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FEBS Letters 337 (1994) 33–38

FEBS 13495

FEBS LETTERS

Replication of adenovirus DNA in vitro is ATP-independent

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Received 10 November 1993

Abstract

Using a reconstituted system for adenovirus DNA replication we tested the requirements for ATP and divalent cations. At the standard Mg^{2+} concentration ATP stimulated initiation 5 to 10-fold. However, this effect was caused by complexing Mg^{2+}. At the optimal Mg^{2+} concentration ATP was not required for initiation or elongation. Besides Mg^{2+} also Mn^{2+}, Ca^{2+} and Ba^{2+} were shown to support initiation whereas for elongation only Mg^{2+} was accepted. Since Mn^{2+} could efficiently be used for DNA chain elongation on synthetic templates we hypothesize that Mg^{2+} is essential for the transition of initiation to elongation.

Key words: Adenovirus; ATP; DNA polymerase; DNA replication; Divalent cation

1. Introduction

Replication of adenovirus DNA requires three virus encoded proteins, the precursor terminal protein (pTP), DNA polymerase (pol) and DNA binding protein (DBP). Two cellular transcription factors, Oct-1 and NFI, enhance initiation of DNA replication up to 200-fold by binding the auxiliary origin, and for enhancement their DNA binding domains (POU domain and NFI-BD, respectively), suffice [1–3]. These five replication proteins have been overproduced and purified to homogeneity. This enabled reconstitution both of the protein-primed initiation reaction (pTP–dCMP complex formation) and DNA chain elongation, using either native adenovirus DNA containing the terminal protein (TP–DNA) or origin containing plasmids as templates.

In such a reconstituted system, full replication activity requires, in addition to the five replication proteins, dNTPs and Mg^{2+} whereas ATP has been reported to stimulate the reaction under standard conditions. The stimulatory effect on complete DNA replication equals the stimulation of pTP–dCMP complex formation [4–6] suggesting that initiation is the ATP-dependent step. However, using subviral particles as template it was observed that especially elongation is stimulated by ATP [7], and using synthetic templates is was found that ATP stimulates the purified DNA polymerase [8]. The mechanism by which ATP stimulates initiation or elongation is not known. Employing templates in which the origin is partially or completely single stranded no stimulation by ATP was observed suggesting that ATP might be required for origin opening [5,9,10]. Furthermore, none of the proteins involved in replication has ATPase activity, moreover, non-hydrolysable ATP analogues such as ATPγS, AMP-PNP and AMP-PCP also stimulate the reaction [10]. This shows that ATP hydrolysis is not required.

We have investigated the role of ATP during adenovirus DNA replication in more detail. Surprisingly, we find that the stimulatory effect of ATP is only due to complexing of Mg^{2+} and that with optimal, low Mg^{2+} concentrations ATP is not required. Related to this we investigated the requirements for divalent cations both during initiation and elongation and found significant differences.

2. Materials and methods

2.1. Isolation of replication components

The pTP–pol complex was isolated as described previously [11]. The protein complex was more than 80% pure as determined by silver staining. One unit (U) of pTP–pol equals 1.2 μg. NFI-BD and the Oct-1 POU domain were purified as described [12] and were more than 95% pure. One binding unit (bu) corresponds to 1.4 ng for NFI-BD and 1 ng for the Oct-1 POU domain. Unit definitions are described elsewhere [12]. DBP was purified to homogeneity as described [13]. Adenovirus type 5 DNA–TP complex was purified from virions using guanidinium hydrochloride as described previously [14,15]. When indicated the DNA–TP complex was digested with *Xho*I, generating fragments of different lengths with TP containing fragments of 5,788 bp and 6,144 bp.

2.2. In vitro pTP–dCMP formation, partial and complete elongation

Formation of the pTP–dCMP initiation complex was performed in a reaction volume of 15 μl containing 25 mM HEPES/KOH (pH 7.5), 1 mM dithiothreitol, 0.2 μM [α-^{32}P]dCTP (3,000 Ci/mmol), 0.5 bu NFI-BD, 0.5 bu Oct1-POU, 6 mU pTP–pol, and as templates either

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SSDI 0014-5793(93)E1412-F

25 ng undigested TP–DNA, or 50 ng *Eco*RI/*Ava*II digested pHRI plasmid DNA, generating an adenovirus origin containing fragment of 1,530 bp with the origin at one extreme [16]. Mg^{2+} and ATP concentrations were as indicated. Incubation was for 1 h at 37 °C. NaCl concentrations were 50 mM and 40 mM for TP–DNA and plasmid DNA, respectively. For partial elongation of the pTP–dCMP complex with 25 nucleotides the reaction conditions were identical but dATP, dTTP and ddGTP were present at a concentration of 10 μM. Reactions were stopped by addition of 15 μl stopmix giving final concentrations of 75 mM sodium pyrophosphate, 10 mM EDTA and 100 μg/ml BSA. When plasmid DNA was used as template, Ca^{2+} was added to a final concentration of 2 mM, 10 units micrococcal nuclease were added and samples were incubated for another 15 min at 37 °C. Samples were processed further as described [17].

For complete DNA replication, nucleotide concentrations were 40 μM for dATP, dGTP and dTTP, and 2.5 μM for [α-^{32}P]dCTP (5.5 Ci/mmol) and 3 mU pTP–pol were used. The amount of template used was 18 ng for *Xho*I-digested TP–DNA and 30 ng for plasmid DNA. Incubation was at 37°C for 30 min in the case of TP–DNA or 60 min with plasmid DNA. Products were electrophoresed through a 1% agarose/0.1% SDS gel in 0.5 × TBE/0.1% SDS for 16 h at 2 V/cm or for 2 h at 20 V/cm. Gels were dried and autoradiographed and the results were quantified by scanning of the autoradiographs using an LKB ultrascan XL.

2.3. Assay of DNA polymerase activity with poly(dT)·oligo(dA) and primed M13 DNA

In the single stranded M13 template used (ssMXE-2) the first 1,338 nt of the adenovirus top strand have been inserted [18]. An oligonucleotide containing the first 55 bases of the adenovirus bottom strand was annealed as a primer. DNA synthesis on primed single stranded MXE-2 DNA was performed in 15 μl reaction volumes for 60 min at 37°C. Reaction mixtures contained 25 mM HEPES/KOH (pH 7.5), 1 mM dithiothreitol, 200 mU pTP–pol, 2.5 μM [α-^{32}P]dCTP (5.5 Ci/mmol), 40 μM of dGTP, dATP and dTTP and 0.35 μg single-stranded MXE-2 DNA. Reactions were stopped by adding 3 μl stopmix (20% sucrose, 5% SDS, 0.05% bromophenol blue) and products were resolved on a 8% polyacrylamide/0.1%SDS gel. Gels were dried and autoradiographed and the results were quantified by scanning of the autoradiographs using an LKB ultrascan XL. DNA synthesis on poly(dT)-oligo(dA) was performed in 15 μl reaction volumes for 30 min at 37°C containing 25 mM HEPES/KOH (pH 7.5), 1 mM dithiothreitol, 30 mU pTP–pol, 2.5 μM [α-^{32}P]dATP (25 Ci/mmol) and 250 ng poly(dT) + 250 ng oligo(dA). Reactions were stopped by adding 75 μl stopmix (20 mM EDTA, 0.1 mg/ml BSA) and 40 μl 50% TCA. After 15 min on ice DNA was precipitated by Eppendorf centrifugation for 30 min. Precipitates were washed with 1% TCA and [α-^{32}P]dAMP incorporation was measured by Cerenkov counting.

3. Results

3.1. Adenovirus DNA replication is ATP-independent

Using a reconstituted system consisting of purified proteins we studied viral DNA replication as a function of the Mg^{2+} concentration, at different ATP concentrations. Employing the natural viral TP–DNA complex, digested with *Xho*I, as a template a high level of replication was observed, indicating several initiation rounds as shown by the appearance of single-stranded origin-containing B and C fragments (Fig. 1A). At a standard ATP concentration of 2 mM used in many previous experiments an optimum Mg^{2+} concentration of 2–4 mM was obtained in agreement with earlier data [4,6,9,19,20] (Fig. 1A, lanes 16 and 17, see also Fig. 1B). Without Mg^{2+} no replication was observed. The Mg^{2+} optimum was highly dependent of the ATP concentration, however, and shifted to lower values at lower ATP concentrations. Without ATP, an optimum of 0.5–1 mM Mg^{2+} was obtained. Remarkably, the absolute level of replication was identical indicating that with appropriate Mg^{2+} concentrations replication is independent of ATP. Qualitatively similar results were obtained when a TP-free linearized origin containing plasmid was used as template (Fig. 1C,D), albeit that the absolute replication

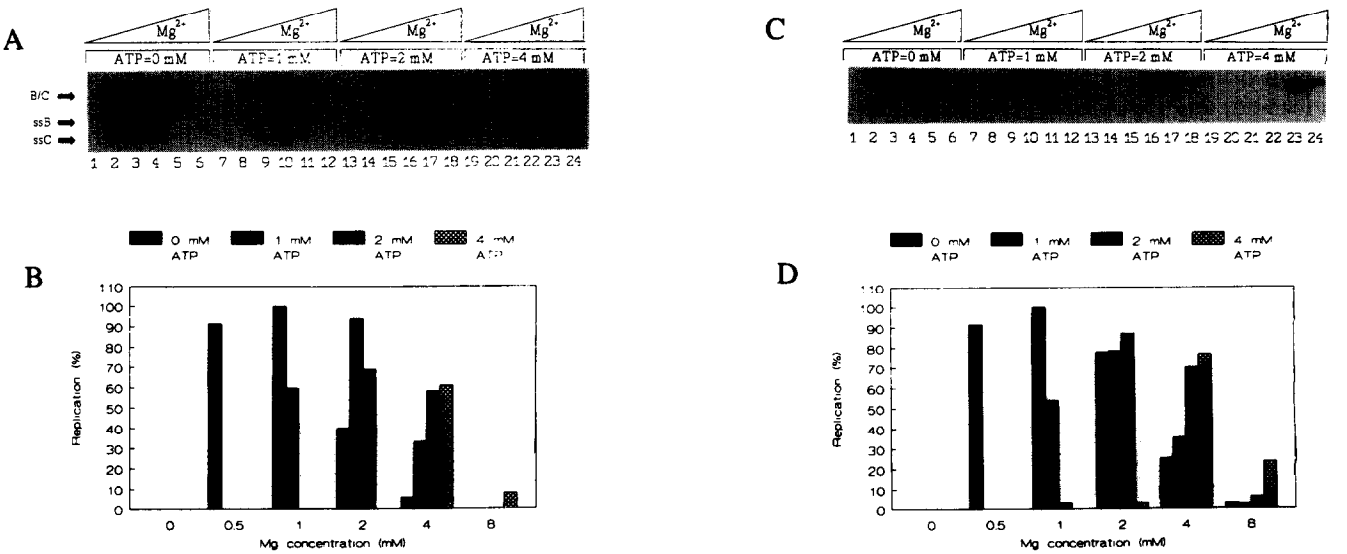

Fig. 1. Optimal Mg^{2+}-concentrations for replication depend on ATP. (A) In vitro DNA replication using TP–DNA as a template leading to replication of origin containing fragments B and C. (B) Histogram of the data in (A). The value in lane 3 was arbitrarily set at 100%. (C) In vitro DNA replication using plasmid pHRI as a template leading to a 1,530 bp product coupled to pTP (pTP-1530). The exposure time of the film shown here was at least 50 times longer than the film shown in 1A. (D) Histogram of the data in (C). ATP concentrations are as indicated in the figure. Mg^{2+} concentrations are 0 mM (lanes 1, 7, 13, 19), 0.5 mM (lanes 2, 8, 14, 20), 1.0 mM (lanes 3, 9, 15, 21), 2 mM (lanes 4, 10, 16, 22), 4 mM (lanes 5, 11, 17, 23) and 8 mM (lanes 6, 12, 18, 24)

levels are much lower than with the natural template. This agrees with the template-stimulating effect of the terminal protein [14,21–25]. Thus, although ATP appears to stimulate 5–10-fold at 4 mM Mg^{2+}, this effect is apparently caused by a reduction of the free Mg^{2+} concentration due to Mg^{2+}–ATP complex formation.

Since DNA chain elongation is responsible for the majority of incorporation of the radioactive precursor and since this process does not require ATP hydrolysis we tested initiation, as this step is presumed to be ATP-dependent. Initiation was measured by the formation of a pTP–dCMP complex (Fig. 2). Interestingly, results were similar as for the complete replication system, albeit that the Mg^{2+} optimum was slightly higher (1–2 mM). Initiation levels were never higher with ATP than without ATP at the optimal Mg^{2+} concentration (Fig. 2, compare lanes 3, 10, 17 and 23; see also Fig. 2B). In agreement with this, non-hydrolysable ATP analogues (ATPγS, AMP-PCP, AMP-PNP) and ADP influenced DNA replication in an identical fashion as ATP, namely by reducing the effective Mg^{2+} concentration leading to higher incorporation (results not shown).

3.2. *Mg^{2+} is essential for DNA chain elongation but not for initiation*

In the absence of ATP, we tested various divalent cations (Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Mn^{2+}) as possible substituents for Mg^{2+} during initiation. Employing TP–DNA as a template, Mg^{2+} could only be replaced by Ca^{2+} and Mn^{2+}. At optimal concentrations the relative efficiencies were 27% (Ca^{2+}) and 102% (Mn^{2+}) (Fig. 3A). Initiation with plasmid DNA was even less selective, as Ba^{2+} could also substitute for Mg^{2+}. Mn^{2+} was even more efficient than Mg^{2+}. Values obtained were 48% (Ba^{2+}), 14% (Ca^{2+}) and 330% (Mn^{2+}) (Fig. 3C). This indicates that the presence of the terminal protein restricts the use of cations thus contributing to selectivity. To study initiation and elongation in the same experiment we added, in addition to dCTP, also dATP, dTTP and ddGTP. This leads to a partial elongation product of 26 nucleotides, stopping after the first G-residue (pTP-26N). Due to the low dCTP concentration (0.2 μM) employed here, still some initiation products can be seen [17] which enables a direct comparison of initiation and elongation in one experiment. Remarkably, neither Ca^{2+} nor Ba^{2+} or Mn^{2+} could support the elongation reaction to any extent. Thus, partial elongation can only be sustained by Mg^{2+}. The different requirements for cations during initiation and elongation may indicate structural changes in the metal or dNTP binding sites of the DNA polymerase occurring during the shift from initiation to elongation. Mg^{2+} was also essential using the intact replication system. Replication levels were undetectable (< 0.1%) using any other cation (not shown).

To study whether the restriction to Mg^{2+} as divalent cation during elongation is an intrinsic property of the DNA polymerase we used oligonucleotide-primed templates rather than the protein-priming system. Employing oligo(dA)-poly(dT) as a template and in the absence

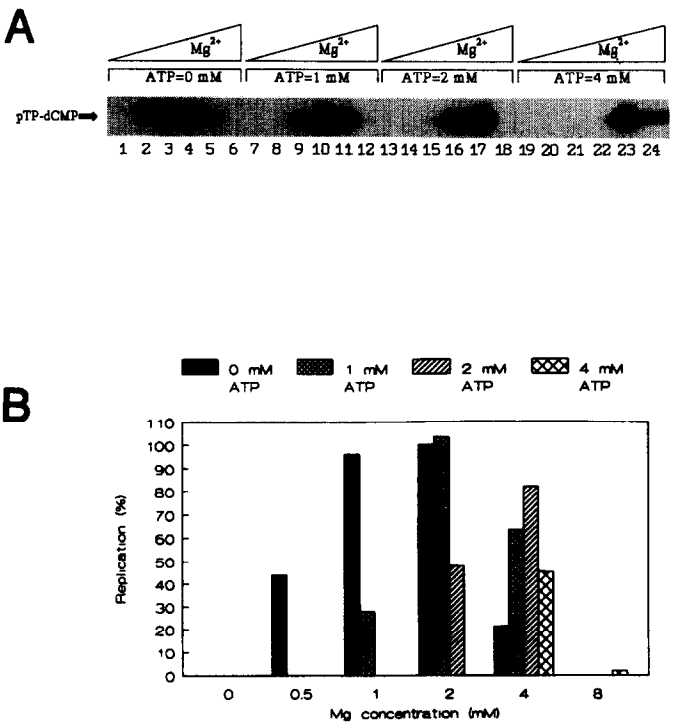

Fig. 2. ATP is not required for initiation. (A) In vitro pTP–dCMP formation using TP–DNA as a template. (B) Histogram of the data in (A). Mg^{2+} concentrations are as in Fig. 1.

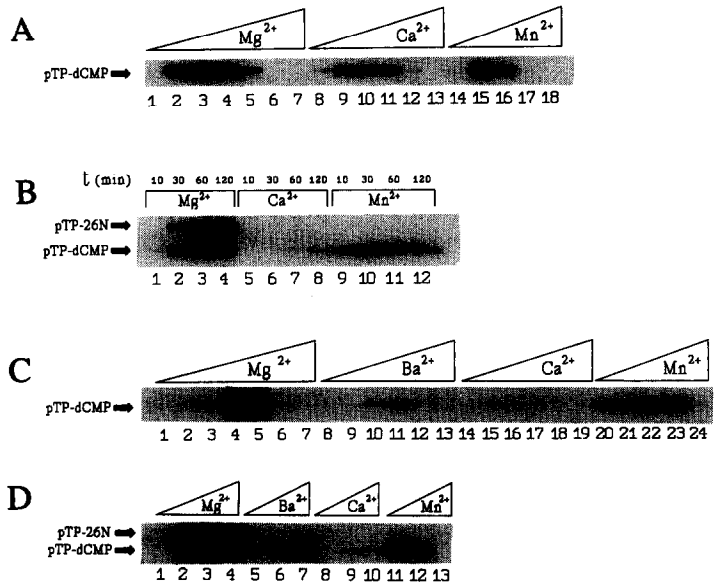

Fig. 3. In vitro pTP–dCMP formation and partial elongation using various cations. (A) pTP–dCMP complex formation using TP–DNA as template. Lanes 1–7 contain 0.15, 0.6, 1.5, 3, 6, 12 and 24 mM Mg^{2+}, lanes 8–13 contain 0.6, 1.5, 3, 6, 12 and 24 mM Ca^{2+}, and lanes 14–18 contain 0.15, 0.6, 1.5, 3 and 6 mM Mn^{2+}. (B) Partial elongation using Mg^{2+}, Ca^{2+} and Mn^{2+} as cations. Lanes 1–4 contain 1.5 mM Mg^{2+}, lanes 5–8 contain 3 mM Ca^{2+}, and lanes 9–12 contain 0.6 mM Mn^{2+}. (C) pTP–dCMP complex formation using plasmid pHRI as template. Lanes 1–7 contain 0, 0.2, 0.5, 1, 2, 4 and 8 mM Mg^{2+}, lanes 8–13 contain 0.2, 0.5, 1, 2, 4 and 8 mM Ba^{2+}, lanes 14–19 contain 0.5, 1, 2, 4, 8 and 12 mM Ca^{2+}, and lanes 20–24 contain 0.1, 0.2, 0.5, 1 and 2 mM Mn^{2+}. (D) Partial elongation using plasmid pHRI as template. Lanes 1–4 contain 0, 0.5, 1, and 2 mM Mg^{2+}, lanes 5–7 contain 0.5, 1 and 2 mM Ba^{2+}, lanes 8–10 contain 0.5, 1 and 2 mM Ca^{2+}, and lanes 11–13 contain 0.5, 1 and 2 mM Mn^{2+}.

of ATP and DBP an optimum of approximately 0.5 mM Mg^{2+} was obtained whereas at high Mg^{2+} concentrations ($\geq$ 5 mM) DNA synthesis was blocked completely, similar to viral DNA replication (Fig. 4A). However, in contrast to viral replication, Mn^{2+} could be used very efficiently as a co-factor and even 5-fold higher maxima were obtained. Thus, using this template elongation is not restricted to Mg^{2+}, a property which is shared with other DNA polymerases [26,27].

In the natural viral system, the adenovirus DBP is essential for DNA chain elongation. Since DBP is known to change some properties of the DNA polymerase, such as the K_m for dNTP [17] and drug sensitivity [11], we investigated whether the requirement for divalent cations might also be changed by DBP, possibly leading to a selective use of Mg^{2+}. Therefore we included DBP in the DNA synthesis reactions using poly(dA) · oligo(dT) as template. However, as shown in Fig. 4B this did not change the cation sensitivity and again Mn^{2+} could be used efficiently. Since with this template DBP hardly stimulated DNA synthesis we also used primed M13 DNA. Here DBP stimulated polymerization up to 100-fold (Fig. 4C) but again, Mn^{2+} could be used efficiently.

4. Discussion

We show here that, under optimal Mg^{2+} concentrations, ATP is not required for adenovirus DNA replication when assayed in a reconstituted system. Previously reported stimulation by ATP most likely was caused by complexing Mg^{2+}. Whether this reflects the situation in vivo is difficult to establish. We cannot exclude that the concentrations of the various replication proteins used in vitro bypass the need for an ATP-dependent function in vivo. Such a situation has been reported for T4 DNA replication, where excess gp45 in vitro overrules the need for the 41/61 gene products, which contain ATPase activity and are required for the assembly of a functional initiation complex [28]. However, as estimated for the most abundant adenovirus replication protein, DBP, intracellular concentrations are even higher than those employed in vitro in this study [29]. Moreover, a search for additional stimulatory factors for adenovirus DNA replication that might have ATPase activity have been negative so far. This makes the presence of an ATP-dependent function in vivo less likely.

In many DNA replication systems unwinding of DNA in front of a replication fork is performed by helicases that require ATP-hydrolysis [30]. However, no helicase is needed in the elongation phase of adenovirus replication. Recently we have shown that DBP by itself stimulates DNA unwinding when the duplex has a single-stranded extension [31], which suggests that DBP is the unwinding protein during chain elongation. Thus unwinding occurs independent of ATP and is optimal at low Mg^{2+} concentrations, similar to the conditions required for in vitro adenovirus DNA replication [31].

In many systems origin unwinding during initiation requires ATP hydrolysis. For instance, the helicase activ-

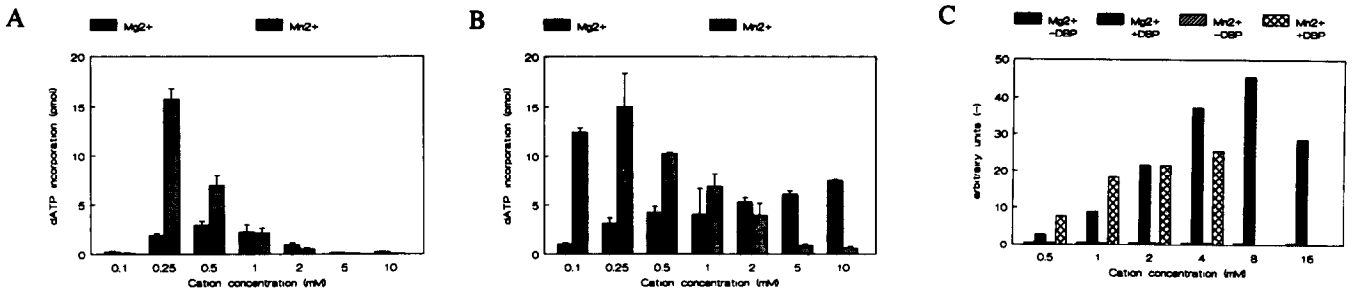

Fig. 4. DNA chain elongation by the Ad DNA polymerase can occur in the presence of Mn^{2+} independent of DBP. (A) DNA polymerase assay with poly(dT) · oligo(dA) as template in the absence of DBP. Cation concentrations are as indicated. The result shown is the average of at least 3 independent observations. Standard deviations are indicated. (B) The same experiment as in (A), but in the presence of 1 μg DBP. (C) DNA polymerase assay with Mg^{2+} and Mn^{2+} using primed single-stranded M13 DNA as template in the absence and presence of 1 μg DBP.

ity of SV40 T-Ag is responsible for origin opening as is dnaB helicase activity in oriC. Why does initiation in adenovirus occur so efficiently in the absence of ATP? Although we do not yet know how adenovirus origin unwinding occurs, it is clear that initiation only takes place if the origin sequences are at, or close to, a molecular end [14,21]. Possibly the location of the origin at the molecular end facilitates origin opening, whereby the combined action of NFI, Oct-1, DBP, and pTP–pol suffices for origin unwinding. Alternatively, the energy released during formation of the initiation complex may be used somehow for further origin opening and progression to the elongation steps.

We show that Mn^{2+} as well as Ba^{2+} and Ca^{2+} can replace Mg^{2+} during initiation, whereas elongation occurs only in the presence of Mg^{2+}. Several explanations are possible for this observation. One is, that only Mg^{2+} can be used by the DNA polymerase for elongation. However, using synthetic templates we observed that Mn^{2+} can be used efficiently by adenovirus DNA polymerase, similar to other DNA polymerases including those that are capable of protein-priming. Another possibility is that only Mg^{2+} can support the displacement reaction. We have tried to test this using a partially double-stranded template containing a primer followed by a single-stranded gap and a second double-stranded region, which would be displaced upon displacement synthesis. Unfortunately, due to the limited length of the duplex region, DBP displaced the double-stranded regions already without DNA synthesis which made it impossible to test this hypothesis. Yet other possibilities are that Mg^{2+} is necessary for the transition from initiation to elongation, or that a conformational change occurs in the pTP–pol complex when going from an initiation to an elongation mode. This conformational change could lead to a change in the Me^{2+} binding site and the dNTP binding site and might be accompanied by dissociation of pTP, which inhibits elongation on synthetic templates [8]. Such a change was already suggested by the difference in K_m for dCTP during initiation (0.5 μM) and elongation (1.4 μM) [17]. There are precedents for such a change in metal requirement in other systems as well. For instance, *Eco*RV binds Mn^{2+} better than Mg^{2+} when bound non-specifically on DNA, but when bound at its specific binding site the affinity for Mg^{2+} is many-fold higher and Mg^{2+} is therefore the preferred cation for nuclease activity [32,33]. A binding site for Mg^{2+} requires the correct juxtaposition of the functional groups that coordinate the metal, from both the protein and the DNA [34,35]. Such a mechanism may also apply to the presumed change in Ad DNA polymerase. Thus, assuming that the same dNTP and Me^{2+} binding sites are used during initiation and elongation, as has been demonstrated for bacteriophage φ29 [36], we hypothesize that the Me^{2+} binding site changes such that after the first initiation step only Mg^{2+} can be accepted, explaining that the synthesis of the first 26 nucleotides is completely Mg^{2+} dependent.

Acknowledgements: We thank D.C. Zijderveld for critical reading of the manuscript. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial support from the Netherlands Organisation for Scientific Research (NWO).

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