Formation in vitro of the pTP–dCMP initiation complex of human adenovirus type 12

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We report the covalent addition of [32P]dCMP to a protein from group A adenovirus 12 (Ad12)-infected human (KB) cells in vitro, using crude extracts. Synthesis of the 60K protein–dCMP complex required a DNA template containing a terminally located adenovirus replication origin; the protein–dCMP bond was alkali-labile but acid-stable. We therefore conclude that this product is the Ad12 terminal protein precursor (pTP)–dCMP initiation complex for DNA replication. Synthesis of Ad12 pTP–dCMP was specific for dCTP but was stimulated by dATP. In contrast to Ad2, the Ad12 initiation reaction required ATP. Antipeptide antiserum targeted to Ad DNA polymerase inhibited Ad12 pTP–dCMP synthesis in vitro, providing evidence that Ad DNA polymerase catalyses dCMP addition to pTP during initiation.

Replication of the linear, dsDNA of adenovirus (Ad) has been investigated extensively using group C serotypes 2 and 5, and a model has been developed based on in vitro and in vivo studies (reviewed in Kelly et al., 1988; Challberg & Kelly, 1989). Replication is initiated at either terminus with the covalent attachment of dCMP to a serine residue in the viral terminal protein (TP) precursor (pTP), a reaction which requires the Ad DNA polymerase (DNApol) and a cellular CCAAT-binding protein, nuclear factor I (NFI). The reaction is further stimulated by a cellular octamer binding protein, ORP-C. Elongation of the nascent chain is catalysed by Ad DNApol and also requires the viral DNA-binding protein and a cellular type I DNA topoisomerase, NFII.

The overall features of the DNA replication model are probably common among all Ad serotypes but there may be some serotype-dependent variation in the details of replication; for example, group E Ad4 DNA replication is not dependent on NFI (Hay, 1985). In light of the limited information concerning the properties of group A Ad12 DNA replication (Shiroki et al., 1974; Chowrira & Lucher, 1990), we have started to investigate the in vitro DNA replication properties of Ad12. The present report describes some of the characteristics of the in vitro initiation reaction for Ad12.

Initiation reactions were conducted at 37 °C in 20 μl volumes containing viral DNA template [150 ng Ad DNA with TP covalently attached to the 5' termini (Ad DNA-pro) or 0.4 μg plasmid DNA], 4 mM-ATP, 0.15 mM-[γ-32P]dCTP (> 3000 Ci/mmol) and crude cell extract, in 25 mM-HEPES pH 7.5, 5 mM-MgCl₂, 1 mM-DTT. Following SDS-PAGE, a 60K reaction product was detected when infected nuclear extract was used as a source of both viral replication proteins and cellular nuclear factors in the initiation reaction mixture (Fig. 1 a); product synthesis varied with the m.o.i. (Fig. 1 a). Infected nuclear extract could be replaced in the assay with infected cytoplasmic extract (for viral proteins) plus mock-infected nuclear extract (for nuclear factors) (Fig. 1 b to d). The mobility of the initiation product is in close agreement with that of a previously reported Ad12 67K in vitro translation product which was tentatively identified as pTP (Esche & Siegmann, 1982).

The nature of the 60K initiation product was similar to that of the Ad2 pTP–dCMP initiation complex (Lichy et al., 1981) in the following respects (Fig. 1 b). (i) Label was incorporated via a covalent bond because it survived preparation for SDS gel electrophoresis. (ii) The product contained protein because it was sensitive to Pronase but resistant to DNase I. (iii) Label was not removed by alkaline phosphatase treatment, indicating that the protein was modified by addition of dCMP rather than phosphate. (iv) The protein–dCMP bond was resistant to HCl but was cleaved by KOH.

The 60K initiation complex was only synthesized when the DNA template was Ad DNA-pro or cloned DNAs containing a terminally located Ad12 replication origin (Fig. 1 c). We conclude that the product is the Ad12
pTP–dCMP initiation complex based on the influence of Ad12 m.o.i. on the reaction, the similarities in product characteristics to Ad2 and Ad5 pTP–dCMP and the requirement for terminally located Ad origins of replication.

The initiation reaction was maximal at pH 7.5 (Fig. 1d) and was unaffected by aphidicolin (Fig. 1d). However, initiation required MgCl2 and ATP when the template used was either plasmid DNA (Fig. 1d) or Ad DNA-pro (not shown). In contrast, the Ad2 initiation reaction with Ad DNA-pro is stimulated by ATP but is unaffected by ATP with deproteinized Ad DNA (Lichy et al., 1981; Kenny & Hurwitz, 1988).

The Ad12 initiation reaction showed a dependence on dCTP concentration between 0.025 μM and 0.3 μM (Fig. 2a) and was specific for dCTP. Synthesis of pTP–dCMP was undetectable after the addition of 50 μM unlabeled competitor dCTP to the reaction but up to 150 μM unlabeled dGTP, dTTP or dATP did not compete for the incorporation of label into pTP–dCMP (Fig. 2b). The marked stimulation of initiation by dATP (Fig. 2b) may be due to an allosteric effect of (d)ATP (Kenny & Hurwitz, 1988).

Synthesis of pTP–dCMP was more dependent on the concentration of nuclear proteins from mock-infected extracts than on the concentration of viral proteins from infected cytoplasmic extracts (Fig. 2c). Under standard conditions, 0.1 to 0.2 pmol dCTP/mg of cytoplasmic protein was incorporated into pTP–dCMP.

Since the carboxy terminus of Ad DNApol is important for its activity (Chen & Horwitz, 1989), we
raised rabbit antiserum to a highly conserved peptide sequence (PNPRNEEVC) near the C terminus of Ad12 (Shu et al., 1986). Antiserum to this peptide (Ab6) recognized a 110K protein from Ad12-infected extracts when used in immunoblot analysis of non-denaturing gels (Fig. 3a); the specificity of the reaction was confirmed by peptide competition analysis (Fig. 3a). The antiserum also detected a 140K Ad2-specific protein under the same electrophoresis conditions (J. Zhao & L. A. Lucher, unpublished observations). Since non-denaturing gels were used, each of these proteins is most likely to be a 1:1 complex of Ad DNApol and pTP (Enomoto et al., 1981). The apparent Mr of 110K for Ad12 DNApol:pTP is lower than expected but may be due to our electrophoresis conditions because the Ad2 DNApol:pTP has shown an Mr of 180K in glycerol gradients (Enomoto et al., 1981).

We tested Ab6 for its effects on two Ad12 DNApol activities. First, the general activity of Ad12 DNApol was determined by the virus-specific, aphidicolin-resistant incorporation of [3H]TTP into activated calf thymus DNA. This activity was inhibited by Ab6 but not by prebleed serum (Fig. 3b). The aphidicolin-resistant cellular DNApol activity was not inhibited by Ab6 (Fig. 3b). Second, synthesis of Ad12 pTP-dCMP was completely blocked by Ab6 but not by prebleed serum (Fig. 3c). Inhibition of the reaction was due to anti-DNApol antibodies because inhibition by Ab6 was prevented by peptide competition (Fig. 3c).

Insertional mutagenesis studies suggest that essential regions of Ad DNApol are scattered throughout its primary structure (Chen & Horwitz, 1989); the C-terminal 13% of Ad2 DNApol contains two such regions, 150 and 102 residues from the C terminus. The inhibition of Ad12 DNApol by Ab6 (the target sequence is 14 residues from the C terminus) is consistent with the importance of the C terminus for Ad DNApol activity.

It is currently hypothesized that attachment of dCMP to pTP during initiation is catalysed by Ad DNApol, although autocatalysis by pTP cannot be ruled out (Challberg & Kelly, 1989). Immune IgG raised against purified Ad5 TP inhibits Ad5 initiation in vitro (Sussenbach & van der Vliet, 1983) but the recognition of multiple epitopes by the IgG makes it difficult to distinguish between steric hindrance of DNApol by antibodies bound to pTP and inhibition of pTP autocatalysis. Since Ab6 recognizes a defined DNApol sequence, inhibition by Ab6 suggests that catalysis is carried out by DNApol rather than pTP. It is possible that Ab6 exerts steric hindrance to block autocatalysis by pTP but inhibition by this antiserum of TTP incorporation into activated calf thymus DNA, a reaction which presumably does not involve pTP, lends support to the idea of DNApol catalysis of Ad initiation.

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References


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