mM } \text{Mn}^{2+}$; ▲, $\text{Mg}^{2+} + 5 \text{ mM } \text{Mn}^{2+}$; ●, Mn^{2+} . The embryo-extract protein concentration was 3.5 mg./ml.

Fig. 3. Effect of pH on the incorporation of $[^3\text{H}]\text{dTTP}$ into polydeoxyribonucleotide in the presence of either 9 mM- MgSO_4 (O) or 9 mM- MnSO_4 (●). The embryo-extract protein concentration was 3.8 mg./ml.

dTTP, the thymidine-triphosphatase activity is abolished by the presence of ATP and either Mg^{2+} or Mn^{2+} . As measured by incubating [^{14}C]polydeoxyribonucleotide in complete reaction mixtures lacking only labelled precursor, the deoxyribonuclease activity of the extracts is minimal. Under these conditions no more than 5% of the polynucleotide becomes acid-soluble after 1 hr. in the presence of either Mg^{2+} or Mn^{2+} . However, the extracts do contain some nuclease activity since a 30 min. preincubation of the primer DNA with an embryo extract causes a 40% increase in the subsequent rate of incorporation of [3H]dTTP. This increase in priming ability is similar in amount

to that which can be produced by the action of endonuclease on primer DNA (Keir *et al.* 1962; Furlong, 1965). Replacement of Mg^{2+} with Mn^{2+} during preincubation has no effect on the amount of stimulation produced.

Effect of 5'-phosphodiesterase on the reaction product. As it had previously been shown (G. D. Birnie & S. M. Fox, unpublished work) that the rate-limiting step in the incorporation of [^{14}C]thymidine into polydeoxyribonucleotide by mouse-embryo extracts, in the presence of either Mg^{2+} or Mn^{2+} , is at the level of DNA nucleotidyltransferase, [^{14}C]thymidine was used in place of [3H]dTTP to synthesize simultaneously two samples of [^{14}C]polydeoxyribonucleotide. Both samples, one of which was isolated from a reaction mixture containing Mg^{2+} , the other from a mixture containing Mn^{2+} , were treated with snake-venom 5'-phosphodiesterase as described by Adler, Lehman, Bessman, Simms & Kornberg (1958). The rates at which ultraviolet-absorbing material and radioactivity were rendered acid-soluble were measured. Table 2 shows that, for both the products of the Mg^{2+} - and Mn^{2+} -stimulated reactions, acid-soluble nucleotides and radioactivity are released at the same rate during digestion of the polynucleotide. Since 5'-phosphodiesterase acts by stepwise removal of nucleoside 5'-monophosphates from the nucleoside end of the polynucleotide (Adler *et al.* 1958), it is concluded that the label is randomly distributed along the polynucleotide chains.

Table 1. *Effect of Mg^{2+} and Mn^{2+} on the priming of DNA nucleotidyltransferase by denatured and native DNA*

Incorporation of [3H]dTTP, in the presence of $MgSO_4$ or $MnSO_4$, was measured with standard assay mixtures containing the primer DNA indicated. The embryo-extract protein concentration was 3.5 mg./ml.

DNA primer	[3H]dTTP incorporated (counts/min.)	
	In Mg^{2+} mixture	In Mn^{2+} mixture
None	40	154
Heated DNA (65 $\mu g.$)	318	2766
Native DNA (65 $\mu g.$)	176	818

Table 2. *Action of 5'-phosphodiesterase on [^{14}C]polydeoxyribonucleotide products from Mg^{2+} - and Mn^{2+} -stimulated reactions*

Reaction mixtures containing [^{14}C]polynucleotide, 1 mg. of 5'-phosphodiesterase, 50 μ moles of $MgCl_2$ and 200 μ moles of tris-HCl buffer, pH 8.5, in a total volume of 5.0 ml., were incubated at 37°. Samples (0.5 ml.) were removed at the times indicated and mixed with 0.5 ml. of ice-cold 0.4 N-HClO₄. The mixtures were centrifuged at 1500g for 15 min., and radioactivity and E_{260} were measured in the supernatant solutions. The acid-insoluble pellet was washed with 0.2 N-HClO₄ and its radioactivity was measured as described in the Experimental section. The total extinction of the [^{14}C]polynucleotide was determined after hydrolysis with N-HClO₄.

Time of incubation (min.)	Mg^{2+} product*			Mn^{2+} product†		
	% of total extinction	% of total radioactivity	Specific activity (counts/min./E unit)	% of total extinction	% of total radioactivity	Specific activity (counts/min./E unit)
15	30.2	29.3	64	32.1	32.4	254
30	54.0	46.1	64	52.9	47.3	244
45	58.8	52.9	70	55.9	54.2	274
60	66.4	58.8	54	57.8	57.4	292
90	66.4	62.7	58	62.2	65.6	289
120	71.3	66.6	62	69.2	69.8	273
180	70.0	73.5	70	83.3	76.8	238

* Mg^{2+} product: 800 $\mu g.$ (1020 counts/min.) of [^{14}C]polydeoxyribonucleotide isolated from a reaction mixture containing Mg^{2+} (see the Experimental section).

† Mn^{2+} product: 770 $\mu g.$ (4040 counts/min.) of [^{14}C]polydeoxyribonucleotide isolated from a reaction mixture containing Mn^{2+} (see the Experimental section).

DISCUSSION

The DNA-nucleotidyltransferase activity of mouse-embryo extracts is similar to the DNA nucleotidyltransferase in both crude and partially purified preparations from other mammalian tissues in its dependence on the four complementary deoxyribonucleoside 5'-triphosphates, primer DNA and a bivalent cation (Bollum & Potter, 1958; Mantsavinos & Canellakis, 1959a,b; Mantsavinos, 1964; Furlong, 1965; Shepherd & Keir, 1966). However, the mouse-embryo enzyme does differ significantly from these other DNA nucleotidyltransferases in that maximum activity of the enzyme is observed with Mn^{2+} at pH 6.4.

The optimum pH of the mouse-embryo enzyme is significantly lower than that of DNA-nucleotidyltransferase preparations from other mammalian tissues. With either Mg^{2+} or Mn^{2+} , maximum activity of mouse-embryo DNA nucleotidyltransferase occurs near pH 6.5 whereas maximum activity of other DNA nucleotidyltransferases is reported to occur close to pH 7.5 (Bollum & Potter, 1958; Mantsavinos & Canellakis, 1959b; Smellie *et al.* 1959; Bollum, 1960; Mantsavinos, 1964; Furlong, 1965).

Mg^{2+} and Mn^{2+} stimulations of DNA nucleotidyltransferase have been compared with enzyme preparations from several mammalian tissues. The synthesis of polydeoxyribonucleotide by crude preparations from Ehrlich ascites-tumour cells (Smellie *et al.* 1959) and by purified preparations from calf thymus (Bollum, 1960) and Landschütz ascites-tumour cells (Shepherd & Keir, 1966) is stimulated more effectively by Mg^{2+} than by Mn^{2+} . Purified DNA nucleotidyltransferase from regenerating rat liver is not activated by Mn^{2+} although a combination of Mn^{2+} and Ca^{2+} does partly activate this enzyme (Mantsavinos, 1964). Mg^{2+} and Mn^{2+} are equally effective activators of crude preparations from mouse leukaemic cells (Mantsavinos & Canellakis, 1959a) and partly purified preparations from Walker carcinosarcoma tissue (Furlong, 1965). In contrast, Mn^{2+} stimulates the incorporation of [^{14}C]thymidylate into polynucleotide by crude extracts of regenerating rat liver to a much greater extent than Mg^{2+} , and a mixture of Mg^{2+} and Mn^{2+} is even more effective (Mantsavinos & Canellakis, 1959b). Mouse-embryo extracts resemble these regenerating-liver preparations in their response to Mn^{2+} (though not in their optimum pH), except that combinations of Mg^{2+} and Mn^{2+} are less effective than Mn^{2+} alone.

The marked difference between the stimulatory effects of Mg^{2+} and Mn^{2+} on the incorporation of

[3H]dTTP into polydeoxyribonucleotide by mouse-embryo extracts is not caused by Mn^{2+} inhibiting the degradation of precursor dTTP or product polynucleotide, or by Mn^{2+} stimulating the activation of primer DNA. Also, there is no difference between the products of Mg^{2+} - and Mn^{2+} -stimulated reactions so far as the distribution of the incorporated label in the polynucleotide chains is concerned. Thus it appears that, in contrast with previously described DNA nucleotidyltransferases, the mouse-embryo enzyme is more effectively activated by Mn^{2+} than by Mg^{2+} , particularly below pH 7. Since mouse embryos are an excellent source of rapidly dividing tissue, these enzyme preparations are of particular interest in view of the suggestion (Keir, 1965) that DNA nucleotidyltransferase may exist in either a 'native' or an 'altered' form and that the ratio between the two forms in a cell depends on whether the cell is actively making DNA or not.

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