EFFECTS OF DIVALENT CATIONS ON THE CONVERSION OF BACTERIOPHAGE M13 AND ϕ X174 DNAs TO THEIR REPLICATIVE FORMS BY EXTRACTS OF *ESCHERICHIA COLI*

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The conversion of single-stranded circular DNA of bacteriophage M13 or ϕ X174 to the double-stranded form by cell-free extracts of *Escherichia coli* requires Mg^{2+} as a cofactor. In this report, it was examined whether any other divalent cation could substitute for Mg^{2+} as a cofactor at the replication of these DNAs. The results showed that Mn^{2+} , Co^{2+} , Sr^{2+} , and Ba^{2+} could replace Mg^{2+} . The replacement, however, induced some abnormal synthesis of DNA. The DNA synthesized with Mn^{2+} or Sr^{2+} contained several interruptions in the synthesized strand. The synthesis of DNA seems to be initiated at several points on the identical template. The DNA synthesized with Mn^{2+} , Co^{2+} , or Sr^{2+} had plaque-forming ability, and the product synthesized with Mn^{2+} , or Co^{2+} but not with Sr^{2+} , showed high frequencies of mutation. Although the synthesis of ϕ X174 DNA was not inhibited by rifampicin when Mg^{2+} was used as a cofactor, it was inhibited when Mn^{2+} or Sr^{2+} was used instead of Mg^{2+} .

All known DNA polymerases [EC 2.7.7.7] require Mg^{2+} as divalent cation for activity (1). Manganese ion which is a potent mutagen (2–4) could usually substitute for Mg^{2+} for their activities. The fidelity of DNA replication was reduced with Mn^{2+} (5–8). SIROVER and LOEB (9, 10) suggested that all of the metal mutagens or carcinogens would affect the activities of DNA polymerases and induce the perturbations in the fidelity of DNA synthesis.

The effects of Mn^{2+} and some other metals were examined with DNA polymerase I of *Escherichia coli* (*E. coli*). The results suggested that the mutagenic metals stimulated the incorporation of non-complementary nucleotides into DNA (6, 8, 11, 12). BERG (13) discovered that ribonucleotide residues could be incorporated into DNA chains by DNA polymerase I of *E. coli*, when synthesis was carried out in the presence of Mn^{2+} in place of Mg^{2+} .

KORNBERG and GEFTER (14), and LIVINGSTON and RICHARDSON (15) reported that Mn^{2+} could substitute for Mg^{2+} for the activity of purified DNA polymerase III which is an essential enzyme for the replication of DNA (16, 17), but the analysis of the product has not been carried out.

Cell-free extracts prepared from *E. coli* cells lacking DNA polymerase I can convert single-stranded circular DNA of phage M13 or ϕ X174 to the double-stranded circular form in a reaction which requires DNA polymerase III and other host proteins (18–20). We have used such a system to investigate the effects of divalent cations on the synthesis of phage DNA. The results show that the synthesis of DNA was observed with Mn^{2+} , Co^{2+} , Sr^{2+} , and Ba^{2+} . The DNA synthesized with Mn^{2+} or Sr^{2+} had some breaks in the products. When plaque-forming activities of these products were assayed on the cells treated with $CaCl_2$, enhanced mutation frequencies were observed with the DNA synthesized with Mn^{2+} and Co^{2+} .

MATERIALS AND METHODS

Bacterial and phage strains. *E. coli* H560 (*polA*, *endI*, *thy*, and *str*) was obtained from R. Okazaki and was used to prepare the cell-free extracts. *E. coli* HfrC was used to grow phage M13. *E. coli* HF4704 (*su*, *hcr*, *thy*, and ϕX^s) and *E. coli* HF4714 (*su*⁺, *thy*, ϕX^s) which were used to grow ϕ X174am3, and ϕ X174am3 were obtained from C. C. Richardson. Phage M13 was obtained from D. Pratt.

Chemicals. Divalent metal salts: $MgCl_2$, $CaCl_2$, $CrCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, $SrCl_2$, $CdCl_2$, $BaCl_2$, $HgCl_2$, and $Pb(NO_3)_2$ were best-grade chemicals purchased from Wako Pure Chemical Industries, Ltd. Cesium sulfate was purchased from Nakarai Chemicals, Ltd. Ribo- and deoxyribonucleoside triphosphates and lysozyme were purchased from Seikagaku Kogyo Co., Ltd. Nucleotides were purified by chromatography on DEAE-Sephadex A25 as described by MASAMUNE and RICHARDSON (21). All nucleotides were purified as ammonium salts. Rifampicin was given by M. Kumagai (Institute of Microbial Chemistry, Tokyo). [3H]-thymidine (20Ci/mmol) and [3H]-deoxythymidine triphosphate (60Ci/mmol) were the products of New England Nuclear.

Growth of phages and preparation of their DNA. Phages M13 and ϕ X174am3 were grown as described previously (22, 23). Phage M13 labelled with [3H]-thymidine was prepared as described previously (23). The DNAs were extracted with phenol from these phages and were dissolved in 10 mM Tris-HCl buffer (pH 7.6) containing 0.1 mM EDTA. The final concentration of these DNAs was adjusted to 2 μ mol of nucleotide per ml. Final concentration of [3H]-DNA was 0.26 μ mol/ml, 1.7×10^5 cts/min/ μ mol. The concentration of DNAs of phages M13 and ϕ X174 was calculated from the absorbance measured at 260 nm (22).

Preparation of cell-free extract. A cell-free extract from *E. coli* H560 was

prepared following the method of W. T. WICKNER *et al.* (19).

Conversion of single-stranded DNA to double-stranded form. The reaction mixture (50 μ l) contained 20 mM Tris-HCl buffer (pH 7.6), 30 mM NaCl, 250 μ M each of ATP, CTP, GTP, and UTP, 500 μ M each of dATP, dCTP, dGTP, and 250 μ M of [3 H]-dTTP (30 cts/min/pmol), 5 nmol of phage DNA and 25 μ l of cell extracts. Various concentration of divalent cations were added to the above reaction mixture. After incubation at 30° for 30 min, the reaction was stopped by the addition of 1 ml of 1 N HCl-0.1 M pyrophosphate. The acid-insoluble radioactivity was determined as described previously (23). The effect of rifampicin on the synthesis of DNA was examined by adding 1 μ l of rifampicin (0.5 mg/ml) to the reaction mixture prior to the incubation.

Quantification of the concentration of magnesium in the extracts of E. coli H560. The concentration of magnesium in the cell-free extracts of *E. coli* H560 was quantified following the standard procedure of atomic absorption spectrophotometry (flame method) using a Hitachi 170-10 atomic absorption spectrophotometer at 285.2 nm. The measurements were performed with the help of Dr. K. Hayakawa in our Department.

Velocity sedimentation and density gradient centrifugation.

Sucrose gradient centrifugation: Analysis of DNA by sucrose gradient centrifugation was performed as described previously (23).

Cs₂SO₄-HgCl₂ density gradient centrifugation: The analysis of DNA with Cs₂SO₄-HgCl₂ density gradient centrifugation was performed following the method of OISHI (24). After the synthesis of DNA for 30 min at 30°, 20 μ l of 0.2 M EDTA and 20 μ l of sodium lauroyl sarcosinate (10% solution) were added to the reaction mixture (200 μ l) which was then extracted several times with chloroform-isoamyl-alcohol (24:1, v/v) to remove proteins. The water layer was dialysed against 20 mM Na₂SO₄-10 mM Na₂B₄O₇ buffer (pH 9.4). After dialysis, 50 μ l of the sample (3 nmols of DNA) and 50 μ l of phage T7 DNA (60 nmol) dissolved in the same buffer were mixed and Cs₂SO₄ dissolved to saturation in the above buffer was added to make the density of the solution ρ =1.626. One-tenth ml of 0.15 mM HgCl₂ was further added to make [Hg²⁺]/[DNA]=0.25. The density of the solution was finally adjusted to ρ =1.570 by the addition of H₂O. The mixture was centrifuged at 35,000 rpm in a Hitachi RP55TA rotor for 40 hr at 5°. The acid-insoluble radioactivities were measured after collecting the samples from the bottom of the tube. [3 H]-M13 DNA (single-strand) and [3 H]-M13 (duplex) which was synthesized with Mg²⁺ in the extracts of *E. coli* H560 under the standard condition (23) were spun in parallel as density markers.

Other methods. *Assay of the plaque-forming ability of DNA synthesized with various metal ions:* DNA synthesized with Mg²⁺, Mn²⁺, Co²⁺, or Sr²⁺ and with M13 DNA as template was centrifuged in neutral sucrose gradient to purify the duplex molecule, the plaque-forming ability of which was measured by plating with *E. coli* HfrC treated with CaCl₂ following the method of COHEN *et al.* (25).

Protein was determined by the method of LOWRY *et al.* (26) using bovine albumin (product of Miles Laboratories, Ltd.) as a standard.

RESULTS

Concentration of magnesium in the extracts of E. coli H560

Since Mg^{2+} is the natural cofactor of DNA polymerase, magnesium in the extracts would disturb the measurements of the activities of metal ions on the synthesis of DNA. The concentration of magnesium in the extracts of *E. coli* H560 grown in L-broth was quantified by atomic absorption spectrophotometry. It was about 6 mM or 0.4 μ mol/mg protein.

Effects of divalent metal salts on the synthesis of DNA

The synthesis of DNA was measured with various divalent metal salts using phage M13 or ϕ X174 DNA as template. The result is shown in Table 1. The synthesis was observed with $MgCl_2$, $MnCl_2$, $CoCl_2$, $SrCl_2$, and $BaCl_2$. Little synthesis was recognized with other salts [$CaCl_2$, $CrCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, $CdCl_2$, $HgCl_2$, and $Pb(NO_3)_2$]. Figures 1a and 1b show the effects of concentration of the former metal salts on the synthesis of DNA with M13 and ϕ X174 DNA as template, respectively. The optimal concentration was specific with each salt and the concentration was almost the same with both templates.

Table 1. The effect of metal salts on the synthesis of DNA in the extracts of *E. coli* polA.

Compound	Concentration of salt (mM)	dTMP incorporated (pmol)	
		M13 DNA	ϕ X174 DNA
None	—	0.0	0.0
$MgCl_2$	7.5	46.5	33.0
$MnCl_2$	5.0	78.0	116.0
$CoCl_2$	1.5	23.0	16.0
$SrCl_2$	10.0	20.7	96.0
$BaCl_2$	7.5	48.4	16.0

The reaction mixture containing various salt and phage M13 or ϕ X174 DNA as template was incubated at 30° for 30 min, and the acid-insoluble radioactivity was measured.

Phage M13 DNA as template (Fig. 1a). The amount of synthesis of DNA with Mn^{2+} exceeded that with Mg^{2+} at the optimal concentration, and the synthesis was strongly inhibited at higher concentration than optimum. The synthesis with Co^{2+} was poor and the optimal concentration was low. The concentration of Sr^{2+} required for its maximal activity was higher than with other metals. All of these syntheses were inhibited by the addition of rifampicin, a specific inhibitor of RNA polymerase (27).

Fig. 1. Effect of the concentration of metal salts (MgCl₂, MnCl₂, CoCl₂, SrCl₂, and BaCl₂) on the synthesis of DNA.

Dependence of the synthesis of DNA on the concentration of divalent cations was measured by the incorporation of [³H]-dTMP into acid-insoluble fraction. The reaction mixture was incubated at 30° for 30 min. a) Phage M13 DNA was used as template. b) Phage ϕ X174 DNA was used as template. Dotted lines (---) are the results with rifampicin of the corresponding symbols. ●, synthesis of DNA with MgCl₂; ○, synthesis of DNA with MnCl₂; ▲, synthesis of DNA with CoCl₂; △, synthesis of DNA with SrCl₂; ×, synthesis of DNA with BaCl₂.

Phage ϕ X174 as template (Fig. 1b). The amount of synthesis of DNA with Mn²⁺ or Sr²⁺ far exceeded that with Mg²⁺ at the optimal concentration. The synthesis was partially inhibited by rifampicin when Mn²⁺ or Sr²⁺ was used as a cofactor, but it was resistant when Mg²⁺, Co²⁺, or Ba²⁺ was used.

It would be noteworthy that no DNA synthesis was detected without addition of any divalent metals with either template, even if extracts supplied about 3 mM magnesium to the reaction mixture.

Effect of concentration of template DNA

The effect of concentration of template DNA on the synthesis of DNA was examined and the results are shown in Figs. 2a and 2b.

Phage M13 DNA as template (Fig. 2a). When Mg²⁺, Co²⁺, Sr²⁺, or Ba²⁺ was used as a cofactor, the amount of DNA synthesis levelled off at the concentration of 20–30 μ M of template. With Mn²⁺, however, the synthesis increased with increasing concentration of template up to 100 μ M.

Phage ϕ X174 DNA as template (Fig. 2b). The amount of DNA synthesized with Mn²⁺ or Sr²⁺ increased with increasing concentration of template. The

Fig. 2. Effect of the concentration of template DNA on the synthesis of DNA. Synthesis of DNA was measured by changing the concentration of template molecule at the optimal concentration of each metal salt (7.5 mM of MgCl_2 or BaCl_2 , 5 mM of MnCl_2 , 1.5 mM of CoCl_2 , and 10 mM of SrCl_2). a) Phage M13 DNA as template. b) Phage ϕX174 DNA as template. The assay conditions of the synthesis of DNA and the symbols of the figure are the same as in Fig. 1. Dotted lines are the results with rifampicin.

syntheses were, however, maxima at 20 μM of template when rifampicin was added to the reaction mixture, and the amounts were reduced to a level comparable with those obtained with Mg^{2+} . The maximal incorporation occurred at 20 μM of template, when Mg^{2+} , Co^{2+} , or Ba^{2+} was used as a cofactor. The addition of rifampicin did not inhibit the synthesis of DNA with these metals.

Kinetics of DNA synthesis

Time courses of DNA synthesis with these metals were examined using 100 μM of M13 and ϕX174 DNA as template. The results are shown in Figs. 3a and 3b.

Phage M13 DNA as template (Fig. 3a). Maximal incorporation of [³H]-dTTP into the acid-insoluble fraction was observed at about 30 min with Mg^{2+} , Mn^{2+} , Co^{2+} , and Ba^{2+} , but with Sr^{2+} , the synthesis of DNA continued slowly for more than 90 min. When Mn^{2+} was used as a cofactor, the rate and extent of DNA synthesis exceeded those with Mg^{2+} .

Phage ϕX174 DNA as template (Fig. 3b). When Mn^{2+} or Sr^{2+} was used as a cofactor, the rate and extent of DNA synthesis were much greater than those with Mg^{2+} . The extra incorporation obtained with these metals was, however, diminished to a level comparable with that gained with Mg^{2+} when rifampicin

Fig. 3. Time course experiments of the synthesis of DNA.

The incorporation of [^3H]-dTMP into acid-insoluble fraction was measured at the indicated times at the optimal concentration of each metal salts and $100\ \mu\text{M}$ of template DNA. a) Phage M13 DNA as template. b) Phage ϕX174 DNA as template. Symbols are the same as in Fig. 1. Dotted lines are the results with rifampicin.

was added.

Centrifugal analysis of M13 DNA synthesized with several divalent cations

i) *Sucrose gradient centrifugations.* DNA synthesized with M13 DNA as template was analyzed with alkaline sucrose gradient centrifugations. The results were shown in Figs. 4a and 4b. DNA synthesized with any metal ion (Mg^{2+} , Mn^{2+} , Co^{2+} , or Sr^{2+}) was the intact linear form of M13 DNA, when the concentration of template was $20\ \mu\text{M}$ (Fig. 4a). When the concentration of template was $100\ \mu\text{M}$, the length of molecules synthesized with Mn^{2+} or Sr^{2+} was shorter (about one-third) than template, but not with Mg^{2+} or Co^{2+} (Fig. 4b).

The short molecules obtained with Mn^{2+} or Sr^{2+} could be due to the hydrolysis of ribonucleotides incorporated into DNA chain during the centrifugation in alkaline solution. The following results suggested that the shortness of the products was genuine and would not be due to the hydrolysis by alkali. The hydrolysis of the products in $0.3\ \text{N}$ NaOH at 37° for 18 hr did not affect the size (Fig. 4b) and their heat denaturation produced the shortness in neutral sucrose gradient spin. Although the lengths of the products with Mn^{2+} or Sr^{2+} were shorter than those of template after denaturation, they showed the intact double-stranded open circular forms when analyzed in neutral sucrose gradient centrifugation under nondenaturing conditions (see Fig. 6b).

ii) *$\text{Cs}_2\text{SO}_4\text{-HgCl}_2$ density gradient centrifugation.* The results shown above

Fig. 4. Alkaline sucrose gradient centrifugation of the DNA synthesized with M13 DNA as template.

DNA synthesized with each metal salt was centrifuged in 5–20% alkaline sucrose gradient which contained 0.2 N NaOH, 0.8 M NaCl, and 1 mM EDTA at 33,000 rpm for 16 hr at 4° with Hitachi RPS55 rotor. After the spin, fractions were collected from the bottom of the tube and acid-insoluble radioactivity was measured as described in MATERIALS AND METHODS. An arrow indicates the position of intact M13 DNA, and the lines numbered 1, 1/2, and 1/3 indicate the relative sizes of the DNA calculated (23). a) Results with 20 μ M of M13 DNA as template. (Results with 20 μ M of ϕ X174 DNA as template were essentially the same as those with M13 DNA. b) Results with 100 μ M of M13 DNA as template. ●, DNA synthesized with MgCl_2 ; ○, DNA synthesized with MnCl_2 ; ▲, DNA synthesized with CoCl_2 ; △, DNA synthesized with SrCl_2 ; ⊙, centrifugation of DNA synthesized with MnCl_2 after 18 hr incubation at 37° in 0.3 N NaOH; △, centrifugation of DNA synthesized with SrCl_2 after 18 hr incubation at 37° in 0.3 N NaOH.

suggested that the molecules synthesized with Mn^{2+} or Sr^{2+} had intact duplex structures and had few gaps, even if the lengths of DNA synthesized were shorter than those of template DNA. In order to confirm the absence of gaps, the molecules synthesized with Mn^{2+} or Sr^{2+} were analyzed with Cs_2SO_4 - HgCl_2 density gradient centrifugation (28). The result is shown in Fig. 5. It is clear that every

Fig. 5. $\text{Cs}_2\text{SO}_4\text{-HgCl}_2$ density gradient centrifugation.

DNA synthesized with $100\ \mu\text{M}$ of M13 DNA and one of the four metal salts was centrifuged in $\text{Cs}_2\text{SO}_4\text{-HgCl}_2$ as described in MATERIALS AND METHODS. An arrow with S.S. indicates the position of single-stranded DNA (---x---) and the arrow with D.S. indicates the position of duplex DNA. Other symbols are the same as in Fig. 4.

DNA had the same density as that of the intact duplex molecule, suggesting the absence of gaps.

Integrity of template DNA

Integrity of template DNA during the synthesis of DNA with Mn^{2+} or Sr^{2+} was examined following the method described previously (23), as the shortness of DNA synthesized with Mn^{2+} or Sr^{2+} might be due to the fragmentation of template molecule during incubation. The result showed that there was essentially no fragmentation of the template with either metal.

Analysis of DNA synthesized with ϕX174 DNA as template

As shown in Table 1, Figs. 1b, 2b, and 3b, the synthesis of DNA was observed with Mg^{2+} , Mn^{2+} , Co^{2+} , Sr^{2+} , and Ba^{2+} . The structures of DNA synthesized with Mn^{2+} were compared with those with Mg^{2+} by alkaline and neutral sucrose gradient centrifugations. The results with $100\ \mu\text{M}$ of template DNA are shown in Fig. 6a. The size of DNA synthesized with Mg^{2+} remained intact whether or not DNA was synthesized in the presence of rifampicin. When Mn^{2+} was used as a cofactor, the lengths of the product were shorter than those of template, as was the case with M13 DNA (Fig. 4b). The main product, however, had intact length when rifampicin was added to the reaction mixture even with Mn^{2+} . The DNA synthesized with Mg^{2+} or Mn^{2+} had intact size when the concentration of

Fig. 6. Centrifugal analysis of DNA synthesized with $100\ \mu\text{M}$ of ϕX174 DNA as template.

An arrow indicates the position of intact M13 DNA, and the lines numbered 1, 1/2, and 1/3 indicate the relative sizes of the DNA calculated. a) DNA was synthesized with MgCl_2 or with MnCl_2 in the presence or absence of rifampicin. It was centrifuged in alkaline sucrose gradient as described in Fig. 4. b) DNA was centrifuged in neutral sucrose gradient as described previously (23). (DNA synthesized with $100\ \mu\text{M}$ of M13 DNA gave essentially the same pattern as shown here with ϕX174 DNA.) ●, DNA synthesized with MgCl_2 ; ---●---, DNA synthesized with MgCl_2 in the presence of rifampicin; ○, DNA synthesized with MnCl_2 ; -○-, DNA synthesized with MnCl_2 in the presence of rifampicin.

template was $20\ \mu\text{M}$ (see Fig. 4a). All of these molecules showed intact double-stranded forms, when centrifuged in neutral sucrose gradient (Fig. 6b).

Induction of mutation by divalent cations

Mutagenic effects of metals were examined with the DNA synthesized with Mn^{2+} , Co^{2+} , and Sr^{2+} . The duplex DNAs of phage M13 synthesized with these metal ions in the extracts of *E. coli* H560 were purified by sucrose gradient centrifugation and the plaque-forming abilities of these molecules were assayed by plating them with *E. coli* HfrC treated with CaCl_2 . The induction of clear plaque mutants was measured from the plaques which appeared on the plates. The meas-

Table 2. Clear plaque mutants in RFII molecule synthesized with various metal salts and phage M13 DNA as template.

Metal salt	I	II	II/I
	Number of plaques per nmol of DNA	Number of clear plaques per nmol of DNA	Mutation frequencies
MgCl ₂	4.1×10^4	1.6×10^2	3.9×10^{-3}
MnCl ₂	2.2×10^4	6.4×10^2	29.4×10^{-3}
CoCl ₂	2.4×10^4	3.8×10^2	15.6×10^{-3}
SrCl ₂	1.6×10^4	1.1×10^2	6.4×10^{-3}

DNA synthesized with various metal salts and phage M13 DNA as template was purified by sucrose gradient centrifugation as described in MATERIALS AND METHODS. The plaque-forming activity of the DNA was measured by plating with *E. coli* HfrC treated with CaCl₂. Clear plaques appeared on the plates were counted as forward mutation.

Measurements of reversion frequencies of amber mutants of phage M13 were unsuccessful due to the high frequencies of spontaneous reversions. The result was summarized in Table 2. Plaque-forming ability of the DNA synthesized with Mn²⁺, Co²⁺, or Sr²⁺ was about half that of the DNA synthesized with Mg²⁺. The DNA synthesized with Mn²⁺ or Co²⁺, but not with Sr²⁺, gave higher mutation frequencies than with Mg²⁺.

DISCUSSION

Even though a fairly high concentration of magnesium was detected in the extracts of *E. coli* H560 grown in L-broth, the replication of phage M13 or ϕ X174 DNA could not be recognized without the addition of divalent metal salts to the reaction mixture. The magnesium in the extracts would tightly bind to the cell components and could not act as a cofactor.

Several divalent cations other than Mg²⁺ could function as a cofactor for the synthesis of DNA (Table 1). It would, however, be hard to conclude that the synthesis of DNA occurred solely with these metal ions, as the extracts contained magnesium. Although the metal salts used in this experiment contained a small quantity of impurities (less than 2%, w/w), the synthesis of DNA observed with some metals would not be due to the impurities, since each metal showed a specific effect on the synthesis of DNA.

Since the gene products required for the conversions of phage M13 and ϕ X174 DNA to their replicative forms were not the same (*I*), the effects of metals were examined with DNA of both phages. The effects of metals other than Mg²⁺ on the synthesis of DNA with M13 DNA as template can be summarized as follows:

i) With any metal salt, the synthesis of DNA would be primed by the RNA synthesized by RNA polymerase, since the synthesis of DNA was inhibited by rifampicin.

ii) With Sr^{2+} , the rate of DNA synthesis was slower than that with other metal ions. With Co^{2+} , the synthesis of DNA was poor and the optimal concentration was low. With Mn^{2+} , the rate and extent of DNA synthesis exceeded those with Mg^{2+} at the optimal concentration. The amount of DNA synthesized increased proportionally with the concentration of template at least up to $100\ \mu\text{M}$.

iii) Analysis of the structures of DNA synthesized with these metals gave the following results: DNA synthesized with Mg^{2+} , or Co^{2+} had intact length. The structure of DNA synthesized with Mn^{2+} , or Sr^{2+} with $100\ \mu\text{M}$ of template was different from that synthesized at $20\ \mu\text{M}$. The product was shorter than template at the former concentration and it had intact size with the latter condition. The production of short molecules with these metals were neither due to the fragmentation of template during the incubation nor the hydrolysis of the products during the alkaline sucrose gradient centrifugation. The short fragments would be produced by the initiations of DNA synthesis at several points on the identical template.

The conversion of phage M13 DNA to its replicative form depends not only on DNA polymerase III but on other proteins (18–20). The effect of metal salts on RNA polymerase which requires divalent cations for its activity will also affect the synthesis of DNA. The effects of divalent cations on the synthesis of RNA in the extracts of *E. coli* H560 under the same condition for the synthesis of M13 DNA was examined. The result showed that the amount of RNA synthesized with Mn^{2+} was much higher than that with other metal salts, when the concentration of template was $100\ \mu\text{M}$. The stimulation of RNA synthesis with Mn^{2+} might be responsible for the multiple initiations of DNA synthesis.

The results with ϕX174 DNA as template were essentially the same as those with M13 DNA, with the exception of the effect of rifampicin. With Mg^{2+} , the synthesis of ϕX174 DNA was initiated by primase encoded by gene “dnaG,” and it was not inhibited by rifampicin (19, 29, 30). With Mn^{2+} or Sr^{2+} , however, the synthesis was not necessarily dependent on dnaG, as demonstrated by the partial inhibition by rifampicin. The synthesis of DNA could be initiated both with primase and with RNA polymerase at several points on the template molecule.

There might be some factors in the extract which could work cooperatively with Mg^{2+} , a natural cofactor, to regulate the initiation of DNA synthesis at a single-specific site per template DNA. When Mn^{2+} or Sr^{2+} was used and the concentration of template molecule was high, however, such regulation did not work and the synthesis could be initiated at several points on the template by RNA polymerase.

A preliminary analysis of DNA synthesized with Ba^{2+} using phage M13 DNA as template by sucrose gradient centrifugation suggested that the synthesis of DNA stopped prematurely, leaving an unreplicated region on the template even at $20\ \mu\text{M}$ of template. Each metal seems to have its own specific effect on the synthesis of DNA.

Several divalent metal ions including Mn^{2+} and Co^{2+} are known to be mutagens or carcinogens (31). The mutagenic effects of divalent cations on the DNA synthesized with M13 DNA as template in the extracts of *E. coli* H560 were examined. The result (Table 2) suggested that Mn^{2+} and Co^{2+} but not Sr^{2+} were mutagenic, probably stimulating the incorporation of mismatched nucleotides into the DNA chain. The interruptions present in the DNA synthesized with Mn^{2+} or Sr^{2+} would have little effect on the plaque-forming activity and the induction of mutations, for the plaque-forming efficiencies of the DNA synthesized with Mn^{2+} , Co^{2+} , or Sr^{2+} were almost the same and the DNA synthesized with Co^{2+} which had no interruption received mutations, whereas the DNA synthesized with Sr^{2+} where interruptions would be present did not show any meaningful increase of mutation frequency.

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