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Primer RNA for DNA synthesis on single-stranded DNA template in a cell free system from *Drosophila melanogaster* embryos

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ABSTRACT A cytoplasmic extract of Drosophila melanogaster early embryos supported DNA synthesis which was dependent on an added single stranded DNA template, ϕX174 viral DNA. The product DNA made during early reaction was about 100 to 600 nucleotides in length and complementary to the added template. After alkali treatment, 70 to 80 per cent of the product DNA chains exposed 5'-hydroxyl ends, suggesting covalent linkage of primer RNA at their 5'-ends. Post-labeling of 5'-ends of the product DNA with polynucleotide kinase and [γ-^{32}P]ATP revealed that oligoribonucleotides, mainly hexa- and heptanucleotides, were covalently linked to the 5'-ends of the majority of the DNA chains. The nucleotide sequence of the linked RNA was mainly 5'(p)ppApA(prN)$_{4-5}$, where tri- (or di-) phosphate terminus was detected by the acceptor activity for the cap structure with guanylyltransferase and [α-^{32}P]GTP. The structure of this primer RNA was comparable to that of the octaribonucleotide primer isolated from the nuclei of Drosophila early embryos.

INTRODUCTION

Oligoribonucleotides, called iRNA, are covalently linked to 5'-termini of the short DNA chains synthesized in vitro and in vivo with papovavirus (simian virus 40 and polyoma virus)-infected and non-infected mammalian cells (1-7). The iRNAs are believed to serve as primers for discontinuous DNA synthesis and characterized by 1) transient linkage to nascent DNA chains, 2) purines at 5'-terminus, 3) random sequences at the RNA-DNA junctions and 4) unique size (9 ±1 residues). Recently, DNA primase activity has been identified in SV40 infected cells (8), and Xenopus eggs (9). And it has been reported that DNA primase activity is associated with purified DNA polymerase α from mouse (10), Drosophila melanogaster (11), Xenopus laevis (12, 13) and human lymphocyte (14). The priming mechanism for discontinuous DNA replication has been well characterized with the in vivo and in vitro systems of bacteriophage T7. In the system, predominantly tetraribonucleotide primers in a characteristic sequence, pppApCpN1pN2 (N1 is mostly C and some A, N2 is rich in A and C), are synthesized by gene 4 protein (T7 primase) (15-18) on the recognition sequence, 3'-C-T-G-N1'-N2'-5', in a template DNA strand (19, 20). Such a

characteristic sequence has not been found in the primer RNA of eukaryotic systems, and it has been proposed that the unique size (9 ±1) itself is an essential character of eukaryotic primers (21). But at present the possibility has not been excluded that some kind of signal sequence or preferred sequence is present on a template DNA. Construction of a completely soluble replication system with simple and defined DNA template and precise structure analysis of the product primer RNA may help the elucidation of the molecular mechanism of the priming reaction in eukaryotic organisms.

In the early developmental stage (0 to 1.5 h after oviposition) of Drosophila melanogaster embryos, DNA replication proceeds at an enormously high rate (22), and transcription of mRNA has not been observed (23, 24). Therefore all enzymes necessary for DNA replication seem to exist abundantly in the cytoplasm of the early embryos. We have constructed a soluble enzyme system for DNA replication with a cytoplasmic extract of Drosophila early embryos which uses ϕX174 viral DNA as a template. The product DNA chains, which were complementary to the template DNA and about 100 to 600 nucleotides in length, were covalently linked to 3'-termini of oligoribonucleotides. The structure of the oligoribonucleotides was mainly 5'(p)ppApA(prN)$_{4-5}$, and was comparable to that of the octaribonucleotide primer isolated from the nuclei of Drosophila early embryos in the same developmental stage.

MATERIALS AND METHODS

Chemicals

Deoxyribonucleoside 5'-triphosphates (dNTPs) and ribonucleoside 5'-triphosphates (rNTPs) were purchased from Sigma, G(5')ppp(5')G and G(5')ppp(5')A from P-L Biochemicals, [Methyl-^{3}H]dTTP (19 Ci/mmol) from Shwarz Bio Research, and [^{32}P]orthophosphate (carrier-free) from New England Nuclear Corp. [γ-^{32}P]ATP was prepared according to the procedure of Walseth and Johnson (25), [α-^{32}P]GTP from GpC and [γ-^{32}P]ATP as described by Wu and Taylor (26), dihydroxyboryl Bio-gel P-60 (boronate gel) by the method of Okayama et al. (27). Nitrocellulose powder was purchased from Nakarai Chemicals Co., Ltd. (Kyoto, Japan) and treated according to Boezi and Armstrong (28).

Enzymes

T4 polynucleotide kinase, E. coli alkaline phosphatase, T4 DNA polymerase and pancreatic DNase I have been described (29). Hog spleen exonuclease has been described (30). Nuclease P1 was purchased from Yamasa Shoyu Co. Ltd., nuclease SW from Seikagaku Kogyo Co. Ltd., and vaccinia guanylyltransferase (capping enzyme) from Bethesda Research Laboratories.

Preparation of cytoplasmic extract

D. melanogaster (Oregon R) embryos of 0 to 3.5 hours after oviposition at 25°C were collected, washed and dechorionated according to the methods of Elgin and Miller (31). They were quickly frozen by liquid nitrogen and stored at -70°C. 3 ml of 2 x C-buffer (C-buffer is 10 mM Hepes (pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT) was added to 3 g of the frozen eggs and thawed at 0°C for 1 h. The egg suspension was homogenized in a Dounce homogenizer (3 strokes with a loose fitting pestle and 3 strokes with a tight fitting pestle) and filtered through a nylon mesh (50 μm pore size) to remove the debris. The filtrate was centrifuged at 1,500 g for 10 min. The supernatant was centrifuged at 27,000 g for 20 min. As the precipitate was not tightly packed at the bottom, three quarters of the supernatant were collected from the top and centrifuged again. An equal volume of glycerol was added to the final supernatant and stored at -20°C (the cytoplasmic extract, 15 mg protein/ml). All the above procedures were performed at 0°C. Although fresh extract was used in each experiment, the extract was enzymatically active at least a month.

In vitro DNA synthesis

Unless otherwise indicated, the reaction mixture (1.0 ml) contained 48 mM Hepes buffer at pH 7.8, 65 mM KCl, 4.3 mM $MgCl_2$, 1 mM EGTA (sodium ethylene glycol bis β-amino ethyl ether N,N'-tetraacetate), 0.3 mM dithiothreitol, 30% glycerol, 100 μM each of dATP, dGTP and dCTP, 5.3 μM ^{3}H-dTTP (9.5 Ci/mmol), 100 μM each of rGTP, rCTP and rUTP, 2 mM ATP, 80 μg of ϕX174 viral DNA (10% of which was linear molecules in genome length) and 0.6 ml of the cytoplasmic extract. The reaction mixture was prepared at 0°C and the cytoplasmic extract was added last of all. Incubation was 3 min at 25°C except where otherwise indicated. The reaction was stopped by the addition of an equal volume of stopping mixture consisting of 2% sodium dodecyl sulphate, 20 mM EDTA and 2 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate). To the control reaction cocktail, which was incubated without template, ϕX174 viral DNA was added right after the stopping mixture was poured.

Isolation and terminal labeling of the short DNA chains

The reaction mixtures were digested with Pronase E (1 mg/ml), and nucleic acid was extracted with phenol. ^{3}H-dTTP was removed by a Sephadex G-100 column chromatography. Nucleic acid excluded from the column was sedimented through a sucrose gradient (5 to 20% in 0.015 M NaCl, 0.0015 M sodium citrate, 1 mM EDTA, 0.1% (w/v) sodium dodecyl sulphate) after heat denaturation and DNA chains shorter than one thousand nucleotides were obtained (30). Free RNA

molecules were removed by repeated chromatography on a nitrocellulose column under the following conditions: Sodium chloride was added to the denatured DNA sample to a final concentration of 3.0 M. The sample was applied on a nitrocellulose column (0.5 ml), the column was washed with 5 ml of 3 M NaCl, 5 mM tris-HCl (pH 7.4) and 1 mM EDTA, and the short DNA chains were eluted with 10 mM tris-HCl (pH 7.4), 0.1 mM EDTA. Overall recovery of the ^{3}H-DNA was 90%.

The 5' termini of the nucleic acids in the nitrocellulose sample were labeled with ^{32}P using [γ-^{32}P]ATP (carrier free) and T4 polynucleotide kinase after removal of terminal phosphates by bacterial alkaline phosphatase (29). [γ-^{32}P]ATP remaining after the reaction was removed by Sephadex G-50 column chromatography.

Selection of the short DNA chains complementary to ϕX174 viral DNA

2 to 5 µg of non-radioactive ϕX174 viral DNA was added to the end-labeled sample, then ethanol precipitated and dissolved in 50 µl of 3 x SSC, 0.1% SDS. The sample was heated at 90°C for 2 min and incubated at 65°C for 1 to 2 hours (Cot ≈ 1). The short DNA chains annealed to ϕX174 viral DNA were collected by chromatography on a Sepharose 2B column (0.7 x 25cm). The re-annealing procedure was repeated again and then the cold ϕX174 viral DNA was removed by the same column right after heat denaturation.

Capping reaction

The nitrocellulose sample was used for the capping reaction. The reaction cocktail (10 µl) containing 50 mM Tris-HCl (pH8.0), 2.5 mM $MgCl_2$, 2 mM dithiothreitol, 2µM [α-^{32}P]GTP (carrier free) and 0.7 unit of vaccinia guanylyltransferase was incubated at 37°C for 30 min. The reaction was terminated by the addition of 1 µl of 0.2 M EDTA. The capped molecules were purified by columns of Sephadex G-50 and nitrocellulose. Removal of the 5'-cap of the sample was performed with periodate oxidation and β-elimination with aniline (33, 34).

Enzyme digestion of nucleic acids

5' ^{32}P-labeled DNA molecules were digested with pancreatic DNase I (100 µg/ml) and the 3'to 5' exonuclease associated with T4 DNA polymerase (5 U/ml) as described by Ogawa *et al.* (29). Part of the DNase digested sample was adjusted to pH 4.5 by the addition of acetic acid and was treated with an RNase mixture containing 67 U/ml, 67 U/ml, 7 µg/ml, and 32 U/ml of RNase T_1, T_2, A, and U_2, respectively, for 30 min at 37°C. Complete digestion of 5' ^{32}P-labeled nucleic acid with nuclease P1 was carried out in a 10 µl reaction mixture containing 10 mM sodium acetate buffer (pH 5.2), 50 µg each of heat denatured salmon sperm DNA and *E. coli* tRNA and 1 µg of nuclease P1 for 2

hours at 37°C. Digestion with nuclease SW was performed as described (15).

Gel electrophoresis

Gel electrophoresis was performed according to Maxam and Gilbert (35) after pre-run for more than 10 h at 1500 V.

Separation of 5'-terminal mono- to tetraribonucleotides

^{32}P-labeled 5'-terminal mononucleotides or capped mononucleotides were obtained by complete digestion with nuclease P1. Mononucleotides were separated by one or two-dimensional chromatography on polyethyleneimine-cellulose plates (Polygram Cell 300 PEI, Macherey, Nagel, Germany) with 0.25 M formic acid and then 0.55 M lithium formate (pH 3.2) in the first dimension and with 0.55 M LiCl, 0.15 M ammonium borate in the second dimension when necessary. Capped mononucleotides were separated on a PEI plate with 4.0 M lithium formate (pH 3.4). 5' ^{32}P-labeled oligoribonucleotides from *in vitro* and *in vivo* samples were digested with nuclease SW. The resulting 5'-terminal di- to tetranucleotides from the *in vitro* sample were fractionated by chromatography through DEAE-Sephadex A25 column in the presence of 7M urea. Dinucleotides were then separated by chromatography on a PEI plate with 0.6 M lithium formate (pH 3.5), 7M urea. Tri- and tetraribonucleotides were mixed and electrophoresed in a 20% polyacrylamide gel in the first dimension. The oligoribonucleotides in the gel were printed *in situ* to a PEI plate as described by Randerath *et al.* (36) and separated with 0.6 M lithium formate (pH 3.5), 7 M urea in the second dimension. Oligoribonucleotides from the *in vivo* sample after digestion with nuclease SW were chromatographed through a column of boronate gel to remove contaminant of oligodeoxyribonucleotides and then separated by the two dimensional procedure as described above.

RESULTS

øX174 viral DNA dependent DNA synthesis by cytoplasmic extract

Fig. 1 shows the time course of *in vitro* DNA synthesis using cytoplasmic extract from early embryos of *Drosophila melanogaster* as enzyme sources and øX174 viral DNA as a template. Clear dependence of the reaction on the externally added template was observed. At a certain concentration of the extract and a high concentration of ATP (2 mM), DNA synthesis continued linearly for more than 2 hours at 25°C. But it ceased at 30 min when the concentration of ATP was 0.2 mM or lower (data not shown). No dependency of this reaction on other ribonucleoside triphosphates was detected, possibly because of their presence in this crude cytoplasmic extracts. The reaction

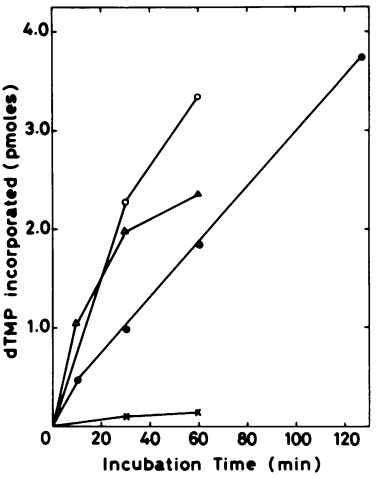

Fig. 1. Template dependent DNA synthesis by the cytoplasmic extract of Drosophila melanogaster embryos

The reaction mixtures (0.1 ml) contained 20 μM each of dATP, dGTP, dCTP and ^{3}H dTTP (0.95 Ci/mmol), 1.3 μg of ϕX174 viral DNA, other ingredients as described in Materials and Methods and 0.14 mg (—●—), 0.28 mg (—○—) or 0.56 mg (—△—) of proteins from the cytoplasmic extract. Minus template mixtures (—x—) contained 0.28 mg of the proteins but no ϕX174 viral DNA. The reaction was stopped by the addition of an equal volume of 10% TCA, 20 mM Na-PPi, and the ^{3}H-dTTP remaining after the reaction was removed by centrifugation. Acid insoluble radioactivity was counted.

was inhibited by aphidicolin (70% inhibition at 20 μg/ml) and was not inhibited by α-amanitin (100 μg/ml).

Short DNA chains with alkali-labile termini were synthesized

The priming event of DNA synthesis was analyzed using the product DNA chains made during brief incubation time (3 min. at 25°C) with excess of ϕX174 viral DNA template to avoid possible nuclease attack of the template DNA during incubation. In a 1 ml standard reaction mixture described in Materials and Methods, 30 pmoles of [^{3}H] dTTP were made acid insoluble during the incubation, whereas less than 0.05 pmoles of [^{3}H] dTTP when the template DNA was eliminated from the reaction. ϕX174 viral DNA in the reaction mixture scarcely received endonuclease attack during 3 min incubation (data not shown). As shown in Fig. 2A the product DNA chains were mainly 100 to 600 nucleotides long, and they co-migrated with ϕX174 viral circular and linear DNA template when electrophoresed in a neutral agarose gel (Fig. 2B). These results indicate that short DNA chains were synthesized on a ϕX174 viral DNA template during the incubation and some kind of primers for DNA synthesis must have been formed prior to synthesis of the short DNA chains. To investigate the possibility of RNA priming, we analyzed the covalent linkage of RNA to the 5'-ends of the product DNA chains by the spleen exonuclease assay developed for the detection of RNA-linked nascent DNA chains (30). The assay is based on the facts that RNA-linked DNA exposes a 5' hydroxyl (5'-OH) end after an alkali digestion of RNA moiety and spleen exonuclease only degrades 5'-OH terminated polynucleotides. As shown in Fig. 3, 70 to 80% of the ^{3}H-labeled product DNA chains became digestable after alkali treatment. The result

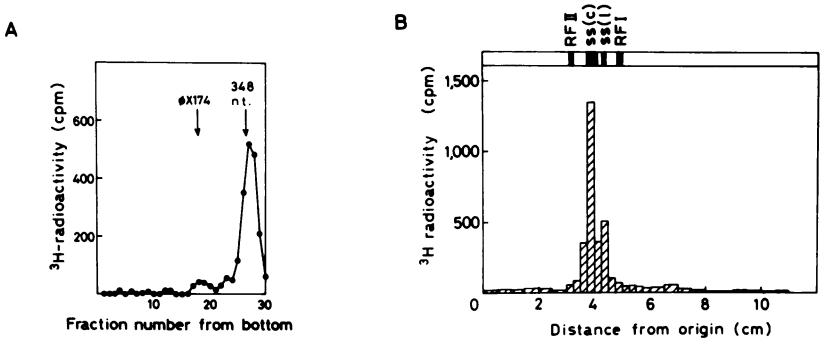

Fig. 2. Size distribution of product DNA in denatured and native states
^{3}H-labeled DNA synthesized by the 3 min reaction in the 1 ml standard reaction mixture containing 1 μg/ml of α-amanitin, was extracted and excluded from a sephadex G-100 column as described in Materials and Methods. (A) An aliquot of the excluded fraction was sedimented through a 5% to 20% linear gradient of alkaline sucrose (0.1 M NaOH, 0.9 M NaCl, 1 mM EDTA) for 3.0 h at 50,000 rpm in a Beckman SW 56 Ti rotor and the distribution of the acid-insoluble ^{3}H-radioactivity was determined. The arrows indicate the positions of ϕX174 viral circular DNA and a 348-nucleotides DNA. (B) Another aliquot of the excluded sample was electrophoresed in a 0.8% agarose gel in 50 mM Tris-borate (pH 8.3), 2.5 mM EDTA for 3 h at 100V. The gel was stained with ethidium bromide and the bands for ϕX174 viral circular (ss-c) and linear (ss-l) DNA in the sample were detected by ethidium bromide fluorescence. ϕX174 RFI and RFII DNA were electrophoresed in the next lane of the gel. The loci of these four size markers are shown schematically at the top of Fig. B. The gel was cut in every 0.5 or 1.0 cm width and ^{3}H radioactivity was counted.

suggests that RNA primers are covalently linked to the 5'-ends of the majority of the reaction products.

Isolation and structure analysis of primer RNA

To isolate and characterize the primer RNA, we purified short DNA chains from the reaction products, labeled their 5'-termini with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P]ATP and selected the chains complementary to ϕX174 viral DNA by a repetition of the re-annealing procedures as described in Materials and Methods. The content of alkali labile radioactivity of the end-labeled short chains thus obtained was found to be about 75%, a value which agrees with the results of the spleen exonuclease assay (Fig. 3). When subjected to pyknographic analysis, the end-labeled molecules were distributed broadly at the position of DNA density and none was found at the RNA density, which indicates that this sample did not contain free RNA molecules (data not shown). From the end-labeled chains prepared from the sample incubated without the template, no alkali labile radioactivity was obtained after the re-annealing procedures, though a small amount of alkali resistant

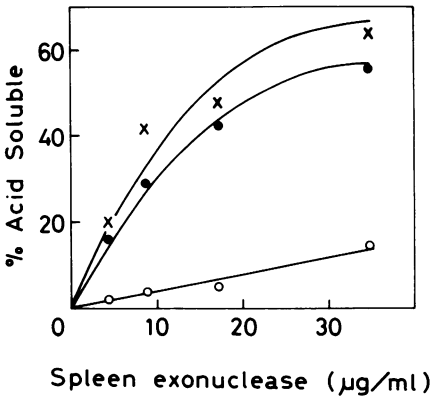

Fig. 3. Spleen exonuclease digestion of the alkali treated product DNA

^{3}H-labeled DNA chains synthesized as described in the legend to Fig. 2 were purified up to the step of neutral sucrose gradient sedimentation as described in Materials and Methods. Short DNA chains thus obtained were further purified by centrifugation to equilibrium in Cs_2SO_4. The purified DNA was phosphorylated with polynucleotide kinase and non-radioactive ATP, and treated with 0.15 M NaOH for 20 h at 37°C. Then the sample was divided into 3 portions. (A) was phosphorylated again, (B) was untreated and (C) was de-phosphorylated with bacterial alkaline phosphatase. And (A), (B) and (C) were digested with various amount of Hog spleen exonuclease. (A): —o—; (B): —●—; (C): —x—.

radioactivity was obtained, which seemed to be a contaminant DNA fragments originating from the cytoplasmic extract.

The purified end-labeled chains were digested with pancreatic DNase I and the 3' to 5' exonuclease of T4 DNA polymerase and the digests were electrophoresed in a 20% polyacrylamide gel. Two major bands with the mobility corresponding to $(pA)_7$- to $(pA)_8$-size markers appeared in the gel for the sample made with the template (Fig. 4A lane 4) but not for the sample without the template (Fig. 4B lane 1). These bands consisted of oligoribonucleotides, because they completely disappeared after RNase digestion (Fig. 4A lane 6). In addition, Fig. 4A lane 4 shows that small amount of oligoribonucleotides shorter than heptanucleotide were also present. Small amount of nonaribonucleotide (one tenth of octaribonucleotide) was also present, although the band was too faint to be seen in Fig. 4A lane 4. As we have shown previously (32), the speed of cleavage of RNA-linked DNA molecules by the 3' to 5' exonuclease associated with T4 DNA polymerase is a thousand fold faster for the DNA portion than for the RNA-DNA junction or for the RNA portion. The major RNA primers, therefore, are expected to be liberated with one residue of deoxyribonucleotide covalently linked at their 3' ends by the digestion. We have analyzed whether the 3'-termini of the octa- and heptanucleotides consisted of ribonucleotide or deoxyribonucleotide. The DNase digest of the purified sample was applied to a column of dihydroxyboryl Bio-gel P-60 (boronate gel), which retains the molecules with 2',3' cis-diol structure

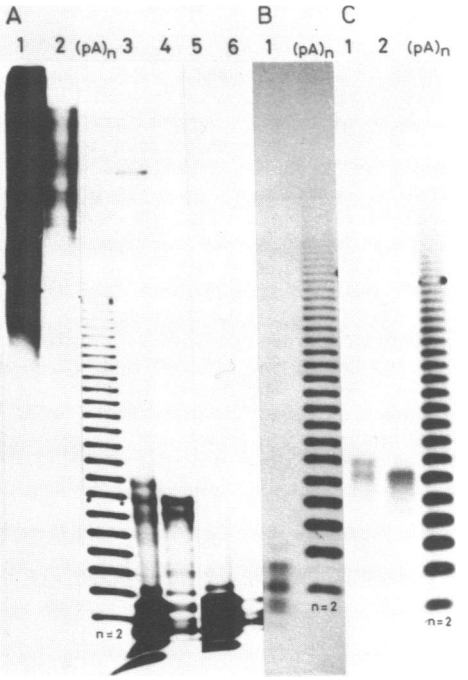

Fig. 4. Detection of oligoribonucleotides covalently linked to 5' termini of the end-labeled short DNA chains
(A) Lanes 2, 4 and 6 show the analyses with the end-labeled short DNA chains obtained from in vitro sample and lanes 1, 3 and 5, those prepared from the nuclei of early embryos (1 to 3 hours after oviposition). The in vivo sample was purified by sedimentation through a neutral sucrose gradient and repeated chromatography on nitrocellulose columns, end-labeled with ^{32}P and then further purified by repeated equilibrium sedimentation in Cs_2SO_4 density gradients (Kitani, Yoda and Okazaki, in preparation). Each of the sample was divided into 3 portions and treated as follows: (lanes 1 and 2) without treatment; (lanes 3 and 4) digested with pancreatic DNase I and the 3' to 5' exonuclease of T4 DNA polymerase as described in Materials and Methods; (lanes 5 and 6) digested with RNase mixture as described in Materials and Methods after digestion with the DNases. After the treatments, the samples were lyophilized, suspended in de-ionized formamide and electrophoresed in a 20% polyacrylamide gel for 3 h at 2000V. After electrophoresis, the gel was autoradiographed with XRP-1 x-ray film (Kodak) for 3 days at -70°C in the presence of an intensifying screen. Over-exposed bands around $(pA)_2$ size marker in lanes 3 and 5 were dinucleotides liberated from the 5'-termini of RNA-free DNA molecules by digestion with the 3' to 5' exonuclease of T4 DNA polymerase.
(B) Short DNA chains in the reaction mixture which was incubated without ϕX174 viral DNA template were labeled with ^{32}P and purified as shown in Materials and Methods. The purified sample was digested with the DNases and electrophoresed (lane 1). (C) The DNase treated sample in vitro after removal of di- and trinucleotides with a column of DEAE-cellulose was chromatographed on a boronate gel column as described by Seki and Okazaki (1979). One-seventh of the passed fraction (lane 1) and all of the retained fraction (lane 2) were electrophoresed. The ladders in A, B and C are the [5'-^{32}P]oligoriboadenylate size markers, which were prepared by the partial digestion of [5'-^{32}P]-polyriboadenylate with nuclease SW. n=2 indicates the dark band above it.

characteristic of ribonucleotide (15). The passed and the retained fractions were electrophoresed in a 20% polyacrylamide gel. As shown in Fig. 4C, octanucleotide was detected only in the passed fraction whereas the hepta-nucleotide was detected in the both retained and passed fractions. Therefore, the structure of the octanucleotide may very well be $(prN)_7pdN$ and the

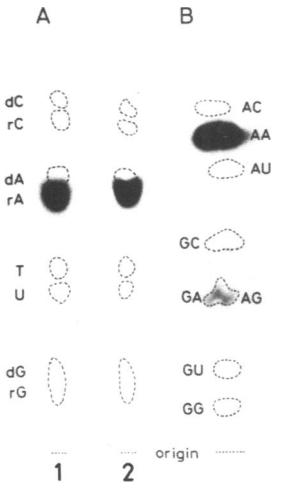

Fig. 5. Separation of 5' terminal mono- and dinucleotides of primer RNA made in vitro
(A) ^{32}P-labeled hepta- and octaribonucleotides shown in Fig. 4A lane 4 were eluted from the gel and digested with nuclease P1. The resulting mononucleotides were chromatographed on a PEI plate as described in Materials and Methods. Lanes 1 and 2 show the analyses of hepta- and octaribonucleotides, respectively. (B) ^{32}P-labeled octaribonucleotides shown in Fig. 4A lane 4 were digested with nuclease SW and the digest was fractionated by chromatography through DEAE Sephadex A 25 column in the presence of 7M Urea. The dinucleotide fraction was then chromatographed on a PEI plate as described in Materials and Methods and autoradiogram was taken. The dotted circles indicate the spots of optical density markers. The tri- and tetranucleotide fractions were combined and analyzed as shown in Fig. 8A.

heptaribonucleotide detected in the retained fraction may be produced by the complete removal of the DNA portion. The heptanucleotide in the passed fraction may be in the structure of $(prN)_6pdN$.

Hexa- and heptanucleotides start with pApA sequence

The bands corresponding to 7 and 8 nucleotides long shown in Fig. 4A lane 4 were cut out, the oligonucleotides were eluted from the gel and 5' mono- and dinucleotides were analyzed. All the 5'termini were composed of ribonucleotides, and the nucleotide compositions for both bands were rG, 4%; U, 0%; rA, 96%; and rC, 0% (Fig. 5A). The nucleotide sequences of the 5' dinucleotides of the octaribonucleotides, which were obtained by digestion with nuclease SW, were pApA, 94% and pApG(pGpA), 5% with other sequences scarcely detectable (Fig. 5B). The same result was obtained from the 5'-dinucleotides of the heptaribonucleotides. The relative amount of the radioactivity obtained as dinucleotides after digestion with nuclease SW was 26% for the octaribonucleotides and 45% for the heptaribonucleotides. Because nuclease SW has little base preference (37), it can be concluded that most, if not all, of the penultimate base of the primer RNA consisted of rA residue.

Detection of intact primer RNA

Guanylyltransferase, capping enzyme, transfers 5'$\overset{*}{p}$G ($\overset{*}{p}$ is symbol for [^{32}P]phosphate group) from 5'ppp$\overset{*}{G}$ specifically to the 5' di- or triphosphate terminus of RNA to form the sequence G(5')$\overset{*}{p}$pp(5')N----- (38). Thus intact primer RNA is expected to be selectively labeled with ^{32}P by the enzyme reaction. To detect intact primer RNA, the sample was reacted with vaccinia guanylyltransferase and [α-^{32}P]GTP and purified as described in Materials and

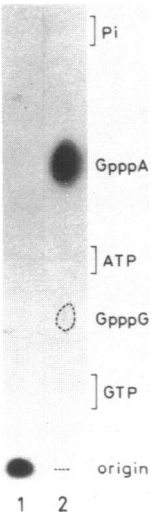

Fig. 6. Detection of the cap structure
The sample after the capping reaction was digested with bacterial alkaline phosphatase (lane 1) or nuclease P1 (lane 2). The digests were separated on a PEI plate as described in Materials and Methods. The dotted circles indicate the spots of optical density markers.

Methods. The amount of ^{32}P-radioactivity recovered after the capping reaction was roughly equivalent to 30% of the RNA-DNA molecules in the sample. The radioactive ends were resistant to bacterial alkaline phosphatase (Fig. 6 lane 1) and ^{32}P-labeled cap structure, composed of 95% in GpppA and 5% in GpppG, was liberated from the ends by digestion with nuclease P1 (Fig. 6 lane 2). The nucleotide composition of the capped termini fully corresponded with that of the 5' ends of hexa- and heptaribonucleotide primers (Fig. 5A). When the capped sample was digested with pancreatic DNase I and the 3' to 5' exonuclease of T4 DNA polymerase, two major bands with mobility corresponding approximately to $(pA)_9$- and $(pA)_{10}$-size markers were generated (Fig. 7A lane 1). This regions corresponded to $Gpp(pA)_7$- and $Gpp(pA)_8$-size markers (Fig. 7B). To ascertain the covalent linkage of deoxyribonucleotide at the 3' termini of these capped molecules, the 5' cap was removed by β-elimination, DNA portion was then digested with pancreatic DNase I and the 3' to 5' exonuclease of T4 DNA polymerase and the digests were fractionated through a boronate gel column. The passed fraction, which was expected to contain molecules having triphosphates at 5' ends and monodeoxyribonucleotides at 3' ends, was electrophoresed in a 20% polyacrylamide gel (Fig. 7A lane 2). The results showed that the hexa- and heptaribonucleotide primers carried triphosphate structure at the 5' termini. In addition, a small amount of primer RNA of penta- to mononucleotides and octanucleotide was also present.

From these results it is concluded that the oligoribonucleotides with characteristic structure of $5'(p)ppApA(prN)_{4-5}$ served mainly as primers for

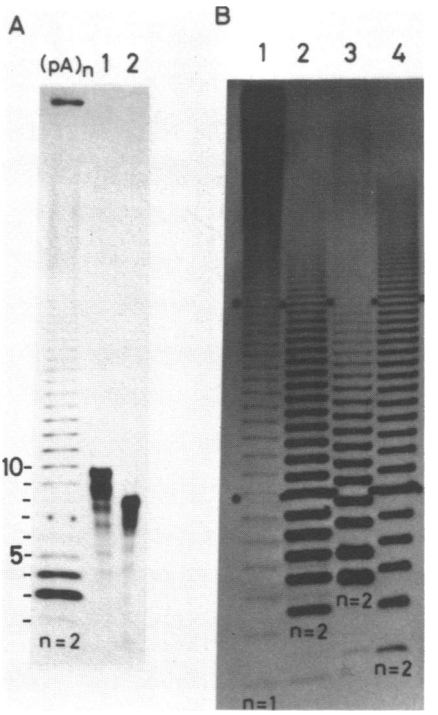

Fig. 7. Chain length of the capped primer RNA

(A) The sample after the capping reaction was divided into two portions. One was digested with the DNases as shown in Fig. 6 and electrophoresed in a 20% polyacrylamide gel (lane 1). The other was de-capped by β-elimination with aniline after periodate oxidation, digested with the DNases, and applied to a column of boronate gel. The passed fraction (molecules with deoxynucleotide at 3' end) was electrophoresed (lane 2). The ladder is the [5'-^{32}P]oligoriboadenylate size marker which was prepared by the reaction with T4 polynucleotide kinase and [γ-^{32}P]ATP of oligoriboadenylate, the digest of polyriboadenylate with nuclease SW. n=2 indicates the faint band above it. (B) Comparison of the mobility of various size markers with different 5' terminal structure in 20% polyacrylamide gel electrophoresis. Lane 1: p(Ap)$_n$ prepared by boiling of [5'-^{32}P]polyriboadenylate in re-distilled water for 30 min. Lane 2: (pA)$_n$ prepared by the partial digestion of [5'-^{32}P]polyriboadenylate with nuclease SW. Lane 3: Gpp(pA)$_n$ prepared by the following procedures. Polyriboadenylate carrying 5'triphosphate terminus, synthesized with E. coli RNA polymerase, was reacted with vaccinia guanylyltransferase and [α-^{32}P]GTP. The capped polyriboadenylate was partially digested with nuclease SW. Lane 4: pp(pA)$_n$ prepared by the partial digestion with nuclease SW of polyriboadenylate carrying 5'[γ-^{32}P]triphosphate terminus. n=1 or n=2 in each lane indicates the band just above it.

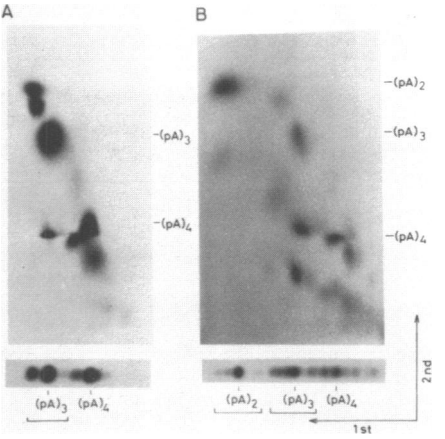

Fig. 8. Two dimensional separation of the 5' terminal oligonucleotides of the in vitro and in vivo RNA primers

Figure (A) shows the 5' terminal tri- and tetraribonucleotides of in vitro primer RNA. The sample prepared as described in Materials and Methods and the legend of Fig. 5B was electrophoresed in a 20% polyacrylamide gel in the first dimension (the lower plate). The vertical bars indicate the positions of 5'-^{32}P labeled oligoriboadenylate size markers. The ribonucleotides in the gel were in situ transferred to a PEI plate, and separated as described in Materials and Methods in the second dimension (the upper plate). The horizontal bars indicate the positions of oligoriboadenylate optical density markers. A bracket at the bottom of the figure indicates the area of trinucleotides. Figure (B) shows the 5'terminal oligoribonucleotides of primer RNA. ^{32}P-labeled octaribonucleotides shown in Fig. 4A lane 3 were eluted from the gel and digested with nuclease SW. The digest was chromatographed through a column of boronate gel to remove the contaminant of free DNA molecules and the retained fraction (molecules with ribonucleotide at 3' end) was used for two dimensional separation as described above. Brackets at the bottom of the figure indicate the areas of di- and trinucleotides.

the template dependent DNA synthesis in this in vitro system.

Comparison with the primer RNA obtained from the nuclei of early embryos

Does the structure of this in vitro primer RNA mimic the characteristic of the in vivo primer RNA? For the purpose of examining this point, we purified the in vivo primer RNA and analyzed the base sequence of the 5' terminus. Short DNA chains were purified from the nuclei of early embryos, digested with pancreatic DNase I and the 3' to 5' exonuclease of T4 DNA polymerase (Kitani, Yoda and Okazaki, in preparation), and electrophoresed in a 20% polyacrylamide gel. As shown in Fig. 4 lane 3, the in vivo primer RNA was octaribonucleotide, which was longer than the in vitro primer by one or two nucleotides. The in vivo and in vitro RNA primers were eluted from the gel and digested with SW nuclease. The digests were analyzed by two

Table 1. The frequency of $(pA)_{1-3}$ sequences appearing at the 5' ends of *in vitro* and *in vivo* RNA primers.

Source of primer RNA	Frequency of $(pA)_n$ sequence at the 5' ends (%) n=1	n=2	n=3
in vitro	96*	94*	45***
in vivo	78**	64***	20***

* These values were taken from the data shown in Fig. 5. ** This value was obtained by the nuclease P1 digestion of the materials in hepta- to nonanucleotide bands in Fig. 4A lane 3. The digests were analyzed by two dimensional chromatography on a PEI plate as described in Materials and Methods. The nucleotide compositions of the 5'-termini for the hepta- to nonanucleotides were essentially the same and rG, 16%; U, 4%; rA, 78%; and rC, 2%. *** These values were calculated from the data shown in Fig. 8 as 100 x r_1 x r_2, where r_1 designates the ratios in the first dimension of the radioactivities at $(pA)_2$ or $(pA)_3$ positions to those for total dinucleotides or trinucleotides, respectively. The area for dinucleotides or trinucleotides in the first dimension is indicated with brackets at the bottom of Fig. 8. r_2 designates the ratio of radioactivities again comigrated with $(pA)_2$ or $(pA)_3$ markers in the second dimension to those at $(pA)_2$ or $(pA)_3$ positions in the first dimension, respectively. The radioactivities were determined by the densitometer tracings of the autoradiograms.

dimensional procedures shown in Fig. 8. The 5' terminal trinucleotides from the *in vitro* primer were separated into 4 distinct spots (Fig. 8A). Among them the spot corresponding to a $(pA)_3$ optical density marker was the major one and occupied about 45% of the total, and the major spot of the 5' terminal tetranucleotides also corresponded to $(pA)_4$ optical density marker. The *in vivo* primer RNA was less biased to rA residues as shown in Fig. 8B, although the spots corresponding to $(pA)_{2-4}$ markers were detected among the major spots on each of the 5' terminal di-, tri- and tetranucleotides. Table 1 summarizes the frequency of $(pA)_{1-3}$ sequences appearing at the 5' ends of *in vitro* and *in vivo* RNA primers. It is suggested that at least the 5' side of the *in vivo* primer tends to be biased to rA residues and this tendency becomes more conspicuous in the *in vitro* primer RNA.

DISCUSSION

The analyses of the ^3H-labeled DNA synthesized in the present *in vitro* system have revealed the following: (1) Short DNA chains about 200 nucleotides long were synthesized using ØX174 viral circular DNA as a

template. (2) The major part of 5'-termini of the product DNA chains exposed 5'-OH ends by alkali treatment, which suggests that they were synthesized using RNA primers.

To analyze more directly and more precisely the structure of 5'-termini of the nascent short DNA chains, we labeled them with ^{32}P using polynucleotide kinase and [γ-^{32}P]ATP. Although this method introduces very high radioactive labeling into the 5' termini, it does not distinguish between the nascent molecules and non-nascent ones. So it is indispensable to show the distinct evidence that ^{32}P-labeled DNA molecules are newly-synthesized ones. The purified ^{32}P labeled RNA-linked DNA molecules are concluded to be the nascent ones, judging from the following evidence: (1) RNA-linked DNA molecules were not detected in the cocktail which was incubated without template. (2) The relative amount of alkali-labile 5' termini in the purified terminally-labeled DNA chains agreed with that of RNA-linked molecules in the ^{3}H-labeled product DNA determined by the spleen exonuclease assay. (3) The amount of RNA-linked DNA molecules was well in accordance with what should be estimated from the amount of ^{3}H-radioactivity in the nascent molecules if the chain length of the nascent DNA fragments were about 200 nucleotides long. The covalent linkage of ^{32}P labeled oligoribonucleotides to the 5'-termini of the short DNA chains is obvious from (1) the appearance of oligonucleotides with DNase digestion, (2) their disappearance by successive digestion with RNase and (3) the covalent linkage of deoxymononucleotide to the 3'-termini of these oligoribonucleotides after DNase digestion. These results indicate that the oligoribonucleotides served as primers for the *in vitro* DNA synthesis.

The structure of the primer RNA can be summarized as 5'(p)ppApA(prN)$_{4-5}$. A tri- (or di-) phosphate terminus was detected by the acceptor activity for the cap structure with guanylyltransferase and [α-^{32}P]GTP. The size was well defined, mainly hepta- and hexanucleotides. A small amount of G residue was also detected at the 5' terminus (G/A = 1/24). These characteristics are principally similar to those of the primer RNA from the early embryos of *Drosophila melanogaster* (Kitani, Yoda and Okazaki, in preparation). The characteristic structure of the primer RNA excludes the possibility that some RNA fragments pre-existing in the cytoplasmic extract may have served as primer for this *in vitro* system. The insensitivity of the reaction to α-amanitin (100 μg/ml) suggests that primase may have participated in the formation of primer RNA in this reaction.

There are two conspicuous differences between the RNA primers made *in vitro* and those made *in vivo*. The first is their size. *In vivo* primer RNA of

Drosophila melanogaster was octaribonucleotide and the same size primer RNA was also found in sea urchin embryos (Hozumi and Okazaki, in preparation), which even suggests that the octaribonucleotide primer for DNA synthesis may be common in eukaryotic systems. On the other hand, the major RNA primers synthesized in this in vitro system were shorter than the in vivo primer by one or two nucleotides (hepta- and hexanucleotides). In addition, a small amount of penta- to mononucleotides or octanucleotide was detected by the capping reaction (Fig. 7 A). Conaway and Lehman reported primase activities associated with Drosophila DNA polymerase α (11). The primers synthesized in their system with M13 DNA template were in unique size, but contained 15 residues (39). Some factors which define the size of primer RNA in vivo might be missing or not fully functional in the in vitro systems. Primers made by primase activities associated with DNA polymerase α from mouse and Xenopus (40, 12) and those made by crude extracts from SV40 infected cells (8) and Xenopus embryos (9) were reported to be around a decanucleotide in length. Primer RNA made by human lymphoblastoid cell extracts on poly(dIT) template is reported to be hepta- to nonanucleotides (14). The second difference between in vivo and in vitro primer RNA is the base sequences. The base sequence of the primer RNA made in vitro was considerably more biased to rA residues at 5' side than that made in vivo (Table 1). This may suggest that primase prefers to start at dT rich sequence on a template. In the reaction in vitro, template strands are in single-stranded form so that primer synthesis will be started first at the most preferred sites and DNA synthesis primed by such sites will quickly convert the weaker primer sites into the inactive duplex form. DNA synthesis in vivo, on the other hand, is coupled with unwinding of double stranded DNA. Thus, the site selection by primase in vivo is made on a restricted template region which is exposed as a single stranded state at a given moment. The site selection in vivo would be influenced not only by the sequence available but also by a topological state of the each site at the replication fork. The concentration of rATP (2 mM) was 20 times higher than that of other rNTPs (0.1 mM) in this in vitro system. Therefore, the further possibility cannot be excluded that this higher concentration might influence the site selection for priming reaction.

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