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# Mouse DNA polymerase α-primase terminates and reinitiates DNA synthesis 2–14 nucleotides upstream of $C_2A_{1-2}(C_{2-3}/T_2)$ sequences on a minute virus of mice DNA template

**(primase consensus formula/telomere replication/linear eukaryotic chromosome/discontinuous DNA synthesis)**

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**ABSTRACT** **The distribution of termination and initiation sites in a 5081-nucleotide minute virus of mice DNA template being copied by a highly purified mouse DNA polymerase α–DNA primase complex in the presence of GTP has been examined. The 3′-hydroxyl termini (17 in all) were clustered at six sites that were located 2–14 nucleotides upstream of $C_2A_2C_2$, $C_2AC_3$, or $C_2A_2T_2$ sequences. When either [α-$^{32}$P]- or [γ-$^{32}$P]GTP was included in the DNA polymerase reaction mixtures, nascent DNA became radiolabeled. Analysis of the $^{32}$P-labeled material following treatment of the DNA with tobacco acid pyrophosphatase, bacterial alkaline phosphatase, or ribonuclease T1 revealed the presence of oligoribonucleotide chains averaging 5–7 nucleotides long and beginning with 5′ GTP residues. Eight presumptive DNA primase initiation sites were located opposite $C_4$ or $C_5$ sequences 3–9 nucleotides upstream of one of the three closely related hexanucleotides $C_2A_2C_2$, $C_2AC_3$, and $C_2A_2T_2$. RNA–DNA junctions were found 3–10 nucleotides downstream of DNA primase initiation sites. The results indicate that hexanucleotides having the general formula $C_2A_{1-2}(C_{2-3}/T_2)$, herein referred to as ψ, are involved in promoting termination of DNA synthesis and/or *de novo* initiation of RNA-primed DNA chains by DNA polymerase α-primase.**

Highly purified preparations of the eukaryotic replicative enzyme DNA polymerase α contain a tightly bound oligoribonucleotide polymerase that is generally referred to as primase (1, 2). Primase is distinguished from RNA polymerases by the fact that the length of the oligoribonucleotide synthesized is restricted (4–10 nucleotides) and by its ability to synthesize mixed oligoribo-deoxyribonucleotide chains (3–5). Primase purified from *Drosophila melanogaster* embryos appears to comprise polypeptides of $M_r$ 50,000 and 60,000 that are tightly bound to a $M_r$ 182,000 DNA polymerase catalytic subunit (6). An apparently homogeneous primase preparation from mouse hybridoma cells, free of DNA polymerase activity, consists exclusively of polypeptides of $M_r$ 46,000 and 56,000 (7). Immunological evidence has been obtained for a tight association of primase activity with DNA polymerase α from (murine) Ehrlich ascites cells (8) and from KB cells (9). These physical and enzymological properties of DNA polymerase α-primase are entirely consistent with a role for this enzyme in the discontinuous RNA-primed DNA synthesis that occurs predominantly, but not exclusively, on the lagging strand of the DNA replication fork in mammalian cells (10, 11).

The ability of highly purified preparations of mouse DNA polymerase α to copy the 5081-nucleotide linear single-stranded DNA genome of minute virus of mice (MVM), an autonomous parvovirus, has been described (12, 13). This simple viral DNA molecule contains self-complementary sequences, 115 and 206 nucleotides long, at its 3′ and 5′ termini, respectively; these regions contain the *cis*-dominant genetic elements that comprise the origin for viral DNA replication (14, 15). During the late S or early $G_2$ phase of the cell cycle, the viral genome is converted to a double-stranded replicative form (RF) DNA by cellular enzymes, possibly including DNA polymerase α (16). Viral DNA replication proceeds through linear monomeric and dimeric replicative intermediates (17). In the present study, specific hexanucleotide sequences in the viral genome were correlated with primase initiation sites. This observation has led us to propose the existence of a primase consensus formula that we have called ψ. Alternatively, these could be termination signals, thus indirectly stimulating primase at these locations.

## MATERIALS AND METHODS

**Materials.** DNA polymerase α-primase was purified from Ehrlich ascites mouse tumor cells and MVM DNA was isolated from purified virus particles (strain MVMp) as described (12, 15). Restriction endonucleases, DNA polymerase I (Klenow fragment), tobacco acid pyrophosphatase (TAP), and bacterial alkaline phosphatase (BAP) were from Bethesda Research Laboratories or Boehringer Mannheim. Ribonuclease T1 was from Sankyo. Nucleotides were from Sigma except for guanosine 5′-triphosphate 3′-phosphate, which was from P-L Biochemicals. Radioactive nucleotides were from New England Nuclear except for [γ-$^{32}$P]GTP, which was from ICN. Polyethyleneimine-cellulose plates were from Machery & Nagel (Brinkmann).

**DNA Polymerase α-Primase Reactions.** Standard reactions were carried out in a total volume of 16 μl containing 50 mM Tris·HCl (pH 7.5), 4 mM $MgCl_2$, 6 mM KCl, 8 mM dithiothreitol, 1.6 mM 2-mercaptoethanol (from enzyme addition), 27% (vol/vol) glycerol, bovine serum albumin at 400 μg/ml, 16 μM (each) dATP, dGTP, dCTP, dTTP, either 0.1 mM, 0.2 mM, or 1.0 mM GTP, 10 ng of MVM DNA, 0.5 μCi of [α-$^{32}$P]dTTP, and 1.0 unit of DNA polymerase α-primase. Incubations were at 37°C for 2 hr and reactions were terminated by the addition of 1/5th vol of agarose gel electrophoresis sample buffer (50 mM EDTA/2 M urea/2% $NaDodSO_4$/1 M sucrose/0.1% bromophenol blue).

For the isolation of radiolabeled MVM DNA, standard reaction mixtures were scaled up 20-fold and 25 μCi of [α-$^{32}$P]dATP and 40 μCi of [α-$^{32}$P]dGTP was added. Radiolabeled primase products were prepared by scaling up standard reaction mixtures 40-fold and adding 0.6 mCi (0.1 mM) of either [α-$^{32}$P]- or [γ-$^{32}$P]GTP. Preparative-scale reaction mixtures were prewarmed (37°C, 5 min) prior to addition of DNA polymerase α-primase. Samples were adjusted to 5 mM

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Abbreviations: MVM, minute virus of mice; RF, replicative form; TAP, tobacco acid pyrophosphatase; BAP, bacterial alkaline phosphatase; kb, kilobase(s).

EDTA, diluted with 1 vol of doubly distilled $H_2O$ or 10 mM Tris·HCl, pH 7.5/0.1 mM EDTA (TE buffer), and extracted once with phenol (equilibrated with Tris·HCl, pH 8.0) and once with diethyl ether. The DNA was precipitated at −30°C in the presence of 70% ethanol/0.3 M sodium acetate, pH 5.5, and tRNA (100 μg/ml), dissolved in TE buffer, and further purified by gel filtration on Sephadex G-50 (7.5-ml bed volume).

**Enzyme Digestion.** Restriction enzymes (2 units per 20-μl reaction mixture) were used according to the specifications provided by the supplier. Prior to treatment with either TAP, BAP, or ribonuclease T1, RF DNA samples were denatured at 96°C for 3 min. TAP digestion (2 units, 37°C, 60 min) was carried out in 50 mM sodium acetate, pH 5.5/10 mM 2-mercaptoethanol/1 mM EDTA. BAP treatment (70 units, 45°C, 10 min) was carried out in 50 mM Tris·HCl, pH 8.0. Ribonuclease T1 digestion (5 units, 37°C, 30 min) was carried out in 50 mM Tris·HCl, pH 8.0.

**Polyacrylamide Gel Electrophoresis.** Polyacrylamide slab gels (0.4 mm × 40 cm) contained 8% acrylamide (acrylamide/bisacrylamide/19:1), 8 M urea, and 0.1 M Tris borate (pH 8.3). The mixture was degassed and polymerized by the addition of ammonium persulphate and $N,N,N^1,N^1$-tetramethylethylenediammine. Electrophoresis was carried out at 1.7 kV for 1.5–3 hr in 0.1 M Tris borate (pH 8.3). Gels were exposed to x-ray film (Kodak XAR-5) for 1–10 days at −70°C with an intensifying screen.

## RESULTS

**Polypeptide Composition of Highly Purified Mouse DNA Polymerase α-Primase.** Using a purification scheme related to one described previously (12), we obtained a DNA polymerase α-primase preparation that contained major polypeptides of $M_r$ 220,000, 182,000, 67,000, 55,000, and 47,000 (Fig. 1). A detailed description of the purification and biophysical properties of the enzyme is beyond the scope of this report and will be described elsewhere. The results described herein could be obtained either with the enzyme fraction shown in Fig. 1 or with the slightly less well purified enzyme described previously (12).

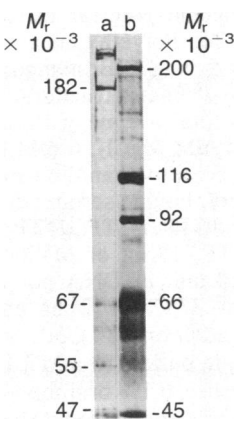


FIG. 1. Polypeptide composition of highly purified DNA polymerase α-primase. DNA polymerase α-primase was purified from 60 g of frozen Ehrlich ascites mouse tumor cells. The enzyme was precipitated from an S-100 fraction with ammonium sulfate as described (12) and purified by chromatography on DEAE-cellulose, phosphocellulose, and hydroxyapatite followed by glycerol gradient sedimentation. Samples (0.1–0.2 μg enzyme protein) were analyzed by electrophoresis under reducing conditions in $NaDodSo_4$/polyacrylamide gels and visualized by silver staining. Lanes: a, DNA polymerase α-primase; b, BioRad protein molecular weight standards [from top to bottom: myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin].

**Termination and Reinitiation at Preferred Sites on MVM DNA.** When MVM single-stranded DNA was incubated with DNA polymerase α-primase in the presence of either GTP or the four rNTPs, the entire template was copied, resulting in a DNA product that comigrated with authentic 5-kilobase-pair RF DNA in agarose gels run under nondenaturing conditions. However, under alkaline conditions, major discrete DNA fragments of ≈9.0, 7.7, 7.5, and 2.1 kilobases (kb) were observed when GTP was included in reaction mixtures (Fig. 2), and additional minor fragments of 1.1 and 0.9 kb were also present (data not shown). The ≈9.0-kb DNA species was present in samples prepared in the presence of 0.1 mM GTP but absent in those prepared in the presence of 1.0 or 4.0 mM GTP. At relatively low GTP concentrations (0.01 mM), a single 10-kb DNA species was observed (data not shown). Fragments of ≈7.7, 7.5, 5.8, 5.6, and 2.1 kb, as well as DNA fragments ranging from 0.5 to 1.5 kb, were seen when reactions were carried out in the presence of the four rNTPs (Fig. 2). These data are consistent with the formation of partially completed hairpin molecules resulting from termination of DNA synthesis ≈0.6, 0.8, 2.5, 2.7, and 4.0 kb from the 3′ hairpin terminus of the 5.0-kb template strand. The absence of a 10.0-kb DNA species (even in overexposed autoradiograms) suggests that the frequency of termination at these sites approaches 100%. Inspection of the DNA sequence at these locations (±100 nucleotides) revealed the presence of multiple closely related hexanucleotide sequences, namely, $C_2A_2C_2$, $C_2AC_3$, or $C_2A_2T_2$ (14). To more firmly establish a correlation between these DNA sequences and specific termination, further analysis was done to map the termination sites at single-nucleotide resolution.

**High-Resolution Mapping of Major Termination Sites.** The 7.5-, 7.7-, and 9.0-kb DNA species were purified as a mixture by sedimentation in alkaline surcrose gradients (15) and allowed to renature at neutral pH. Following cleavage with appropriate restriction enzymes, the DNA was denatured and analyzed by electrophoresis in 8% polyacrylamide/8 M urea gels. Fragments of labeled DNA present in digests of the 7.5- to 9.0-kb DNA but absent from digests of complete RF DNA, synthesized from viral DNA by *Escherichia coli* DNA polymerase I (Klenow fragment) were judged to be due to termination. From the size of the labeled DNA fragment and the known location of the restriction enzyme cleavage site, the precise location of a particular 3′-hydroxyl terminus could be determined. To map termination sites to the nucleotide, it was necessary to generate DNA fragments of less than ≈100 nucleotides. Wherever possible, enzymes with a single cleavage site in MVM DNA were used and assignments were confirmed by cleavage with more than one enzyme. The results of these analyses are shown in Fig. 3 and the termination sites deduced from them are summarized in Table 1.

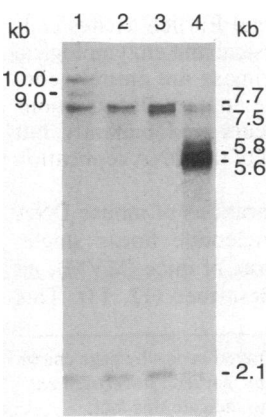


FIG. 2. Detection of specific termination/initiation sites. MVM RF DNA synthesized by DNA polymerase α-primase in the presence of either GTP or all four rNTPs was analyzed by alkaline agarose gel electrophoresis. Horizontal 1% alkaline agarose slab gels (15×30 cm) were formed in 30 mM NaOH/2 mM EDTA. Electrophoresis was carried out at 200 mA for 16–20 hr. Gels were neutralized, dried, and placed on x-ray film at −70°C with an intensifying screen. Lanes: 1–3, 0.1, 0.2, and 1.0 mM GTP, respectively; 4, 1 mM (each) ATP/GTP/CTP/UTP.

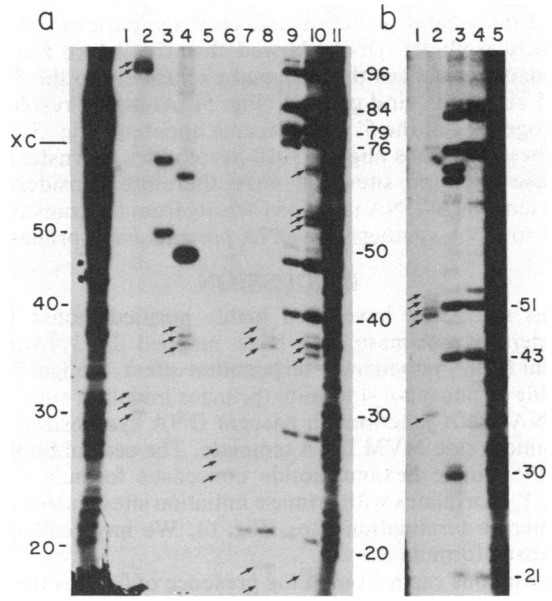


FIG. 3. Mapping of the termination sites at single-nucleotide resolution. DNAs were cleaved with various restriction enzymes, denatured at 96°C in 66% formamide, and analyzed by electrophoresis in 8% polyacrylamide 8 M urea gels. (*a*) Lanes: 1, 3, 5, 7, and 9, RF DNA; 2, 4, 6, 8, and 10, 7.5- to 9.0-kb DNA synthesized in the presence of 0.1 mM GTP. Restriction enzymes were as follows. Lanes: 1 and 2, *Hae* III; 3 and 4, *Mbo* I; 5 and 6, *Bgl* I; 7 and 8, *Pvu* II; 9 and 10, *Alu* I. Lane 11 and the left-most lane (unmarked) contained DNA chain-length markers. (*b*) Lanes: 1 and 3, RF DNA; 2 and 4, 7.5- to 9.0-kb DNA synthesized in the presence of 1.0 mM GTP. Restriction enzymes were as follows. Lanes: 1 and 2, *Hae* II; 3 and 4, *Alu* I; 5, DNA chain-length markers. Numbers at the side of the figure refer to the sizes of DNA fragments in nucleotides. XC, xylene cyanol FF.

Most of the termination sites were found in clusters of three or four. One such cluster, at nucleotides 2901, 2902, and 2903, was assigned on the basis of the single *Bgl* I cleavage site in MVM DNA, and this assignment was confirmed by *Hae* III and *Pvu* II cleavage. An additional termination cluster was tentatively assigned based on *Pvu* II cleavage to nucleotides 2880, 2881, and 2882. As expected, *Alu* I digestion yielded three corresponding fragments. A third set of termination sites was assigned to nucleotides 2593, 2594, and 2595 on the basis of *Mbo* I and *Hae* III cleavage. The assignments are consistent with the release of the aforementioned 7.5- and 7.7-kb (±200 nucleotide) DNA species from intact RF DNA under alkaline conditions. Termination sites assigned to nucleotides 3749, 3750, and 3751 on the basis of *Alu* I cleavage most likely account for the ≈9.0-kb DNA species; *Alu* I fragments corresponding to these latter termination sites were not observed when DNA samples lacking the 9.0-kb species were examined (Fig. 3*b*). Certain termination sites, such as those at nucleotides 2329, 2342, 2343, 2344, and 2345, appeared only in DNA samples prepared at relatively high GTP concentration [i.e., 1.0 mM (Fig. 3*b*)]. Thus, at high resolution, 17 3′-hydroxyl termini were found at specific locations in the nascent complementary DNA strand of MVM RF DNA, signifying that DNA polymerase α-primase had terminated DNA synthesis at these locations. We also note that all 17 termination sites were located 2–14 nucleotides upstream of one of three closely related hexanucleotide sequences—namely, $C_2A_2C_2$, $C_2AC_3$, or $C_2A_2T_2$ (see *Discussion*).

**Characterization of Oligoribonucleotides in Nascent MVM DNA.** To explain the termination of DNA synthesis at specific locations in MVM RF DNA, we examined the possibility that oligoribonucleotides had been synthesized *de novo* at or near these locations and subsequently used as primers for DNA synthesis. DNA was synthesized in the presence of either [α-$^{32}$P]- or [γ-$^{32}$P]GTP as the only radiolabeled substrate and the products of the reaction were filtered through a Sephadex G-50 column. $^{32}$P-labeled material (presumptive RNA-primed nascent DNA chains) eluting in the void volume of the column was heat denatured and then treated with either TAP, BAP, or ribonuclease T1. The products of digestion were then analyzed by PEI thin-layer chromatography in 1.0 M LiCl. In the case of DNA synthesized in the presence of [γ-$^{32}$P]GTP, TAP and BAP treatment resulted in quantitative release of the $^{32}$P label as $^{32}PP_i$ and $^{32}P_i$, respectively. By contrast, ribonuclease T1 released a $^{32}$P-labeled polyphosphorylated guanine nucleotide that comigrated with authentic guanosine 5′-triphosphate 3′-phosphate. Taking into account the known specificity of ribonuclease T1, this material was tentatively identified as $^{32}$pppGp(2′,3′), indicating that oligoribonucleotide chains are initiated with 5′ GTP residues. In the case of DNA synthesized in the presence of [α-$^{32}$P]GTP, ribonuclease T1 released radiolabeled pppGp(2′,3′) and Gp(2′ or 3′). The latter compound comigrated with 3′ dGMP on polyethyleneimine-cellulose and contained 2.4 times as much radiolabel as the pppGp(2′,3′). Since pppGp(2′, 3′) has twice as many phosphate atoms labeled in the α-position as Gp(2′ or 3′), the value of 2.4 suggested that the oligoribonucleotide chains were seven nucleotides long. BAP released 21% of the radiolabel as $^{32}P_i$, suggesting a chain length of five residues. The oligoribonucleotides therefore appear to consist of five to seven nucleotide residues.

Table 1. Summary of termination and initiation sites and RNA–DNA junctions

| Restriction enzyme | Fragment size(s) | Cleavage site* | Site on DNA |
|---|---|---|---|
| | Termination sites | | |
| *Bgl* I | 25, 26, 27 | 2876 | 2901, 2902, 2903 |
| *Pvu* II | 17, 18, 19 | 2863 | 2880, 2881, 2882 |
| | 37, 38, 39 | 2863 | 2901, 2902, 2903 |
| *Hae* III | 30, 31, 32 | 2870 | 2901, 2902, 2903 |
| | 94, 95† | 2500 | 2594, 2595 |
| *Mbo* I | 37, 38, 39 | 2556 | 2593, 2594, 2595 |
| *Alu* I | 58, 59, 60 | 3691 | 3749, 3750, 3751 |
| | 17, 18, 19 | 2863 | 2880, 2881, 2882 |
| | 37, 38, 39 | 2863 | 2901, 2902, 2903 |
| *Hae* II | 37 | 2292 | 2329 |
| | 50, 51, 52 | 2292 | 2342, 2343, 2344, |
| | 53 | | 2345 |
| | Initiation sites | | |
| *Hin*dIII | 54, 53 | 2650 | 2597, 2598 |
| | 245, 235† | 3993 | 3751, 3761 |
| *Alu* I | 56, 55 | 2652 | 2597, 2598 |
| | 88, 78 | 3838 | 3751, 3761 |
| | 245† | 3128 | 2884 |
| | 230† | 3128 | 2901, 2902, 2903 |
| *Hin*fI | 54, 53, 52 | 2954 | 2901, 2902, 2903 |
| | 71 | 2954 | 2884 |
| | 67 | 2662 | 2597 |
| | RNA–DNA junctions | | |
| *Hin*dIII | 48, 47 | 2650 | 2603, 2604 |
| *Hin*fI | 59 | 2662 | 2604 |
| | 66, 64, 63 | 2954 | 2888, 2890, 2891 |
| | 61, 60 | | 2893, 2894 |
| | 48, 46, 44, | 2954 | 2906, 2908, 2910 |
| | 43 | | 2911 |
| *Alu* I | 50, 49 | 2652 | 2603, 2604 |

*Cleavage occurs between the nucleotide shown and the one following it.
†Fragment size approximate.

**High-Resolution Mapping of Primase Initiation Sites.** Presumptive primase initiation sites were mapped using MVM RF DNA that had been synthesized in the presence of either [$\alpha$-$^{32}$P]- or [$\gamma$-$^{32}$P]GTP. The assumption was made that initiation sites would lie close to the sites established for termination, and restriction enzymes were chosen so as to cleave the DNA downstream (on the 3′ side) of these sites. Also, since the fragments terminated in 5′ GTP residues, it was necessary to dephosphorylate the 5′ ends using TAP in order to compare them with 5′-monophosphorylated size markers. Fragments of DNA ranging in size from 53 to 1100 nucleotides were seen in *Hin*dIII, *Hin*fI, and *Alu* I digests (Fig. 4) and these were labeled with [$\alpha$-$^{32}$P]- as well as with [$\gamma$-$^{32}$P]GTP, indicating that in each case oligoribonucleotide synthesis had been initiated *de novo* with 5′ GTP residues. Additional minor fragments were also present. From these data, eight major primase initiation sites were assigned on the basis of fragment size and the known locations of the corresponding restriction enzyme cleavage sites (Table 1).

We conclude from these results that DNA polymerase $\alpha$-primase exhibits strong site specificity for initiation of oligoribonucleotide primers. We note also that the primase initiation sites are located near the major termination sites three to nine nucleotides upstream of $C_2A_2C_2$, $C_2AC_3$, or $C_2A_2T_2$ hexanucleotide sequences (see *Discussion*).

**High-Resolution Mapping of RNA–DNA Junctions.** The $^{32}$P-labeled 2.1-kb nascent DNA fragment was purified by sedimentation in alkaline sucrose gradients and hybridized with the 5-kb MVM template strand. To determine the position of the 5′ terminus of the hybridized 2.1-kb fragment, hybrid DNA was cleaved with *Hin*dIII, *Hin*fI, or *Alu* I; the resulting cleavage products were denatured and then analyzed by electrophoresis in 8% polyacrylamide/8 M urea gels. Comparison of the overall cleavage pattern with that obtained from RF DNA showed that the 2.1-kb fragment contained sequences that mapped exclusively to the 5′ half (≈2.5 kb) of the viral genome (Fig. 5). At higher resolution, heterogeneity at the 5′ ends became apparent (Fig. 5; Table 1). These positions aligned 3–10 nucleotides downstream of primase initiation sites and were therefore considered to represent RNA–DNA junctions arising from the transition of RNA to DNA synthesis by DNA polymerase $\alpha$-primase.

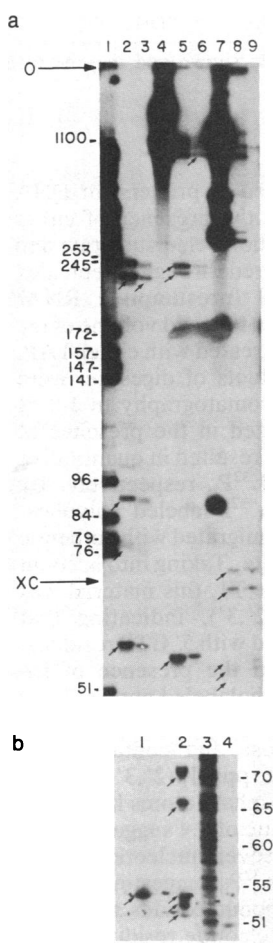


FIG. 4. Mapping of the primase initiation sites at single-nucleotide resolution. DNAs labeled with either [$\alpha$-$^{32}$P]- or [$\gamma$-$^{32}$P]GTP were cleaved with various restriction endonucleases, denatured at 96°C in 66% formamide, and analyzed by electrophoresis in 8% polyacrylamide 8 M urea gels. (*a*) Lanes: 1, 4, and 7, RF DNA synthesized with DNA polymerase I, Klenow fragment; 2, 5, and 8, [$\alpha$-$^{32}$P]GTP-labeled DNA; 3, 6, and 9, [$\gamma$-$^{32}$P]GTP-labeled DNA. Restriction enzymes were as follows. Lanes: 1–3, *Alu* I; 4–6, *Hin*dIII; 7–9, *Hin*fI. (*b*) DNA labeled with [$\alpha$-$^{32}$P]GTP was cleaved with various restriction enzymes, denatured at 96°C, treated with TAP, and analyzed as in *a*. Lanes: 1 and 2, *Hin*dIII and *Hin*fI digests, respectively, of [$\alpha$-$^{32}$P]GTP-labeled DNA; 3, DNA chain-length markers; 4, *Alu* I digest of RF DNA synthesized as for lane 1 of *a*. Numbers at the side of the figure refer to the sizes of DNA fragments in nucleotides. XC, xylene cyanol FF; O, origin.

## DISCUSSION

In this study, we have used highly purified mouse DNA polymerase $\alpha$-primase and have mapped 17 3′-hydroxyl termini (DNA polymerase termination sites), 8 oligoribonucleotide 5′-phosphoryl termini (primase initiation sites), and 11 RNA–DNA junctions in nascent DNA synthesized on a 5081-nucleotide MVM DNA template. The central finding is that the simple hexanucleotide consensus formula $C_2A_{1-2}$ ($C_{2-3}/T_2$) correlates with primase initiation sites and/or DNA polymerase termination sites (Fig. 6). We have called this consensus formula $\psi$.

In reactions carried out in the presence of GTP as the only ribonucleotide, initiation by primase occurred exclusively in relatively cytosine-rich regions of the DNA template. Oligoribonucleotides 5–10 nucleotides long were initiated with 5′ GTP residues and although some oligoribonucleotide chains appeared to contain a single dTMP (nucleotides 2889 and 2907) or dAMP (nucleotide 2601) residue, they otherwise consisted of GMP. However, template sequence alone could not explain the strong specificity of site selection by primase for several reasons. The DNA template contains 4 $C_5$ sequences, 7 additional $C_4$ sequences, and 23 additional $C_3$ sequences. The $C_5$ sequences, 2 of the $C_4$ sequences, and 2 of the $C_3$ sequences are located near $\psi$ and were utilized by primase. The latter $C_3$ sequences were found as part of the $\psi$-like sequence of $C_3AC_2$ present within the tetradecanucleotide $C_3AC_2T_2C_3AC_2$ (nucleotides 4759–4772; a copy of $\psi$ is present at position 4771). The remaining 5 $C_4$ and 21 $C_3$ sequences were not located near $\psi$ and were not utilized by primase. Furthermore, a major primase initiation site was

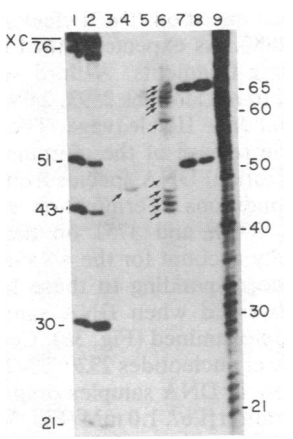


FIG. 5. Mapping of the RNA–DNA junctions at single-nucleotide resolution. The 2.1-kb labeled DNA fragment was hybridized to excess unlabeled MVM DNA (3.8 $\mu$g/ml) at 68°C for 5 hr in 100 $\mu$l of 0.30 M NaOAc/50 mM Tris·HCl, pH 7.5. The hybrid DNA was cleaved with various restriction enzymes, denatured at 96°C in 66% formamide, and analyzed by electrophoresis in 8% polyacrylamide 8 M urea gels. Lanes: 1, 3, 5, and 7, RF DNA synthesized by DNA polymerase I, Klenow fragment; 2, 4, 6, and 8, 2.1-kb hybrid DNA. Restriction enzymes were as follows. Lanes: 1 and 2, *Alu* I; 3 and 4, *Hin*dIII; 5 and 6, *Hin*fI; 7 and 8, *Mbo* I. Lane 9, DNA chain-length markers. Numbers at the side of the figure refer to the sizes of DNA fragments in nucleotides. XC, xylene cyanol FF.

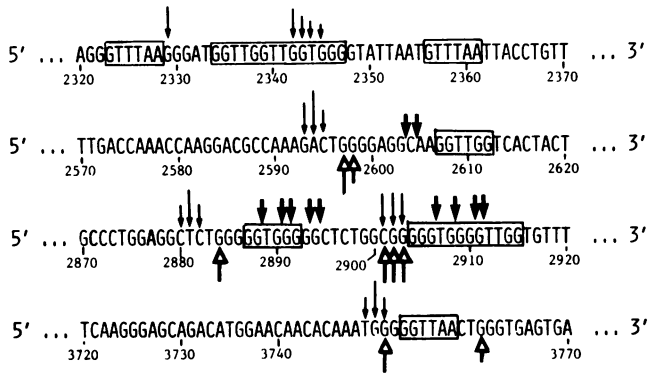


FIG. 6. Summary of termination and initiation sites and RNA–DNA junctions. Positions of termination (↓) and initiation (⌓) sites and RNA–DNA junctions (⌓) in the complementary DNA strand synthesized by highly purified DNA polymerase α-primase with MVM DNA as a template are indicated. The sequences from which the consensus formula, ψ, was derived are boxed. Nucleotides are numbered based on the MVM DNA sequence reported by Astell *et al.* (14).

located within the sequence 3′$AC_4TC$ 5′ (nucleotides 2596–2602), upstream of a copy of ψ, whereas an identical sequence at nucleotides 3148–3154, not located near ψ, was not utilized by primase. Also, the overall pattern of primase specificity was maintained over a 40-fold range of GTP concentration (from 0.1 to 4.0 mM). Analysis of DNA synthesized in the presence of all four rNTPs revealed two additional major termination sites, not seen with GTP, that were tentatively mapped near copies of ψ at nucleotides 539 and 727. Since ψ at these latter positions is not associated with tandemly repeated deoxycytidine residues in the template, and because of the strong positive correlation outlined above, ψ alone appears to specify termination/initiation.

Studies of simian virus 40 (SV40) DNA replicative intermediates (18, 19) and *in vitro* primase reactions with SV40 DNA (20) have revealed initiation by primase at preferred sites that include the ψ-like sequence 3′ $C_3GC_2$ 5′ near the SV40 replication origin, while a more general priming reaction appears to involve preferential initiation opposite thymidine and deoxycytidine residues that are preceded by purine nucleotides. The length of the RNA primers in SV40 replicating intermediates varied from 6 to 10 nucleotides. Thus, the *in vivo* primase reaction appears similar in a number of respects to the *in vitro* reaction described here and may in some cases involve recognition of ψ or ψ-like sequences.

Our results also revealed that DNA synthesis terminated 1–4 nucleotides upstream of a primase initiation site. In several cases, if not all, 3′-hydroxyl DNA termini and 5′-phosphoryl oligoribonucleotide termini were immediately juxtaposed. Since some preparations of DNA polymerase α are unable to fill gaps in DNA of <15 nucleotides (21), this result indicates that termination of DNA synthesis precedes initiation of new RNA-primed DNA chains. The fact that ψ may lie 10 nucleotides downstream of primase initiation sites is consistent with the asymmetric structure of DNA polymerase α-primase (22, 23) and with the ability of primase to synthesize oligoribonucleotides of a discrete chain length of 4–10 nucleotides. Contact with ψ may therefore be made while the enzyme is still polymerizing a DNA chain, 10 base pairs upstream of ψ; binding at ψ could then cause termination. The enzyme could remain bound to the DNA near the termination site despite disengagement from the 3′- hydroxyl terminus of the growing DNA chain. Primase could then synthesize a new oligoribonucleotide immediately adjacent to the termination site, thus displacing the enzyme from ψ and permitting the DNA polymerase catalytic subunit to extend the newly synthesized RNA primer. The fact that ψ-dependent termination/initiation is influenced by the GTP concentration (presumably due to effects on primase) supports the hypothesis that termination and initiation are coupled. Alternatively, the data could be explained if DNA polymerase α-primase is able to fill gaps to completion, in which case ψ may specify priming independently of termination. In any event, our results indicate that primase is activated by ψ.

The MVM genome contains 26 hexanucleotides that conform to the primase consensus formula ψ, including 17, 5, and 4 copies of $C_2A_2C_2$, $C_2AC_3$, and $C_2A_2T_2$, respectively. Twenty copies of ψ are present in the strand of (−) polarity that was used as a template in the present study. Of these, 5 are located within the hairpin termini. Moreover, 4 copies of the ψ-like sequence 3′ $C_3AC_2$ 5′ exist as part of near-perfect tandem repeats—namely, 3′ $C_3AC_3TC_3AC_3$ 5′ (4897–4911) and 3′ $C_3AC_2T_2C_3AC_2$ 5′ (4759–4772) located within or very near the origin for viral RF DNA replication (14, 15, 24). Our results therefore implicate ψ as well as DNA polymerase α-primase in the initiation of MVM RF DNA replication *in vivo*.

Finally, we note that there is a strong resemblance between ψ and the tandemly repeated sequences found at telomeres (ends) in the chromosomes of several lower eukaryotic organisms (25). ψ-Dependent termination of DNA synthesis and/or initiation of RNA-primed DNA chains by DNA polymerase α-primase may therefore play a critical role not only in the replication of the linear parvovirus chromosome but also in the replication of telomeres in linear chromosomes of eukaryotes in general.

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