Primase Activity of Human DNA Polymerase α-Primase

DIVALENT CATIONS STABILIZE THE ENZYME ACTIVITY OF THE p48 SUBUNIT

(Received for publication, March 27, 1998, and in revised form, June 15, 1998)

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DNA polymerase α-primase consists of four subunits, p180, p68, p58, and p48, and comprises two essential enzymatic functions. To study the primase activity of the complex, we expressed cDNAs encoding for the human p58 and p48 subunits either as single proteins or together using Escherichia coli expression vectors. Co-expression of both primase subunits allowed the purification of a heterodimer in high yields that revealed stable primase activity. Purified recombinant p48 subunit showed enzyme activity, whereas purified p58 did not. In contrast to the heterodimer, the primase activity of p48 was unstable. The activity of p48 could be stabilized by the addition of the divalent cations Mg\(^{2+}\) and Mn\(^{2+}\) but not Zn\(^{2+}\). On a poly(dC) template the primase activity was hardly influenced by the monovalent cation potassium. However, by using poly(dT) as a template the recombinant p48 activity was sensitive to salt, whereas recombinant p58-p48 and the bovine DNA polymerase α-primase purified from thymus were less sensitive to the addition of monovalent cations. A complex of bacterially expressed primase and baculovirus-expressed p180 and p68 was assembled in vitro and shown to support replication of simian virus 40 DNA in a cell-free system.

DNA polymerase α and primase are essential proteins of the cellular DNA replication machinery. They are important components of the initiation complex and play a central role in leading and lagging strand synthesis (1–7). Since no known DNA polymerase is able to initiate DNA synthesis de novo, primase synthesizes oligoribonucleotides, called RNA primers, during initiation of genomic DNA replication in eukaryotes (2), interest arose to determine the subunits of the complex that carry the catalytic center of the primase. Each of the four subunits of DNA polymerase α-primase (pol-prim)\(^1\) was proposed to contain primase activity by biochemical and immunological methods (9–16).

When the mouse primase was purified to near-homogeneity, it was found to contain two proteins of 55 and 49 kDa, which were comparable to the molecular masses of the two small subunits of Drosophila pol-prim that showed primase activity after separation from the complex (17, 18). Thereafter, it was proposed that the 48-kDa subunit of the bovine pol-prim is sufficient for primase activity (14). Soon this result was questioned, since cross-linking studies with photoreactive ribonucleotides suggested that the 58–60-kDa subunit of pol-prim might also be involved in the synthesis of oligoribonucleotides (19, 20). The characterization of a 48-kDa protein from baker’s yeast that had primase activity and that was immunologically related to the 48-kDa subunit of pol-prim seemed to provide a satisfactory solution to the issue (21). However, the discussion concerning the function of the primase subunits was revitalized when the cloning and subsequent expression of pol-prim subunits from different organisms led to a controversy whether p48 alone can synthesize the first dinucleotide (initiation activity) and then elongates it or whether it has only elongation activity and requires p58 for the synthesis of the first dinucleotide (22–26).

To address the activity of p48 again, we modified the T7 RNA polymerase promoter system of Escherichia coli (27) to allow the co-expression of multigene complexes from a single plasmid. The p58 and p48 subunits of human pol-prim were expressed either as wild type or as fusion proteins with oligohistidines at their N terminus. Thus, human primase that contained p58 and p48 was expressed and purified in high yields as a stable and active protein complex. Furthermore, for the first time the vectors that are described here allowed expression and purification of eukaryotic p48 in E. coli as a soluble protein and a fully active primase with regard to initiation and elongation. The addition of magnesium and manganese stabilized its primase activity. The bacterial and baculovirus expressed proteins could be assembled in vitro and formed a four-subunit pol-prim complex that is active in DNA replication.

MATERIALS AND METHODS

Enzymes were obtained from Amersham Pharmacia Biotech (Freiburg, Germany), Boehringer Mannheim (Mannheim, Germany), or New England Biolabs (Schwalbach, Germany). Reagents were purchased from Merek unless otherwise indicated (Darmstadt, Germany). [\(^{32}P\)]ATP, [\(^{32}P\)]GTP, [\(^{32}P\)]dATP, [\(^{32}P\)]dGTP, and [\(^{32}P\)]ATP (3000Ci/mmol) were obtained from Amersham Pharmacia Biotech or NEN Life Sciences (Cologne, Germany). Ultrapure rNTPs were purchased from Amersham Pharmacia Biotech; dNTPs were obtained from Boehringer Mannheim; poly(dT) and poly(dC) were purchased from Amersham Pharmacia Biotech; poly(dC,dT) and leupeptin were obtained from Sigma (Deisenhofen, Germany). Trasylol (aprotinin solution) was a generous gift of Bayer AG, Leverkusen, Germany.

Protein Manipulations—Protein concentrations were determined according to Bradford (28) using a commercial reagent with bovine serum albumin as a standard (Bio-Rad, Munich, Germany). SDS-gel electrophoresis was carried out as described (29) with a 10-kDa ladder or

\(^1\) The abbreviations used are: pol-prim, DNA polymerase α-primase; BSA, bovine serum albumin; ssDNA, single-stranded DNA.

\(^{‡}\) This work was supported by Deutsche Forschungsgemeinschaft Grants Gr895/11-1, Na130/6-3, Na190/8-1, and Wi319/11-2 and European Community Contract ERBFMRXCT980125. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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pET11-MCS (33). This yielded the plasmids pET11-MCS and pET15-MCS after verification of their sequence and pET15-MCS to express the primase subunits as unmodified proteins or fusion proteins with 6xHis, respectively.

To co-express the proteins the vector pET11-Hp48 was digested with SpeI and EcoRI, and the vector pET15-Hp58 was cut with XhoI and EcoRI. The DNAs were separated on agarose gels and purified according to standard procedures (33). The fragment containing the coding sequence of human p58 was ligated into vector pET11-Hp48, and the plasmid pET-Hp48-HisHp58 was created that expressed p48 as an unmodified protein and p58 as a fusion protein.

**Expression and Purification of Primase Proteins—E. coli** cells BL21(DE3) containing the expression vectors either pET11-Hp48, pET15-Hp58, or pET-Hp48-HisHp58 were grown in terrific broth at 37 °C (33) to an optical density of 2 measured at 600 nm. After lowering the growth temperature to 23 °C, the expression of recombinant proteins was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were harvested 3.5 h after induction, collected at 3000 g, and washed twice with phosphate-buffered saline (33). Alternatively, p48 was expressed and purified in the presence of divalent cations, as indicated.

Cells that expressed p48 or p58 as single proteins were immediately homogenized in lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 3 mM 2-mercaptoethanol, 0.05 mM leupeptin, and 0.01 mg/ml Trasylol) by sonication. After sonication the homogenate was adjusted to 1% Triton X-100, and then DNA and insoluble proteins were removed by centrifugation at 5000 g.

**Expression cassette of pET11-MCS including its multicloning site**

| Bgl II | Xba I |
|--------|-------|
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | AAGAAATTATTTGGTTAATCTTAAGAGAGAGATATACATTATAGGGGATATGTTGAGGATATAATATCCCTCT |
| (Bcl I) | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |
| Sma I | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |
| Sac I | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |
| Sal I | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |
| Spe I | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |

**Expression cassette of pET15-MCS including its multicloning site**

| EcoRI | Xho I |
|--------|-------|
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GCCGCGCGCGCCGGCATATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |
| (Bcl I) | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |
| Sma I | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |
| Sac I | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |
| Sal I | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |
| Spe I | (Bcl I) |
| GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT | GATAGGAGATGACGCGTCACTGATATTAGGGGATATGTTGAGGATATAATATCCCTCT |

**FIG. 1.** Expression vectors pET11-MCS and pET15-MCS. The vectors pET11-MCS and pET15-MCS were constructed (see “Materials and Methods”) to express single cDNAs and co-express cDNAs of a multisubunit complex in *E. coli* from a single plasmid. Partial restriction maps of pET11-MCS (A, nucleotides 496 to 257) and pET15-MCS (B, nucleotides 555 to 316) showing single cutting restriction enzymes (rare cutting ones are in parentheses) are presented. The oligonucleotides inserted into the plasmids pET11-MCS and pET15-MCS are in lowercase letters.
contained 0.1 mM MnSO₄ (Sigma) or 1 mM MgCl₂, as indicated. To
purify p48 further, the eluted fractions of the metal chelate column were
dialyzed against buffer 3 (50 mM HEPES-KOH, pH 7.8, 0.5 mM dithiothreitol, 7 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mg/ml BSA). The reaction mixtures with poly(dT) as a template had 0.1 mM [α-32P]GTP (5000 Ci/nmol) whereas the mixtures with poly(dC) contained 0.05 mM [α-32P]ATP (1000 Ci/nmol). After addition of 0.2–1 unit of primase, as indicated, the assays were incubated 60 min at 37 °C.

The synthesis products were precipitated with 0.8 M LiCl, 10 μg of sonicated salmon sperm DNA (Sigma), and 2.5-fold volume of ethanol for 15 min on dry ice, washed twice with 75% ethanol/water, dried, redissolved in 45% formamide, 5 mM EDTA, 0.09% xylene cyanol FF, 0.09% brophenol blue at 65 °C for 10 min, heated for 3 min at 95 °C, and separated in denaturing 20% polyacrylamide gels (acrylamide/bisacrylamide ratio, 19:1; 7 x urea) for 3 to 4 h at 600 V with radioactive markers and oligo(dC) (Amersham Pharmacia Biotech) as a marker (35). The reaction products were visualized by autoradiography using x-ray films or PhosphorImager (Molecular Dynamics).

SV40 DNA Replication in Vitro—S100 extracts were prepared from logochromically growing mouse FM3A cells as described previously (26, 34). The replication of SV40 DNA in vitro was performed as described (34). Briefly, the SV40 assay (60 μl) contained 0.6 μg of SV40 T antigen, 0.25 μg of SV40 origin DNA (pUC-HS), 0.6 μg of human RP-A, 300 μg of S100 or depleted S100 extract from FM3A cells in 30 mM HEPES-KOH, pH 7.8, 0.5 mM dithiothreitol, 7 mM magnesium acetate, 1 mM EGTA, pH 7.8, 4 mM ATP, 0.3 mM CTP, GTP, and UTP, 0.1 mM dATP and dGTP, 0.05 mM dCTP and dTTP, 40 mM creatine phosphate, 80 μg/ml creatine kinase, and 5 μCi each of [α-32P]dCTP and [α-32P]dGTP (3000 Ci/nmol). Comparisons between different enzyme preparations were made by adjusting the amounts of enzyme added to equal primase activity as indicated. The primase activity was determined at the time the replication assays were performed. The incorporation of radioactive deoxynucleoside monophosphate (dNMP) was measured by acid-precipitation of DNA and scintillation counting.

RESULTS

Expression and Purification of Recombinant Human Primase in E. coli—The cDNAs of p48 and p58 were expressed separately fused to six histidine residues at their N terminus with the E. coli vectors pET15-Hp48 and pET15-Hp58. After metal chelate chromatography, the purified recombinant p48 subunit reproducibly showed primase activity as determined in the primer elongation assay using Klenow DNA polymerase (Fig. 3A, column 1), whereas the recombinant p58 was inactive (Fig. 3A, column 2). Typically about 1 mg of soluble protein was purified from 500 ml of bacterial culture (Fig. 2A, lanes 1–3). Then affinity purified p48 was applied to phosphocellulose chromatography which yielded a single protein and activity peak (data not shown).

Primase preferentially synthesizes products of short size typically 5–10 nucleotides long (8, 17, 18). To verify the primase activity of the bacterially expressed proteins, the incorporation of NMP was directly determined by separating the synthesis products on denaturing gels and visualizing them by autoradiography (“direct assay”; Figs. 3B and 7). In the direct assay, p58 did not show any primase activity (Fig. 3B, lane 5), and no radioactive products were detectable above background (Fig. 3B, compare lanes 5 and 7). In contrast to this result, p48 efficiently initiated oligoribonucleotide synthesis on different templates such as poly(dC) (Fig. 3B, lanes 1–4; Fig. 7A), poly(dT) (Fig. 7D), poly(dC,dT), and M13ssDNA (data not shown). In addition, in the presence of p48, as well as p58–48 and pol-prl-long, long products were produced on poly(dC) that were not fully resolved by the gel electrophoresis (Figs. 3B and 7, A–C). The observed high amounts of oligo/poly(G) primase products that were detected at the top of the gel might be due to a lower solubility of oligo/poly(G) products. Second, the poly(dC) oligo/poly(G) products are extremely stable and they might quickly reanneal after heating to 95 °C and loading on the gel. Thus, a great portion of the primase products did not move into gel. These interpretations are consistent with the observation made by other investigators.2

By using the plasmid pET-Hp58-HisHp48, co-expression of p48 and p58 yielded high amounts of purified two-subunit primase with high specific primase activity using metal chelate chromatography (8–10 mg per 500-ml culture of bacteria with a specific activity of about 10,000 units/mg; Fig. 2A, lanes 4 and 5; Fig. 3, lane 6; data not shown). Interestingly, the size of products synthesized on poly(dC) by p48 alone and p58–p48 heterodimer varied. The p48 primase synthesized preferentially short products in the range of 5–10 nucleotides and some products longer than 17 nucleotides, whereas the p58-p48 heterodimer predominantly synthesized products that were longer than 17 nucleotides (Fig. 3B, compare lanes 1–4 with lane 6, respectively). The activity of heterodimeric primase was stable for several months at 4 °C, and after dialysis against 30% glycerol it could be stored at −20 or −80 °C. Freezing and thawing was without detectable influence on the activity of p58–p48 primase (data not shown).

2 R. Kuchta, personal communication.
Divalent Cations Stabilize Primase Activity of p48—However, the enzymatic activity of p48 expressed in E. coli was extremely unstable and could only be determined if expression, purification, and enzyme assay occurred within a few hours. Lengthy purification, freeze-thawing of bacterial cell pellets, or low concentrations of p48 resulted in a rapid loss of enzyme activity that could not be prevented by 1 mg/ml BSA, 10% glycerol, or 10% ethylene glycol (data not shown). Within 16 h of storage at 4 °C, the primase activity of p48 decreased by more than 85% of the starting activity (Fig. 4A, compare columns 1 and 2). At concentrations of p48 that were lower than about 0.4 mg/ml, the half-life of primase activity was even shorter, and the activity decreased to background levels within 3 h (data not shown).

The primase activity of p48 was reproducibly stabilized by the presence of 1–10 mM Mg2+ or 0.1–10 mM Mn2+ (Fig. 4A, compare column 2 with columns 3–7). In contrast, Zn2+ (1 μM to 10 mM; Fig. 4A, columns 8–11), and low concentrations of Mg2+ or Mn2+ (1 μM to 0.1 mM or 1–10 μM, respectively, and data not shown) were ineffective. On the other hand, with 10 mM Mg2+ or Mn2+ the enzyme activity of p48 was even detected after 14 days of storage at 4 °C (data not shown).

Since divalent cations stabilized the primase activity of p48, we compared the influence of Mg2+ on the primase activity of recombinant p48, heterodimeric p58-p48, and heterotetrameric pol-prim (Fig. 4B). The enzymes were diluted in buffer without (open bars 1–3) or with (hatched bars 4–6) 10 mM Mg2+, and the activity was measured after incubation at 37 °C. As expected the activity of p48 diminished within 10 min in the absence of Mg2+, and only about 5% activity of the starting activity that was determined before preincubation could be detected (Fig. 4B, column 1). In the presence of Mg2+ the activity of p48 and of the other enzymes was equally stable (Fig. 4B, compare columns 4–6). In contrast to p48, the presence of 10 mM Mg2+ did not stabilize the primase activity of either p58-p48 or pol-prim (Fig. 4B, compare columns 3 and 5 with 6, respectively).

These results prompted us to optimize the production of active p48. The primase activity was measured by the primer elongation assay or directly by analyzing the primase products on denaturing gels. The addition of divalent cations to the purification buffers increased specific primase activity of p48 by a factor of 3 to 4 (Fig. 5, compare columns 1 and 2). The presence of Mg2+ during protein expression and purification raised the specific primase activity by a factor of up to 10 in comparison with the procedure without divalent cations (Fig. 5, compare columns 1 and 3). These findings were supported by direct assays that were performed in parallel (Fig. 3A, lanes 1–4). It is worth mentioning that the addition of divalent cations to the bacteria decreases the level of expression of p48 by...
activity of p48 was readily inhibited by K⁺ in the presence of Mg²⁺ (Fig. 5, columns 1 and 2) or in the presence of additional 0.1 mM Mn²⁺ plus 1 mM Mg²⁺ (column 3). Then the recombinant p48 was purified in the absence of divalent cations (column 1) or in the presence of 0.1 mM Mn²⁺ plus 1 mM Mg²⁺ (column 2 and 3). The primase activity was measured with the Klenow elongation assay using poly(dT) as a template.

The primase activity was reproducibly stimulated by K⁺ up to a concentration of 150 mM (Fig. 7E, lanes 1–5). The bovine pol-prim synthesized products with comparable lengths to p48 (Fig. 7, compare C and F with A and D). The activity of pol-prim was stimulated by K⁺ on poly(dC) and on poly(dT) (Fig. 7, C and F, respectively).

Fig. 5. Specific activity of primase activity of p48 in the presence of Mg²⁺ and Mn²⁺ ions. The influence of Mg²⁺ and Mn²⁺ during expression and purification of recombinant p48 was tested. After the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside, p48 was expressed in the absence of additional divalent cations (columns 1 and 2) or in the presence of additional 0.1 mM Mn²⁺ plus 1 mM Mg²⁺ (column 3). Then the recombinant p48 was purified in the absence of divalent cations (column 1) or in the presence of 0.1 mM Mn²⁺ plus 1 mM Mg²⁺ (columns 2 and 3). The primase activity was measured with the Klenow elongation assay using poly(dT) as a template.

The E. coli-expressed Primase Forms a Complex with p180 and p68 That Is Active in SV40 DNA Replication in Vitro—Using a cell-free DNA replication system is a stringent test for the function of a recombinant replication protein. The heterodimeric primase p58-p48 alone cannot initiate SV40 DNA replication and requires the large subunits of pol-prim (data not shown). Therefore, a four-subunit complex was formed by mixing crude bacterial extracts with insect cell extracts that contain the recombinant human p58-p48 and p180-p68 complexes, respectively. Then the assembled four-subunit pol-prim complex was purified by ion exchange and immunoaffinity chromatography to near-homogeneity (Fig. 2B, lanes 1 and 2). The pol-prim assembled in vitro had high specific DNA polymerase and primase activity (7,000 and 4,500 units/mg, respectively) which was comparable to those of recombinant pol-prim from insect cells that were infected with four baculoviruses (7,600 and 5,100 units/mg DNA polymerase and primase activity, respectively).

The replication activity of the in vitro assembled pol-prim complex was tested in extracts from mouse cells that are non-permissive for SV40. In the absence of human pol-prim the incorporation of radioactive dNMP was just 4 pmol in these extracts (Fig. 8, column 1), and the addition of 1 unit of purified heterodimeric primase did not significantly change the incorporation of dNMPs (Fig. 8, column 2). In the mouse cell extracts the addition of human pol-prim formed in vitro allowed the replication of SV40 DNA in a concentration-dependent manner (Fig. 8, columns 3–5). The incorporation of dNMPs rose from 4 pmol to 13.5, 28, and 35 pmol in the presence of 0.25, 0.5, and 1 unit of pol-prim, respectively (Fig. 8). These results show that human primase that was expressed in E. coli is functional in a eukaryotic DNA replication assay in vitro.
The initiation activity of primase is an essential function to start DNA replication de novo (2). The exact mechanism of initiation is as yet unknown, and it is still under discussion whether p48 alone can initiate primer synthesis on ssDNA or whether it requires a second subunit p58 for the synthesis of the first dinucleotide (14, 21–26).

To study their functions the primase subunits were expressed using modified bacterial vectors that also allow the co-expression of protein complexes. With these plasmids, we were able to express and purify the p48 subunit of human pol-prim that is soluble and shows both initiation and elongation activities. However, depending on the expression and purification conditions the enzymatic activity of p48 is highly labile, since low concentrations of p48 or the absence of divalent cations leads to a fast loss of enzyme activity which could not be stopped by the addition of 1 mg/ml BSA, 10% glycerol, or 10% ethylene glycol. The instability of p48 primase activity observed here confirms earlier findings with bovine and yeast p48 that had highly unstable primase activity (14, 21). The specific requirement of divalent cations for the stability of enzyme activity is underlined by the presence of a metal binding motif in human p48 that is also present in primases from mouse, Drosophila, and bacteriophages T4 and T7 (22, 26, 36–40). In biochemistry the function of divalent cations during catalysis is well characterized and documented (for reviews see Refs. 2 and 41), but only since quite recently is an additional view of their function becoming more and more accepted. Divalent cations like calcium, magnesium, manganese, and zinc might also influence the stability of enzyme activities inde-
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The determined stability of p48 activity allowed us to start characterization of its enzymatic activity and compare it with that of the two-subunit primase and the four-subunit pol-prim. Interestingly, on poly(dT) the cations Mg\textsuperscript{2+} and K\textsuperscript{+} modulated the primase activity of single subunit p48 differently from that of two- and four-subunit enzyme complexes (Figs. 6 and 7, D–F), whereas on poly(dC), the different primase assemblies behaved comparably and had a broad optimum for both cations (Figs. 6 and 7, A–C, Ref. 14). On poly(dT), efficient primer synthesis by p48 required Mg\textsuperscript{2+} concentrations higher than 2.5 mM, whereas the two-subunit primase was highly active with 2.5 mM Mg\textsuperscript{2+}. These results suggest that on poly(dT) p48 might have to overcome a rate-limiting step in the formation of the dinucleotide or its elongation that requires higher Mg\textsuperscript{2+} concentration. Alternatively, the affinity of p48 to Mg\textsuperscript{2+} is too low for the initiation with 2.5 mM Mg\textsuperscript{2+} on poly(dT), and the p58 primase subunit might increase the affinity of p48 for the divalent cation. The further characterization of p48 primase showed that the presence of K\textsuperscript{+} inhibited primase activity of p48 on poly(dT), even concentrations as little as 25 mM were effective, whereas the addition of the monovalent cation stimulated activity of the two- and four-subunit enzyme complexes. Several mechanisms could be the cause of the high sensitivity of p48 primase toward K\textsuperscript{+}, e.g. the binding of p48 to the template poly(dT) or to the substrate ATP might be salt-sensitive. Alternatively, a specific step during the polymerization reaction (either the synthesis of the first nucleotide or the elongation step) could be inhibited by K\textsuperscript{+}. Since recent findings indicate that primase forms a ternary enzyme-template-NTP complex that preferentially contains GTP (52), the different influence of cations on the activity of p48 using poly(dC) or poly(dT) suggests that the p48-poly(dC)-GTP complex might be less sensitive to the concentration of cations than the p48-poly(dT)-ATP complex. Taken together these findings indicate that p48 has a complete primase activity that is able to initiate and elongate. The different salt sensitivity of p48 in comparison to p58-p48 and pol-prim suggests that this activity might be modulated by p58 and the other subunits of the pol-prim complex.

Co-expression of both primase subunits resulted in a highly active and stable primase that could be purified in large quantities. These findings reproduced earlier results that co-expression of mammalian primase subunits had yielded an active primase that was stable for months (23, 24, 26). The observed lengths of products that were synthesized by the two-subunit primase on poly(dC) prompted us to ask whether the recombinant enzyme was able to support DNA replication in vitro. To this end, we produced a chimeric four-subunit DNA polymerase α-primase in vitro that contained the bacterially expressed primase subunits and baculovirus-expressed p180 and p68. This in vitro assembled enzyme complex supports DNA replication in a cell-free system which indicates that the bacterially expressed primase is competent to support DNA replication reactions that require primase. The results presented here suggest some functions for the p58 subunit of pol-prim as follows: p58 might modulate and stabilize the primase activity of p48 either by increasing the affinity of p48 to divalent cations (which would explain the higher Mg\textsuperscript{2+} requirements of the p48 primase in comparison to that of the two- and four-subunit complex). In addition, p58 and divalent cations might selectively protect the inactivation of p48 activity by a specific mechanism, e.g. oxidative stress, which would resemble the selective inactivation of the 3′ → 5′ exonuclease activity of T7 DNA polymerase (44, 50). During rate-limiting steps of oligoribonucleotide synthesis p58 might also act as a stimulatory factor for the catalysis by p48, since the specific activity of the heterodimeric primase was significantly higher than that of p48 alone.

In summary, the vectors pET11-MCS and pET15-MCS allow the simple expression of single proteins and multiprotein complexes in E. coli. Our data showed that p48 can be expressed as a soluble protein, which requires Mg\textsuperscript{2+} and Mn\textsuperscript{2+} for stabilization of its enzyme activity. Furthermore, putative new functions for the p58 subunit of pol-prim emerged. The protein might increase the affinity of p48 to divalent cations; it might act as a stimulatory factor during the rate-limiting step of the dinucleotide synthesis, and p58 as well as specific divalent cations might selectively protect the inactivation of p48 activity by a specific mechanism. In the future, the expression and purification of large amounts of eukaryotic primase will allow detailed biochemical, biophysical, and structural analysis of the initiation reaction and DNA replication.

Acknowledgments—We thank Hella Förster for providing bovine DNA polymerase α-primase and Diana Oppitz and Dirk Bühler for cloning some constructs during their rotation work.

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