RNA Priming Coupled with DNA Synthesis on Natural Template by Calf Thymus DNA Polymerase α–Primase†

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ABSTRACT: A bovine genomic DNA library was surveyed with respect to the template activity for RNA-primed DNA synthesis by calf thymus DNA polymerase α–primase complex. About 7% of the single-stranded DNA clones contained distinct initiation sites consisting of pyrimidine clusters of pyrimidine-rich sequences. The initiation sites were located at or near the 3′-end of the pyrimidine clusters. One of these sequences, containing a 10-mer pyrimidine cluster with major initiation sites, was analyzed in detail. By the successive substitutions of pyrimidines in the cluster with oligodeoxyadenylate [(dA)*n*] in the 5′ to 3′ direction, the minimum length of the initiation sequence was estimated to be as long as the 7-mer. In contrast, when one or three pyrimidines at the 3′-end of the cluster were replaced with (dA)*n*, the priming activity was largely lost, indicating that these pyrimidine residues were indispensable for priming. Furthermore, base substitutions of upstream or downstream sequences outside the pyrimidine cluster also decreased the total priming frequencies. Interestingly, the base substitutions inside or outside of the pyrimidine cluster sometimes caused a shift in the major priming sites. These results indicate that the minimum priming unit of the CTPPS1 template for RNA-primed DNA synthesis consists of a pyrimidine cluster (6-mer) with one purine at its 3′-border and that both the 3′-downstream 6-bases and the 5′-upstream 17-bases modulate the priming by enhancing the priming frequency and/or slightly shifting the sites of initiation of primer synthesis. It was also revealed that the lengths of the product RNA primers became shorter as the length of pyrimidine cluster was shortened by substitution with (dA)*n*. The gel retardation assay further showed that the complex formation between DNA polymerase α–primase and the DNA templates was strongly in competition with poly(dC), poly(dG), and poly(dT) but not with poly(dA). Furthermore, template activities as well as the pyrimidine contents of a series of base-substituted DNA correlated well with their affinities to the enzyme, as measured by both gel retardation assay and their K_m values for the priming reaction. Apparently, DNA polymerase α–primase primarily recognizes the minimum priming unit consisting of a pyrimidine cluster with a purine at the 3′-boundary of purine, but the initiation of primer RNA synthesis can be modified by pyrimidine residues outside of the minimum priming unit.

One of the basic rules of DNA replication is that DNA polymerase cannot start a chain and must rely on a priming device. Every initiation event requires a primer. DNA replication usually initiates by synthesizing RNA primers, in either continuous synthesis in the leading strand or discontinuous synthesis in the lagging strand (Kornberg & Baker, 1992). It has been shown that primer synthesis is carried out by DNA primase (primase), a special kind of RNA polymerase in *E. coli* (Wickner et al., 1973; Bouché, et al., 1975), and that a similar enzyme, eukaryotic primase, was also found in a wide variety of eukaryotic cells (Roth, 1987; Yagura et al., 1982; Conaway & Lehman, 1982; Shioda et al., 1982). Eukaryotic primase has been found to exist in association with DNA polymerase α (Yagura et al., 1982; Conaway & Lehman, 1982). The mechanism of binding of the primase to the template and the initiation of primer RNA synthesis is crucial to the understanding of the regulation of DNA replication. The RNA primer synthesis by eukaryotic primase has been extensively studied using synthetic homopolymers poly(dC) and poly(dT) [for reviews, see Roth (1987), Yagura et al. (1982), Nishizawa et al. (1983), Gronostajski et al. (1984), Holmes et al. (1985), Grosse and Krauss (1985), Yamaguchi et al. (1985), Yoshida et al. (1985), Suzuki et al. (1989), Kuchta et al. (1990), and Podust et al. (1991)]. Studies have also been performed using deoxyheteropolymers including natural templates to determine the sequence specificity of the priming site (Yamaguchi et al., 1985; Davey & Faust, 1990; Sheaff & Kuchta, 1993). However, the sequence specificity of the priming site still remains obscure for the following reasons. First, there have been few analyses of the specificity of priming sites using purified enzyme and natural template. Second, the number of nucleotides as well as the sequence of the minimum priming unit has not been clarified with natural template. Third, the synthesized RNA fragments, which are shorter than a heptamer, can hardly be used as primers for DNA synthesis by DNA polymerase α (Yoshida et al., 1985; Kuchta et al., 1990; Podust et al., 1991); thus this abortive primer synthesis (Podust et al., 1991) should be excluded.

Recently, we found that free primase, which was separated from the calf thymus DNA polymerase α–primase complex, can recognize the initiation sequence for RNA primer synthesis in a manner similar to the DNA polymerase α–primase complex (Suzuki et al., 1993). We also showed that the DNA polymerase α moiety of the complex could modulate the primase action, since the binding of Simian virus 40 T-antigen

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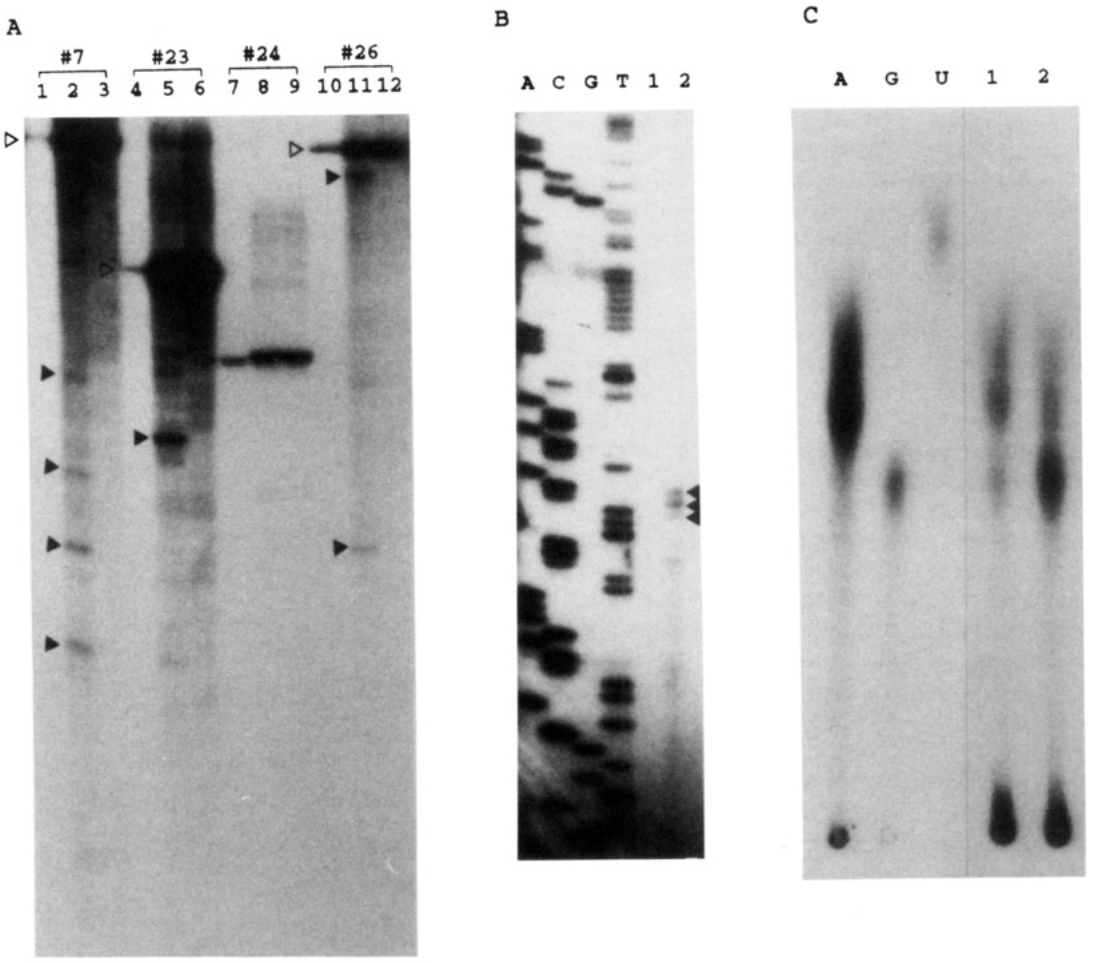

FIGURE 1: Determination of priming sites by the calf thymus DNA polymerase α–primase complex. (A) Screening of the active templates. *Eco*RI–*Hind*III fragments of bovine DNA, cloned in pUC118, were randomly picked up and amplified by the PCR method, as described in Materials and Methods. The amplified single-stranded DNA fragments were then used as templates for *de novo* DNA synthesis by DNA polymerase α–primase complex, and the reaction products were analyzed by 8% polyacrylamide gel electrophoresis containing 8 M urea. The results with four different templates are shown: template 7, lanes 1–3; template 23, lanes 4–6; template 24, lanes 7–9; and template 26, lanes 10–12. In lanes 1, 4, 7, and 10, the templates, ^{32}P-labeled at their 5′-ends, were loaded to show the sizes of full-length templates (open arrowheads). The reaction was performed in the presence (lanes 2, 5, 8, and 11) or absence (lanes 3, 6, 9, and 12) of NTP. Filled arrowheads indicate the bands corresponding to the primase-dependent reaction products. (B) Determination of the priming sites. The reaction was performed with NTP omitted (lane 1) or under the complete conditions (lane 2). The opposite strand for *de novo* DNA synthesis was used as a template for DNA sequencing, to correspond to migrated positions of the reaction products, while the sequence ladders correspond to be initiation positions. A, C, G, and T indicate the lanes of ddATP, ddCTP, ddGTP, and ddTTP, respectively. Filled arrowheads indicate the bands corresponding to the primase-dependent reaction products. (C) Identification of the first ribonucleotides of RNA primers. Unlabeled products of "21-mer" and "22-mer" on the template CTPPS1 (for sizes of the reaction products, see Figure 2A,B) were extracted from the cut pieces of the gel, and ribonucleotides at the 5′-ends of products were labeled with ^{32}P using T4 polynucleotide kinase. The products were then hydrolyzed and subjected to thin-layer chromatography, as described in Materials and Methods. Lanes A, G, and U show the positions of ^{32}P-labeled ribonucleoside monophosphates used as controls. Lanes 1 and 2 represent the 5′-end nucleotides of the 21- and 22-mers, respectively, which were obtained by using CTPPS1 as a template.

to the DNA polymerase α moiety stimulated the primase actiivty in the complex (Savoysky et al., 1993). In the present study, the initiation sites of RNA-primed DNA synthesis by calf thymus DNA polymerase α–primase were surveyed with clones from a bovine DNA library, and a number of distinct priming sequences containing pyrimidine clusters were obtained. Using these distinct sequences and their base-substituted fragments, we analyzed the primer synthesis, which was routinely tested by subsequent DNA synthesis. Systematic base substitutions revealed the minimum length of the pyrimidine clusters necessary for RNA-primed DNA synthesis by measuring the changes in the frequency of DNA synthesis. This frequency correlated well with the length of primer RNA and with the binding affinity of DNA polymerase α–primase. Furthermore, substituting the bases surrounding the priming site and changing the composition of deoxynucleoside triphosphate could also result in modifications in the RNA priming–DNA synthesis reaction.

MATERIALS AND METHODS

Materials. Unlabeled ribonucleoside triphosphate (NTP) and deoxyribonucleoside triphosphate (dNTP) were purchased from Yamasa Shoyu Co. Ltd. (Chiba, Japan). Radioactive compounds were from ICN Pharmaceuticals (CA, USA). T7 DNA polymerase was from Pharmacia (Uppsala, Sweden). T4 DNA polymerase exonuclease was from Takara Shuzo Co. Ltd. (Kyoto, Japan). All other reagents for enzyme assays were from commercial sources.

Enzyme Purification. Immunoaffinity-purified DNA polymerase α–primase complex was obtained as previously described (Tamai et al., 1988).

Preferred Priming Sites. Calf thymus DNA digested by *Eco*RI and *Hind*III was inserted into pUC118 and transfected into *E. coli* JM109. A number of insert-DNAs were amplified by an asymmetric PCR with primers 5′-GTAAAACGACGGCCAGT and 5′-CAGGAAACAGCTATGAC in a reaction mixture (100 μL) containing 10 mM Tris–HCl, pH 8.0, 50

mM KCl, 1.5 mM $MgCl_2$, 100 μM each of dNTP, 2.5 units of Taq polymerase, 3 μg of the one primer and 300 μg of another primer, and 0.1 ng of calf thymus DNA/pUC118. After 30–50 cycles of incubation, DNA was purified by SUPREC-02 (Takara Shuzo Co. Ltd.). The reaction products composed of single-stranded DNA were then subjected to screening for active templates for primase reaction by incubating with 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 50 μM each of CTP, GTP, and UTP, 2 mM ATP, 50 μM each of dCTP, dGTP, and dTTP, 5 μM [α-^{32}P]dATP (296 Bq/pmol), 0.2 unit of T7 DNA polymerase, and 0.04 unit of DNA polymerase α–primase complex. As a control, the DNA was also incubated under NTP-minus conditions. After the reaction products were denatured, electrophoresis was carried out with use of 8% polyacrylamide gel containing 8 M urea, which was then exposed to an X-ray film. When the product bands appeared in the NTP-dependent manner, the DNA clones were taken as positive for preferred priming sites. After the second screening, the NTP-dependent products were loaded on an 8% denatured polyacrylamide gel, next to the lanes of dideoxy-sequencing of the opposite strand. The dideoxy-terminated fragments which migrated at the same positions as the reaction products would correspond to the starting sites of primase.

Thin-Layer Chromatography. After *de novo* DNA synthesis reaction in the presence of DNA polymerase α–primase and CTPPS 1 (see below) without [α-^{32}P]dATP, the products were purified by Sephadex G-50 column chromatography. The 5′-end of the products was dephosphorylated by alkaline phosphatase, followed by phenol–chloroform extraction and ethanol precipitation, after which the 5′-end was labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Each phosphorylated product was then heated at 100 °C for 5 min, separated from the DNA template by 20% polyacrylamide gel containing 8 M urea, extracted, and digested in 0.1 M NaOH for 6 h at 30 °C. The solution was the neutralized and applied on a PEI–cellulose chromatography plate in 1.6 M LiCl. Standard spots of ^{32}P-labeled AMP, GMP, and UMP were obtained with synthetic RNA homopolymers treated in the same way.

Synthetic DNA. A series of DNA was synthesized depending on the preferred sequences, CTPPS 1 and mycSL1 (Suzuki, et al. 1993). The sequences are as follows:

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(positions)          5     10    15    20    25    30
CTPPS1,  5'-GCTATTCCACAAATTCCCTTTCCATCCACC;
PPSM1,   5'-GCTATTCCACAAAaaCCCTTTCCATCCACC;
PPSM2,   5'-GCTATTCCACAAAaaaaCTTTCCATCCACC;
PPSM3,   5'-GCTATTCCACAAAaaaaaaTTCCATCCACC;
PPSM4,   5'-GCTATTCCACAAAaaaaaaaaCCATCCACC;
PPSM5,   5'-GCTATTCCACAAATTCCCTTTCaATCCACC;
PPSM6,   5'-GCTATTCCACAAATTCCCTTaaaATCCACC;
PPSM8,   5'-GCTATTCCACAAATTCCCTTTCCtTCCACC;
PPSM9,   5'-GCTATTCCACAAATTCCCTTTttATCCACC;
PPSM12,  5'-GCTATTCCACAAATTCCCTTTCCAaaaAaa;
PPSM13,  5'-aaaAaaaaAaAAATTCCCTTTCCATCCACC;
mycM3,   5'-GATCCTAATagTCtCATCTCTTATGCGtgaatAagt
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Small letters indicate the substituted sequences. Deoxyribonucleotides were numbered from the 5′-end of each DNA strand. The DNA was chemically synthesized and purified by HPLC. Their purity was more than 98%, as estimated by 15% polyacrylamide gel electrophoresis containing 8 M urea.

Table I: DNA Sequences Which Support Primer RNA Synthesis[a]

	sequence containing priming sites	size of Py-cluster	Py content (%)
1.	5'-CAAATTCCCTTTCCATCCA-3'	10	100
2.	5'-ATCTGTGCTATTCCACAAA-3'	7	85.7
3.	5'-TTTTTTTAATTATTGCTCA-3'	14	78.6
4.	5'-TTGCTCATAATTTTACCCG-3'	11	72.7
5.	5'-TCATAATTTTACCCGTTCT-3'	8	87.5
6.	5'-CCGTTCTCTCCTCCACCCC-3'	11	100
7.	5'-CCCACAAATCATTCGTGAC-3'	6	83.3
8.	5'-TCATTCGTGACTCTGTCAT-3'	11	72.7
9.	5'-CATTTCTTTCCTTCAAAGG-3'	12	100
10.	5'-CTCGCACTATTTTTGCAGA-3'	8	87.5
	(Mean)	9.8	86.7

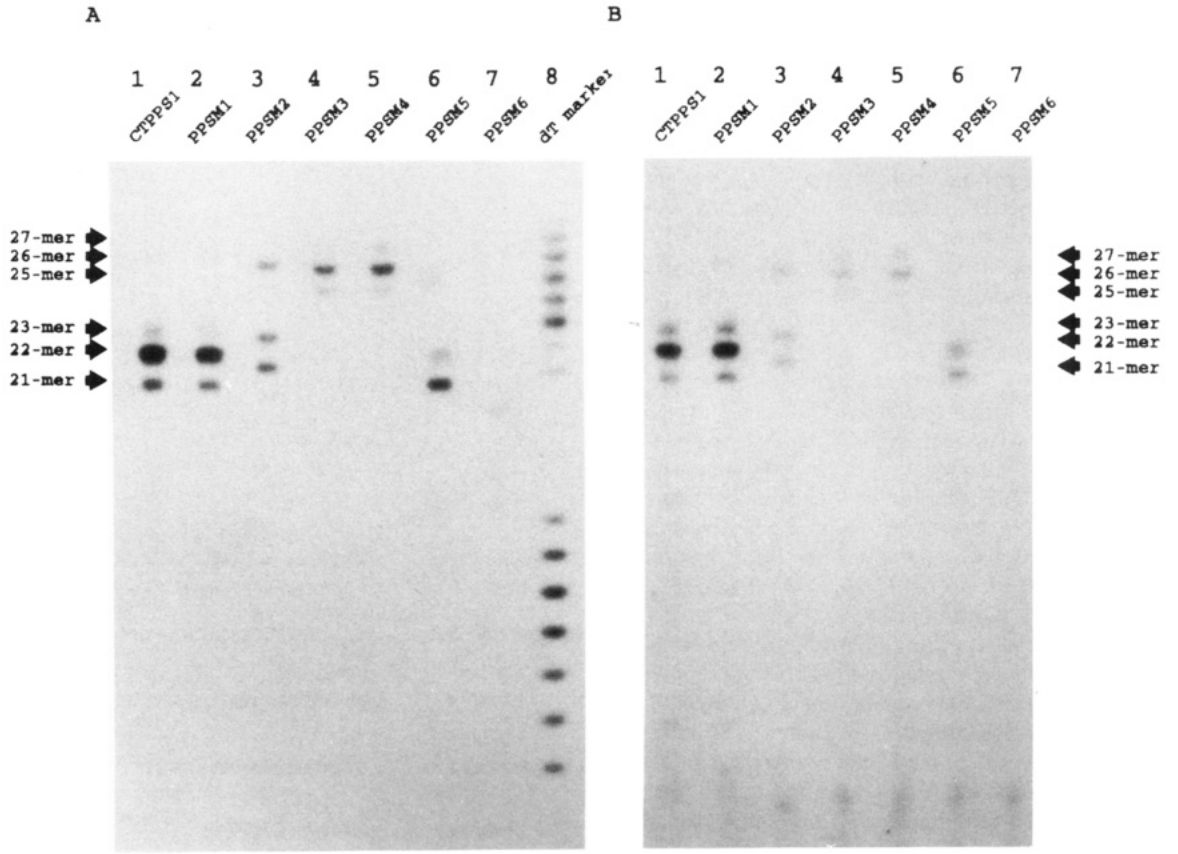
[a] Construction of calf thymus DNA library in pUC118 and amplification of the inserts by PCR were performed as described in Materials and Methods. The preferred priming sequences were determined as described in Figure 1. Ten sequences obtained by random screening are shown. Putative initiation sequences consisting of pyrimidine-rich stretches are shown shaded, while purine bases located near or adjacent to the priming sites downstream of templates are shown in bold letters. Sizes of the pyrimidine (Py) cluster and the content of pyrimidine in each cluster are shown. The initiation bases estimated from the sequencing gel are underlines. The priming sites 1–8 were obtained from template 7, while 9 and 10 were from 23 and 26 (Figure 1), respectively.

The DNA content was measured by the absorption of ultraviolet light (260 nm).

De Novo DNA Synthesis. Standard reaction mixture (25 μL) contained 50 mM Tris–HCl (pH 7.5), 2 mM dithiothreitol, 5 mM $MgCl_2$, 50 μM each of dATP, dGTP, and dTTP, 5 μM [α-^{32}P]dCTP, 2 mM ATP, 50 μM each of CTP, GTP, and UTP, 4 μg/mL of synthetic DNA, and 0.04 unit of calf thymus DNA polymerase α–primase. In some cases, 50 μM dCTP and 5 μM [α-^{32}P]dATP were used in place of 50 μM dATP and 5 μM [α-^{32}P]dCTP. To detect the products by RNA labeling, the same conditions were used except that 50 μM dCTP and 50 μM [α-^{32}P]GTP were used instead of [α-^{32}P]-dCTP and GTP, respectively. After incubation for 60 min at 37 °C, the reaction products were concentrated by ethanol with 1 μg of bovine DNA and then analyzed on 20% polyacrylamide gel containing 8 M urea. In some cases, the reaction products were quantified by the Fuji Image Analyzer Bas 2000 system or the Hamamatsu DVS 3000 system.

Length of RNA Primer. The reaction was carried out in 50 mM Tris–HCl (pH 7.5), 2 mM dithiothreitol, 5 mM $MgCl_2$, 50 μM each of dATP, dGTP, dTTP, and dCTP, 2 mM ATP, 50 μM each of CTP, UTP, and [α-^{32}P]GTP, 4 μg/mL of synthetic DNA, and 0.04 unit of calf thymus DNA polymerase α–primase. The reaction products were extracted by phenol–chloroform followed by ethanol precipitation. After the digestion of DNA by 0.05 unit of T4 DNA polymerase exonuclease in 50 mM Tris–HCl, pH 7.9, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.01% bovine serum albumin, and 50 mM potassium chloride, the remnant RNA was analyzed by electrophoresis on 20% polyacrylamide containing 8 M urea, and the reaction products were quantified by the Hamamatsu DVS 3000 system.

Gel Retardation Assay. CTPPS 1 was labeled with ^{32}P at its 5′-end using T4 polynucleotide kinase. One nanogram of ^{32}P-labeled CTPPS1 and 0.1 μg of DNA polymerase α–primase were incubated on ice for 5 min in the same buffer system used for *de novo* DNA synthesis except that substrates and $MgCl_2$ were omitted. Glutaraldehyde was then added in a final concentration of 0.02%, and the mixture was incubated



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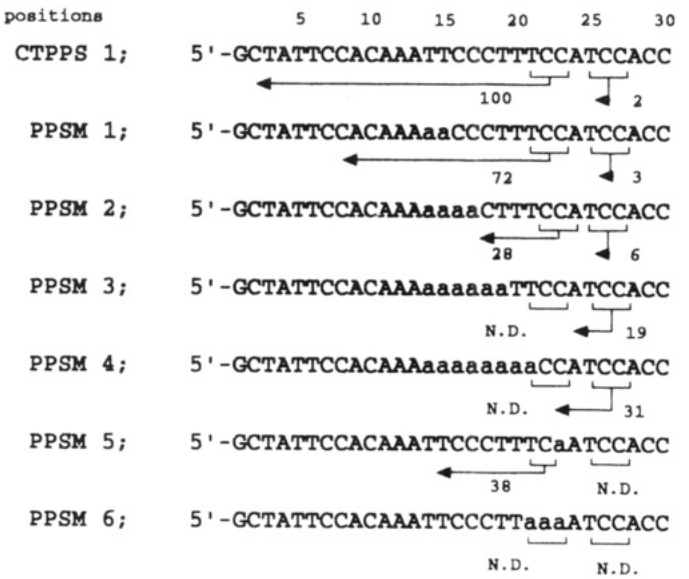

FIGURE 2: Effects of base substitutions in the pyrmidine cluster on the priming frequency of DNA polymerase α–primase. (A) Reaction products were labeled at the elongated DNA portions with [α-^{32}P]dCTP, denatured, and applied on a 20% polyacrylamide gel containing 8 M urea. The templates used were as follow: in lanes 1–7, CTPPS 1, PPSM 1, PPSM 2, PPSM 3, PPSM 4, PPSM 5, and PPSM 6, respectively. Size marker dT12–30 was loaded on lane 8. Arrows indicate the sizes of the reaction products. (B) Reaction products were labeled at primer RNA portions with [α-^{32}P]GTP and applied on a 20% polyacrylamide gel containing 8 M urea, as described in Materials and Methods. The templates used were as follow: 1–7, CTPPS 1, PPSM 1, PPSM 2, PPSM 3, PPSM 4, PPSM 5, and PPSM 6, respectively. Arrows indicate the sizes of the reaction products. (C) The intensity of each band was measured with use of the Hamamatsu DVS 3000 system and is illustrated in scheme. The arrow length and the numbers indicate the relative priming frequencies. N.D. indicates that the intensity was less than 1%.

further at 37 °C for 5 min. The complex formation was analyzed by electrophoresis on 5% polyacrylamide gel. Various amounts of DNA competitors were added at the same time as the ^{32}P-labeled CTPPS1 DNA probe.

Competition Analysis and de Novo DNA Synthesis. Deoxyhomopolymer (0.1, 1, or 10 μg; 1, 10, or 100 times excess over DNA template) was added to the standard reaction mixture for *de novo* DNA synthesis as described above. After the mixtures were incubated for 60 min at 37 °C, the reaction products were concentrated by ethanol with 1 μg of bovine DNA and then analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea. In some cases, GTP was omitted from the UTP-rich reaction mixture to prevent RNA synthesis on the competitor poly(dC).

RESULTS

Screening of Initiation Sites of RNA-Primed DNA Synthesis. A bovine genomic DNA library was constructed in pUC118, and inserts were amplified by means of an asymmetric PCR method into single-stranded (ss) DNA. These ssDNAs were then screened with respect to the template activity for RNA-primed DNA synthesis by calf thymus DNA polymerase α–primase complex. Among 140 strands of ssDNA surveyed, nine strands (7%) gave distinct product bands, which were absolutely dependent on ribonucleoside triphosphates (NTP) in the reaction mixture. Four examples of these analyses are shown in Figure 1A. Apparently, templates 7 (lanes 1–3), 23 (lanes 4–6), and 26 (10–12) gave positive patterns. In contrast, template 24 (lanes 7–9) appears to be negative since no product bands were observed except for NTP-independent incorporation of the template size or larger, which might be due to the end-addition of deoxyribonucleotides to the template DNA. Table I summarizes the results of the random screening of primer RNA synthesis (priming sites) on bovine DNA. In all cases, the start sites of priming sites were pyrimidines. At the 5′-sides of the priming sites, there were pyrimidine-rich sequences with lengths ranging from 6 to 14 bases long. Three of them were completely pyrimidine clusters, while others contained 1–3 purines in pyrimidine-rich stretches. In general, the priming sites were observed at or near the 3′-end of these pyrimidine-rich sequences, and purines (A or G) existed at the 3′-end, adjacent to or near the priming sites. These characteristics agree well with those proposed by another group using partially purified human DNA polymerase α–primase (Yamaguchi et al., 1985). To analyze precisely the sequence specificity, a part of template 7 (designated as CTPPS1) and its base-substituted templates were synthesized because they showed typical characteristics as described above.

Determination of Priming Site on CTPPS 1. CTPPS 1 (5′-GCTATTCCACAAATTCCCTTTCCATCCACC) is rich in pyrimidine residues (22/30) and contains a 10-mer pyrimidine cluster. Initiation positions with this template were determined by measuring the sizes of the reaction products using 7 as a template (Figure 1B) and also by determining the 5′-end nucleotides of two out of three major reaction products found on CTPPS 1 (Figure 1C). The RNA primers are therefore considered to initiate from the pyrimidines at positions 21, 22, and 23 on the synthetic DNA, CTPPS 1 (Figure 2A). Although the intensity of the 21-mer products appeared stronger than that of the 23-mer (Figure 2A), the ratio between these reaction products varied in experiments (see Figures 3 and 7A), while the 22-mer always gave the strongest image.

To determine the priming sites from the length of products, it must be certain that the products could be elongated to the 5′-ends of templates by DNA polymerase α–primase complex under the conditions employed, since the processivity of DNA polymerase α has been reported to be rather low (Tsurimoto & Stillman, 1991). As shown above, CTPPS 1 contains only one guanine base at its 5′-end. Taking advantage of this characteristic, the products were labeled with [α-^{32}P]dCTP at their 3′-ends to detect only the ful-length products (Figure 2A). Alternatively, the products were also labeled with [α-^{32}P]-GTP in their RNA primers (Figure 2B). Patterns of products under these two different conditions were shown to be essentially the same, indicating that the synthesis proceeded to the 5′-ends of the templates. Since CTPPS 1 or its base-substituted derivatives carry only one deoxyguanosine residue in each 5′-end, their initiation frequencies may correspond to the intensities of the reaction products when labeled with

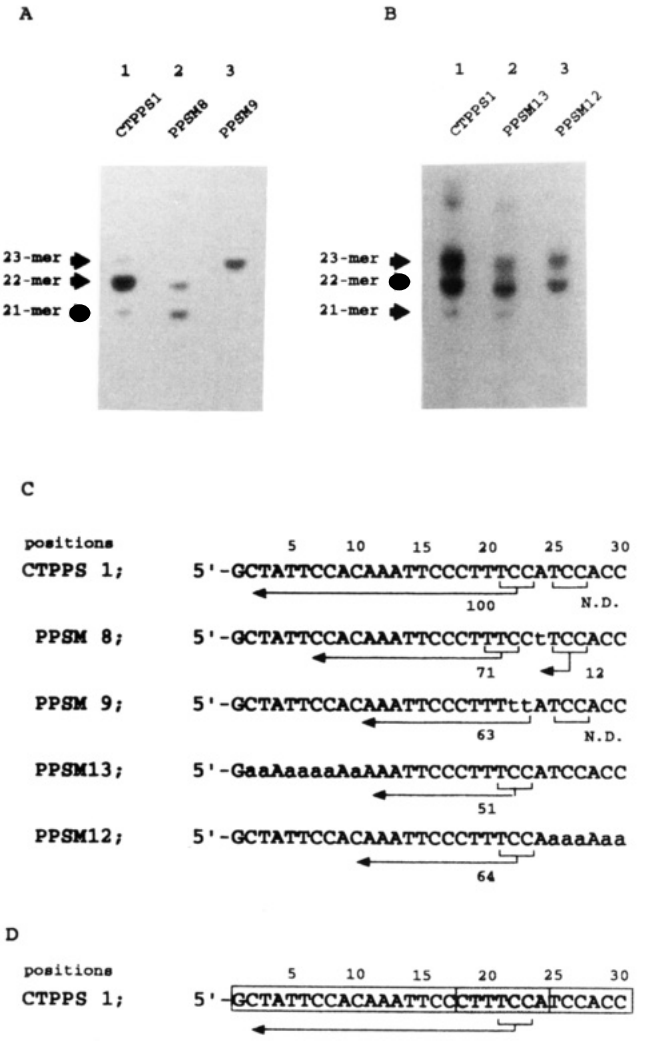

FIGURE 3: Effects of base substitutions at outside of the pyrimidine cluster on the priming frequency. (A) Reaction products were labeled with [α-^{32}P]dCTP and analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea as described in Materials and Methods. The templates used were as follow: 1–3, CTPPS 1, PPSM 8, and PPSM 9, respectively. Arrows indicate the sizes of the reaction products. (B) Reaction was performed as in A with [α-^{32}P]-dCTP. The templates used were as follow: lanes 1–3, CTPPS 1, PPSM 13, and PPSM 12, respectively. Arrows indicate the sizes of the reaction products. (C) The intensity of each band was measured with use of the Hamamatsu DVS 3000 system is and illustrated in scheme. The arrow length and the numbers indicate the priming frequency. N.D. indicates that the intensity was less than 1%. (D) The results of Figures 2C and 3C are summarized. Pyrimidine residues in the open box contribute to increase the priming frequency, while the sequence in the shaded box is essential for both priming frequency and initiation from positions 21, 22, and 23 of the template.

[α-^{32}P]dCTP. Thus, this assay system is highly quantitative, excluding abortive RNA primer synthesis which consists of short nucleotides from which DNA synthesis does not occur (Kuchta et al., 1990; Podust et al., 1991).

Minimum Priming Unit of Pyrimidine Cluster for Priming. When the 10-mer pyrimidine cluster (positions 14–23) in CTPPS 1 containing the priming sites was shortened in the 5′ to 3′ direction by replacement with (dA)*n*, the priming frequencies from positions 21, 22, and 23 decreased drastically as the cluster became shorter than a 6-mer, and there was no initiation from these sites with a tetramer or a dimer (Figure 2A,B, lanes 1–5). Thus, two pyrimidines could be replaced in the 5′ to 3′ direction without any serious effect on the priming frequency. Interestingly, the minor primings at -TCC- (positions 25–27) became predominant after these replacements (Figure 2A, lanes 3–5), although these products

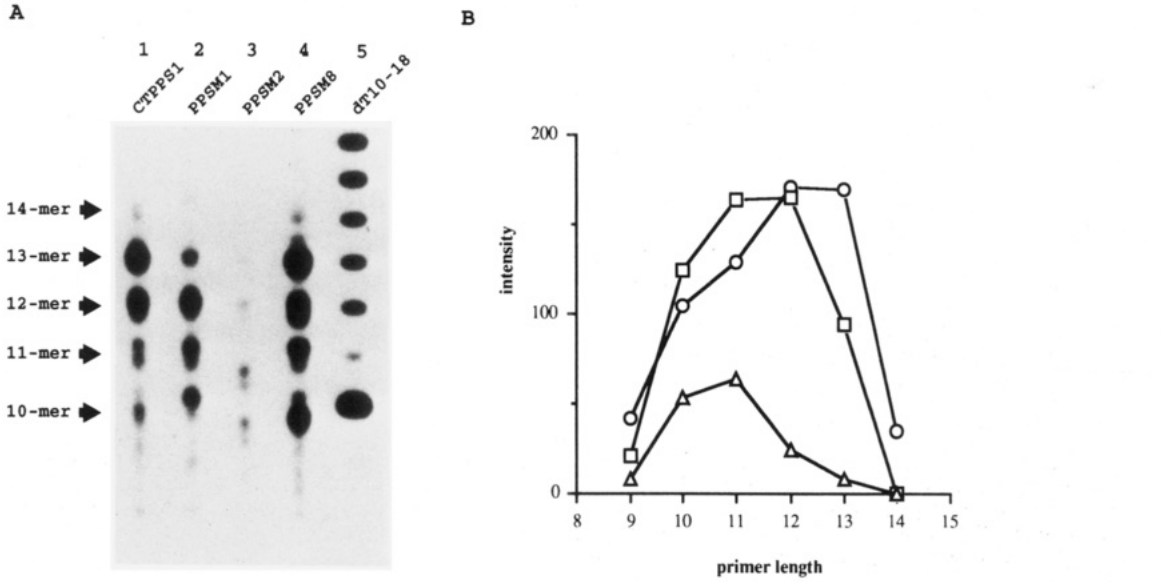

FIGURE 4: Changes in the length of synthesized RNA primers by the base substitution of priming sequence. (A) Reaction products were labeled with [α-^{32}P]GTP. The RNA–DNA products were extracted with chloroform–phenol, followed by ethanol precipitation. The extracted DNA was then digested with T4 DNA polymerase exonuclease for 60 min at 37 °C and loaded on a 20% polyacrylamide gel containing 8 M urea, as described in Materials and Methods. The templates in lanes 1–4 were CTPPS 1, PPSM 1, PPSM 2, and PPSM 8, respectively. In lane 5, ^{32}P-labeled dT10–18 was loaded as a size marker. (B) The results in A were quantified with use of the Fuji Image Analyzer Bass 2000 system. Symbols used are as follows: ○, CTPPS 1; □, PPSM 1; △, PPSM 2.

migrated slightly slower than expected in the gel for unknown reasons. In contrast, when the 10-mer was shortened in the 3′ to 5′ direction in the same way, the priming was strongly suppressed by onely one-base substitution and completely suppressed by three-base substitution (Figure 2A,B, lanes 6 and 7). However, when the -CC- sequence (positions 22, 23) corresponding to the priming sites was substituted with -TT-, the priming was not abolished, but the unique size products initiated from position 23 (Figure 3A, lane 3). These results indicate that the 5′-half of the 10-mer pyrimidines enhances the priming, while the 3′-half is essential for the initiation.

Regulatory Roles of the Downstream Sequence from the Pyrimidine Cluster. As shown in Table I, the priming sites were observed at or near the 3′-end, adjacent to or near the priming sites. In the case of CTPPS 1, the purine is adenine. A single base substitution of this deoxyadenine to deoxythymidine decreased the initiation from positions 22 and 23, and the major initiation site shifted to position 21, with minor initiation from positions 25 and 26 (Figure 3A, lane 2). Therefore, adenine in position 24 was also required for the predominant initiations from positions 21, 22, and 23. Base substitutions with (dA)*n* in the remaining 5′- or 3′-sequence outside the cluster decreased the priming frequency but did not change the initiation positions (Figure 3B). As summarized in Figure 3D, seven residues in the hutched box are essential for the initiation from positions 21, 22, and 23, and pyrimidine residues outside of this box contribute to enhance the priming frequency.

Sequence Substitution Affects the Primer Length. The length of the RNA primer in each product was measured (Figure 4A,B). With CTPPS 1, the 10- to 13-mers of the RNA primers were synthesized before DNA synthesis started, and the major products were 12- and 13-mers. In contrast, with PPSM 1 and 2, although the RNA primers ranged from 10- and 13-mers similar to CTPPS 1, the major products were 11- and 12-mer and 10- and 11-mer, respectively. Apparently, the shorter pyrimidine stretch produced a shorter RNA primer. With PPSM 8, carrying the longest pyrimidine cluster, the length of the RNA primer was distributed in wider range, 10- to 13-mer (Figure 4A). Thus, the lengths of pyrimidine residues also affect the length of RNA primers.

DNA Polymerase α–Primase Binds to the Pyrimidine Cluster. By gel retardation assay, it was clearly shown that DNA polymerase α–primase physically bound to the DNA template, CTPPS 1. Complex formation of DNA polymerase α–primase with CTPPS 1 was not competed by 100 times excess of poly(dA), whereas it was strongly competed by 10 times excess of poly(dC), poly(dG), or poly(dT) (Figure 5A). These data suggest that DNA polymerase α–primase could bind to poly(dC), poly(dG), or poly(dT) but not to poly(dA). This was confirmed by analysis of the reaction products. The addition of homopolymers gave similar competition patterns, *i.e.*, 10 times excess of poly(dC), poly(dG), or poly(dT) abolished the initiation on CTPPS 1 from positions 21, 22, and 23, while poly(dA) did not (Figure 5B). Different from the results of the gel retardation analysis, however, 100 times excess of poly(dA) added into the reaction mixture slightly decreased the product amounts (Figure 5B, lane 8), indicating the DNA polymerase α–primase complex might be able to interact with deoxyadenylate very weakly. A similar pattern of competition was also observed with another template, myc M3 (Figure 5C).

The binding affinity of DNA polymerase α–primase was further tested with the deoxyheteropolymers, CTPPS 1 and its derivatives, which showed various template activities. In the gel retardation assay, DNA polymerase α–primase–CTPPS 1 complex was competed with 25 and 100 times excesses of CTPPS 1, PPSM 2, PPSM 3, and PPSM 4 (Figure 6A). The binding was competed in the following order: CTPPS 1 > PPSM 1 > PPSM 2 > PPSM 3 > PPSM 4. The strengths of competition were nearly proportional to their pyrimidine contents and also to their template activities, except for PPSM 4, which showed a slightly higher template activity than PPSM 3 (Figure 2C). In addition, we determined the K_m value of each template in *de novo* DNA synthesis (Figure 6B). The K_m values for CTPPS 1, PPSM 1, PPSM 2, PPSM 3, and PPSM 4 were 0.45, 0.63, 2.50, 5.56, and 4.55 μg/mL, respectively. Evidently, in agreement with results of the gel retardation assay, as the number of deoxyadenosine residues increases, the K_m value of the template becomes higher, except for PPSM 4, which showed a K_m value lower than that of PPSM 3 (Figure 6A). These results strongly suggest that the

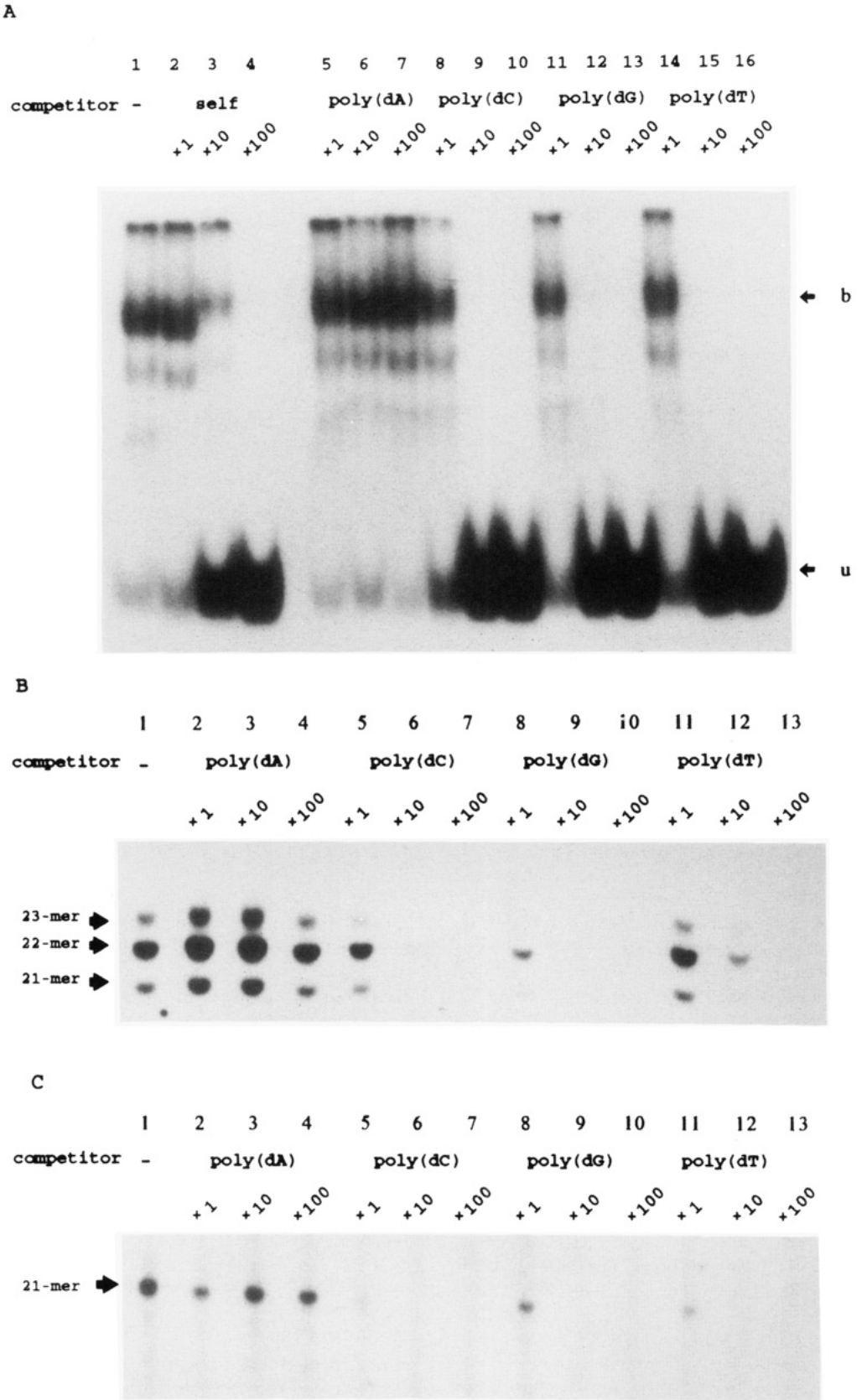

FIGURE 5: Competition in the DNA binding and in the priming reaction of DNA polymerase α–primase complex by synthetic homopolymers. (A) The DNA polymerase α–primase–DNA (CTPPS 1) complex was detected by the gel retardation assay as described in Materials and Methods. No competitor was added in lane 1, and various amounts of competitor DNA (unlabeled) were added as follows: 1, 10, and 100 ng of CTPPS 1 in lanes 2–4, respectively: 1, 10, and 100 ng of poly(dA) in lanes 5 to 7, respectively; 1, 10, and 100 ng of poly(dC) in lanes 8–10, respectively; 1, 10, and 100 ng of poly(dG) in lanes 1–13, respectively; 1, 10, and 100 ng of poly(dT) in lanes 14–16, respectively. The letter u indicates the position of unbound DNA, and the letter b indicates the position of complexed DNA. (B) Competition in *de novo* DNA synthesis on CTPPS 1. To the reaction mixture were added 0.1, 1, or 10 μg of competitor DNA (1, 10, or 100 times excess over DNA template) and 0.1 μg of CTPPS 1, and the mixture was incubated as described in Materials and Methods. No competitor was added in lane 1. Poly(dA) in 1, 10, or 100 times excess was added in lanes 2–4, respectively; 1, 10, or 100 times excess poly(dC) was added in lanes 5–7, respectively; 1, 10, or 100 times excess poly(dG) was added in lanes 8–10, respectively; 1, 10, or 100 times excess poly(dT) was added in lanes 11–13, respectively. (C) Compeition of DNA synthesis on myc M3. To the reaction mixture were added 0.1, 1, or 10 μg of competitor DNA

CAPTION TO FIGURE 5 (CONTINUED)
(1, 10, or 100 times excess over DNA template) and 0.1 μg of myc M3, and the mixture was incubated in Materials and Methods. No competitor was added in lane 1; 1, 10, or 100 times excess poly(dA) was added in lanes 2–4, respectively; 1, 10, or 100 times excess poly(dC) was added in lanes 5–7, respectively; 1, 10, or 100 times excess poly(dG) was added in lanes 8–10, respectively; 1, 10, or 100 times excess poly(dT) was added in lanes 11–13, respectively. The determination of the priming positions on myc M3 was described previously (Suzuki et al., 1993).

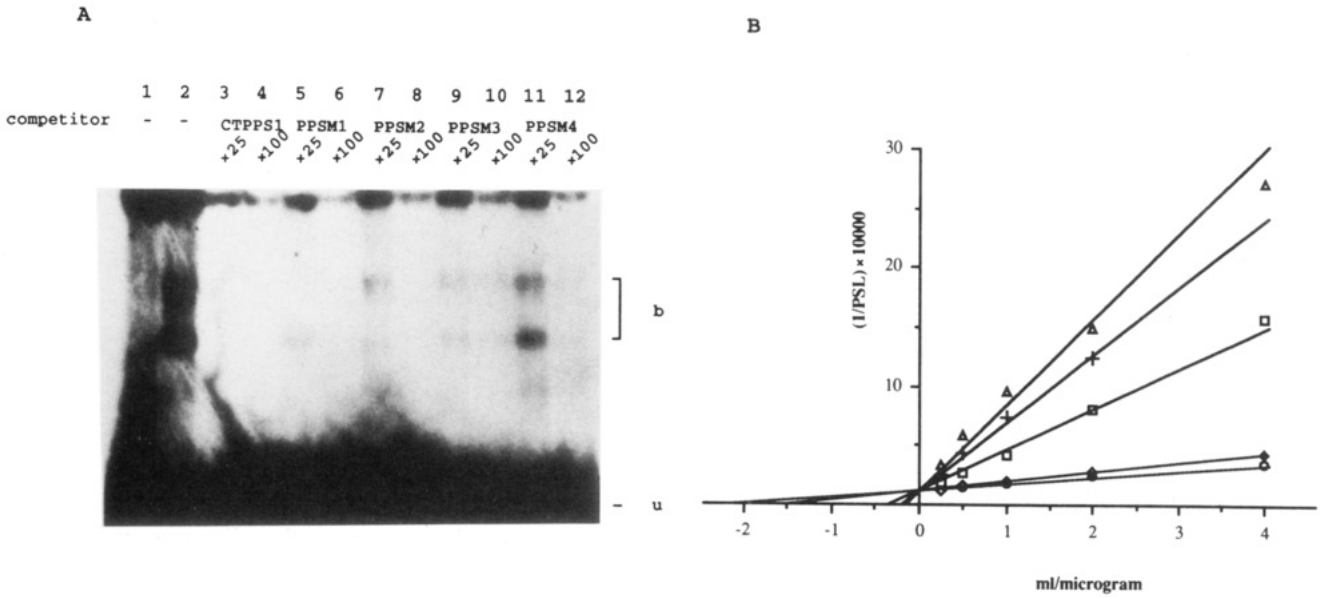

FIGURE 6: Binding affinity of DNA polymerase α–primase to various DNA. A. The gel retardation assay was performed as described in Materials and Methods. The DNA polymerase α–primase–DNA (CTPPS 1) complex was competed by various synthetic DNA. Neither competitor nor DNA polymerase α–primase was added in lane 1; no competitor was added in lane 2. Various amounts of competitor DNA (cold) were added as follows: 25 and 100 ng of CTPPS 1 in lanes 3 and 4, respectively; 25 and 100 ng of PPSM 1 in lanes 5 and 6, respectively; 25 and 100 ng of PPSM 2 in lanes 7 and 8, respectively; 25 and 100 ng of PPSM 3 in lanes 9 and 10, respectively; 25 and 100 ng of PPSM 4 in lanes 11 and 12, respectively. The letter u indicates the position of unbound DNA, and the letter b indicates the position of complexed DNA. (B) the K_m value of each DNA template was determined with use of a Lineweaver–Burk double reciprocal plot. Reaction products were labeled stoichiometrically with [α-^{32}P]dCTP at their 3′-end, electrophoresis was carried out using a 20% polyacrylamide gel containing 8 M urea, and the products were analyzed with use of Fuji Image Analyzer Bass 2000 system. Symbols used are as follows: ○, CTPPS 1; ◇, PPSM 1; □, PPSM 2; △, PPSM 3; +, PPSM 4. From this data, the K_m values for CTPPS 1, PPSM 1, PPSM 2, PPSM 3, and PPSM 4 were found to be 0.45, 0.63, 2.50, 5.56 and 4.55 μg/mL, respectively.

frequency of RNA-primed DNA synthesis is proportional to the binding affnity of the DNA polymerase α–primase to DNA.

NTP Concentration Influences Priming Patterns. Since it has been reported that the priming patterns could be influenced by NTP in the reaction mixture (Yamaguchi et al., 1985; Sheaff & Kuchta, 1993), we analyzed the RNA-primed DNA synthesis at various concentrations of NTP. The concentration of ATP in our standard reaction conditions was 40 times higher (2 mM) than those of other nucleotides (50 μM). When the ATP concentration was decreased to 50 μM, only a small amount of DNA synthesis was observed. However, when UTP or GTP concentration was increased to 0.5 mM, primase started from sites distinct from those under ATP-rich conditions (Figure 7, lanes 4 and 5). Similar changes in priming sites were also observed with another template, myc M3 (Figure 7B). It was noteworthy that the second base from each priming site was always complementary to the ribonucleotide added in excess amount, *i.e.*, GTP or UTP.

We then analyzed the effects of competitor DNA on the reaction products, using various concentrations of NTP. Under ATP-rich conditions, the 21-mer products on myc M3 were eliminated by the addition of poly(dC) but not by poly(dA) (Figure 8A), as evident in Figure 5C. Addition of deoxy-homopolymers also showed almost the same competition patterns under both CTP- and UTP-rich conditions (Figure 8B,C. Since these reaction mixtures contain 50 μM GTP, they may support the small amount of primer synthesis on poly(dC) by DNA polymerase α–primase complex. Since this catalytic process might cause an overestimation of the results of the competition analysis with poly(dC), we omitted GTP from the UTP-rich reaction mixture to prevent RNA synthesis on the competitor poly(dC). As shown in Figure 8D, the 34-mer products were also observed under these conditions, and the reaction products were eliminated by excess amouints of poly(dC), but the reaction was less affected by poly(dA) (Figure 8D).

The competition data indicate that the binding affinity of DNA polymerase α–primase to DNA is independent of the NTP concentrations, although the initiation site of RNA-primed DNA synthesis can be altered by changing the balance of the four NTPs in the reaction mixture.

DISCUSSION

From a bovine DNA library, we obtained nine strands of ssDNA containing distinct initiation sites for RNA-primed DNA synthesis by the calf thymus DNA polymerase α–primase complex. The general characteristics of the sequences around the priming sites were the following: (1) rich in pyrimidines, (2) priming sites were located at or near the 3′-end of pyrimidine clusters, and (3) pyrimidine-rich stretches were 6–14 bases long (Table I).

Although CTPPS 1 gave predominant reaction products in the cloned fragment, DNase I footprinting did not show any specific protected regions around the initiation sites (data not shown). This may be due to the weak interaction between DNA polymerase α–primase and DNA since cross-linking by glutaraldehyde was necessary to detect the DNA polymerase α–primase–template comkplex during the gel retardation assay. Alternatively, the enzyme might migrate along the template and not stay at the initiation sites for a long time,

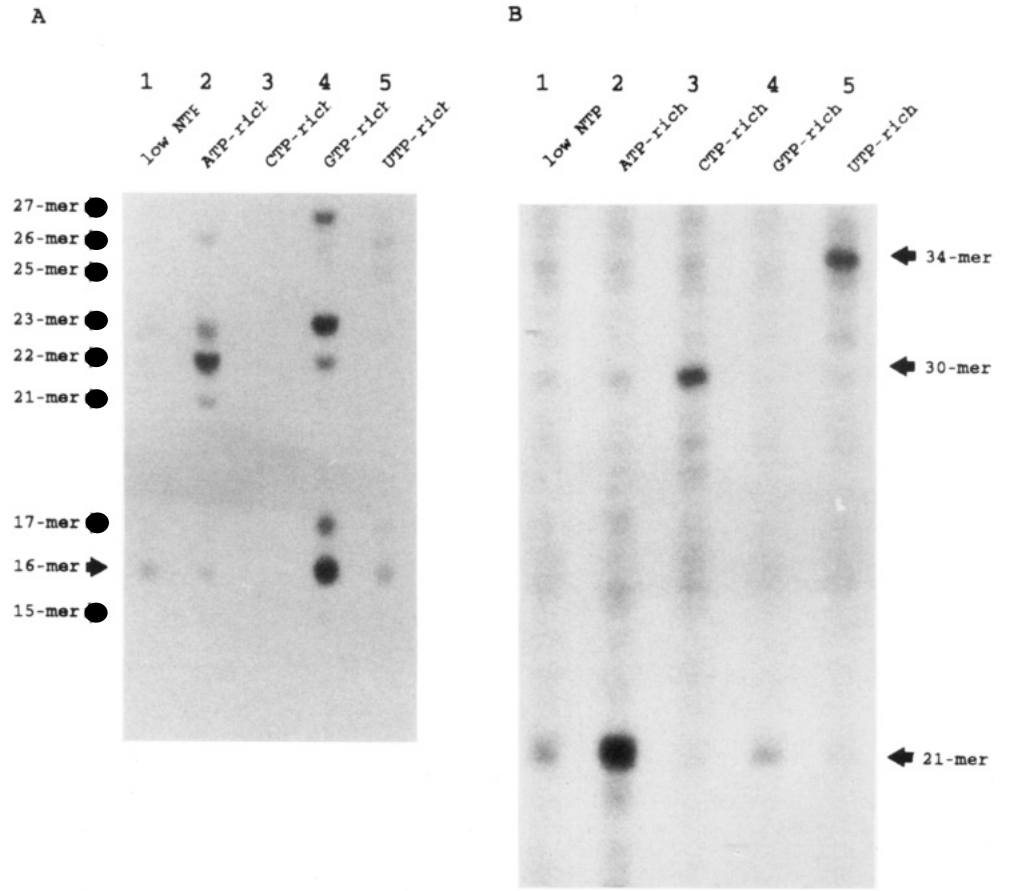

FIGURE 7: Effects of various NTP compositions on the *de novo* DNA synthesis. Reaction was carried out with use of DNA polymerase α–primase complex using CTPPS 1 (A) or myc M3 (B) DNA template, in the presence of various concentrations of NTP (Materials and Methods). The reaction products, labeled with [α-^{32}P]dATP, were analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea. In both A and B, NTP compositions were as follows: lane 1, low (50 μM each) 4 NTP; lane 2, 0.5 mM ATP and 50 μM each of other 3 NTP (ATP-rich); lane 3, 0.5 mM CTP and 50 μM each of other 3 NTP (CTP-rich); lane 4, 0.5 mM GTP and 50 μM each of other 3 NTP (GTP-rich); lane 5, 0.5 mM UTP and 50 μM each of other 3 NTP (UTP-rich). Arrows indicate the sizes of the reaction products.

as was seen with the gene 4 protein of bacteriophage T7, which translocates along the DNA in search of its recognition sequence (Tabor & Richardson, 1981).

Interestingly, the changes in the template sequence affected the lengths of the product RNA primers. The most frequent sizes of product RNA depended on the length of pyrimidine cluster and on the length of (dA)*n* in the replaced region (Figure 4), suggesting that the replaced (dA)*n* stretch may urge the enzyme to stop primer synthesis on PPSM 1 and 2.

Although the main experiments were performed with use of a series of synthetic DNA, the same priming pattern from the positions 21, 22, and 23 on CTPPS 1 was also observed on a longer stretch of template DNA (Figure 1B). With other sequences, the primings were also quite similar between long DNA and short fragments of synthetic DNA (data not shown); thus, the priming pattern on the short fragment may reflect the basic priming rule of the longer stretch of ssDNA. The weaker primings around position 16 on CTPPS 1 compared with those on template 7 (Figures 2A and 1B) may be due to the shorter stretch of the 5′-side pyrimidines of the synthetic DNA.

In this study, one of the preferred sequences, CTPPS 1, was analyzed in detail with respect to its priming activity and its binding affinity to the enzyme by substituting the pyrimidines with deoxyadenines. It has been reported that poly(dA) could neither serve as a template for primase reaction nor bind the primase (Roth, 1987; Suzuki et al., 1993; Holmes et al., 1986). The pyrimidine cluster (10-mer) was shortened by replacement with (dA)*n* in the 5′ to 3′ direction, resulting in a stepwise loss of priming activity. It was revealed that the minimum length of the priming sequence consists of a 6-mer of pyrimidine clusters in the case of CTPPS 1. At the same time, this series of replacements stimulated the minor primings from other pyrimidines located 3′-downstream of the pyrimidine cluster, while largely suppressing the major primings (Figure 2C). The facts that the total priming frequency on PPSM 4 was higher than that of PPSM 3 (Figure 2C) and that the K_m for PPSM 4 was lower than that for PPSM 3 (Figure 6) indicate that this shift of priming sites might be caused by a higher affinity of primase to the sequence 5′-CCATCC-3′ (positions 22–27) in the replaced templates compared to its affinity to the original sequence.

On the other hand, replacement of the bases in the pyrimidine cluster with (dA)*n* in the 5′ to 3′ direction immedately abolished the priming activity, suggesting the importance of the -CC- sequence at the 3′-end of the pyrimidine cluster. When the -CC- was replaced with -TT-, however, the priming activity was well preserved (Figures 2 and 3). These results suggest that pyrimidines are itnerchangeable in this site. Furthermore, almost complete replacement with (dA)*n* in either the 5′-sequence or the 3′-sequence located outside the pyrimidine cluster significantly decreased the priming frequency from the major priming sites (Figure 3C). Davey and Faust (1990) reported that murine DNA polymerase α–primase initiates RNA-primed DNA synthesis preferentially upstream of the 3′-CC(C/A)-5′ motif. With our result from the use of calf thymus DNA polymerase α–primase, however, this motif was not always found downstream of the initiation site. Both the 3′-downstream and the 5′-upstream position of deoxypyrimidines, irrespective of

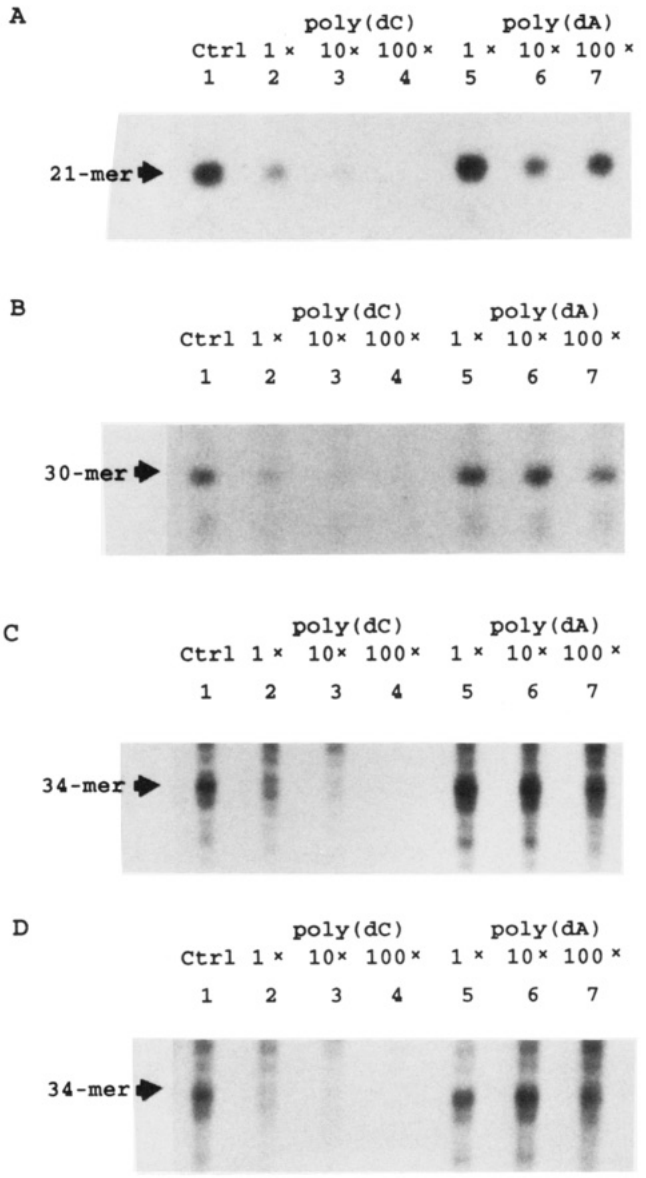

FIGURE 8: Competition in *de novo* DNA synthesis with homopolymers in various NTP compositions. Reaction was performed with use of myc M3 and DNA polymerase α–primase complex, in the presence of various homopolymers. Product was labeled with [α-^{32}P]dATP. A, B, and C correspond to the products under ATP-, CTP- and UTP-rich conditions, respectively (see Figure 7). Lane 1, no competitor; lanes 2–4; 0.1, 1, or 10 μg (1, 10, and 100 times more than the DNA template) of poly(dC) was added, respectively; lanes 5–7, 0.1, 1, or 10 μg (1, 10, and 100 times more than the DNA template) of poly(dA) was added, respectively. (D) Reaction was performed under UTP-rich conditions without GTP in the presence of various homopolymers. Lane 1, complete reaction mixture without competitor; lanes 2–4, 0.1, 1, or 10 μg (1, 10, and 100 times more than DNA template) poly(dC) was added, respectively; lanes 5–7, 0.1, 1, or 10 μg (1, 10, and 100 times more than template DNA) of poly(dA) was added, respectively. Product sizes are indicated on the left sides (A–D).

sequence, appeared to be important, although this result does not rule out the preferential motif outside the initiation site.

The priming mechanism was further studied with respect to enzyme protein–DNA interaction (Figures 5, 6, and 8). The competition experiments on the gel retardation and the product analyses showed that pyrimidine residues could mediate the interaction regardless of NTP balance, whereas deoxyadenosine could hardly do so. With the deoxyheteropolymers, the priming frequencies were proportional to the binding affinities of DNA to enzyme. Moreover, adenine in position 24, located at the 3′-boundary of the pyrimidine cluster, was also required for the predominant initiations from positions 21, 22, and 23 in the case of CTPPS 1. Evidently, this adenine at the 3′-boundary of the pyrimidine cluster was needed to recognize the specify the priming site.

Taking all these results together, it was concluded that both the 3′-downstream 6-bases and the 5′-upstream 17-bases modulate the primings from the minimum priming unit, which consists of a pyrimidine cluster (6-mer) associated with one adenine at the 3′-side in the case of CTPPS 1, by enhancing the priming efficiency and/or slightly shifting the site of initiation.

Although we replaced the pyrimidine bases with (dA)*n*, the priming frequency also decreased with the replacement of pyrimidines in the pyrimidine cluster with dG (Suzuki et al., 1993). Therefore, both dA and dG decrease the priming frequency in the heteropolymer, except in cases wherein they locate 3′-adjacent to the pyrimidine cluster. We have also demonstrated that the primase subunit, free from the α subunit, is able to recognize the same preferred sites (Suzuki et al., 1993).

These results suggest an initiation mechanism for RNA-primed DNA synthesis *in vitro*, in which primase primarily recognizes the minimum priming unit consisting of a pyrimidine cluster with a 3′-boundary of purine. The primase may also interact with the pyrimidine residues of both the 3′-downstream and the 5′-upstream of the minimum priming unit. In the minimum priming unit, the priming site could be determined by the NTP balance. Under the usual conditions wherein ATP is predominant, the priming occurs from one (or two) base(s) downstream (3′) of dT, which is complementary to ATP. Under GTP- or UTP-rich conditions, a shift in priming occurs in the same pattern: priming occurs from one base downstream of dC or dA, which is complementary to each NTP in excess amount.

Our results showing that DNA polymerase α–primase binds to poly(dG) (Figure 5) are contradictory to previous results by Holmes et al. (1986) that DNA polymerase α–primase binds to neither poly(dG) nor poly(dA). The reasons for this discrepancy may be due to the different reaction conditions. However, the interaction between poly(dG) and DNA polymerase α–primase is also not consistent with the fact that this homopolymer could not be used as a template by primase (data not shown). Previously, a number of the initiation sites in the core origin of SV40 DNA were localized to pyrimidines within guanine-rich sequences (Hay & DePamphilis, 1982). It is conceivable, therefore, that the primase activity binds to a guanine-rich sequence *in vivo*. Since the primase by itself cannot start from those guanine-rich sites *in vitro* (Yamaguchi et al., 1985; Suzuki et al., 1993), the initiation *in vivo* may need the help of other factors.

On the other hand, replication protein A(RP-A) may be responsible for the suppression of initiation because it shares the same pyrimidines residue sites as DNA polymerase α–primase, thus inhibiting the priming activity (Collins & Kelly, 1991; Kim et al., 1992). The basic initiation mechanism of RNA-primed DNA synthesis described here may therefore be regulated by other mechanisms, which may activate or inactivate the primase in the replication machinery. The flexible change in the initiation sites of RNA-primed DNA synthesis caused by changes in the composition of NTP suggests another regulation mechanism, *e.g.*, the activation or suppression of priming by modulation of the microenvironment of the potential priming sequences.

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