High expression of functional adenovirus DNA polymerase and precursor terminal protein using recombinant vaccinia virus

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ABSTRACT
Initiation of Adenovirus (Ad) DNA replication occurs by a protein-priming mechanism in which the viral precursor terminal protein (pTP) and DNA polymerase (pol) as well as two nuclear DNA-binding proteins from uninfected HeLa cells are required. Biochemical studies on the pTP and DNA polymerase proteins separately have been hampered due to their low abundance and their presence as a pTP-pol complex in Ad infected cells. We have constructed a genomic sequence containing the large open reading frame from the Ad5 pol gene to which 9 basepairs from a putative exon were ligated. When inserted behind a modified late promoter of vaccinia virus the resulting recombinant virus produced enzymatically active 140 kDa Ad DNA polymerase. The same strategy was applied to express the 80 kDa pTP gene in a functional form. Both proteins were overexpressed at least 30-fold compared to extracts from Adenovirus infected cells and, when combined, were fully active for initiation in an in vitro Adenovirus DNA replication system.

INTRODUCTION
The Adenovirus DNA polymerase (1,2) and the precursor terminal protein (3,4) are essential components of the Adenovirus DNA replication machinery which has recently been dissected in detail. Initiation of DNA replication occurs by the covalent coupling of a dCMP residue to the pTP. The 3'-OH group of this pTP-dCMP complex serves as a primer for further elongation by a strand displacement mechanism. The Ad DNA polymerase performs a dual function both in formation of a pTP-dCMP initiation complex and in the subsequent elongation of the DNA chain (for reviews see 5,6).

The Adenovirus pol and pTP open reading frames map in the E2B region of the genome within a large (22 kb) L-strand transcription unit. Both the pol and pTP transcripts are initiated at the E2A promoter.
(coordinate 75) and share leader segments at coordinates 75, 68 and 39 (7,8). The main body of the Ad5 genes map at coordinates 22.8.-14.1 for pol and coordinates 28.8-23.4 for pTP (9). The exact structure of the splice sites in the mRNA's and therefore the positions of the start codon are unknown.

We have considered two different splice models for the pol and pTP mRNA's which are schematically indicated in Fig. 1A. The first model is based on addition of a non-translated exon from the 39 region. The ATG start codons at positions 8367 and 10,544 within the pol and pTP open reading frames, respectively, would in this case be used for translation initiation resulting in polypeptides of 1056 amino acids for pol and 653 amino acids for pTP (9). Based upon this model pol-CAT or lacZ-pol-CAT fusion proteins were expressed in E.coli which were not enzymatically active (10,11).

The second possibility involves splicing of a putative exon from coordinate 39 which would provide the translation start codon. Computer analysis showed that the only in-frame splice donor site would be at Ad2 position 14,105 providing the ATG start codon at 14,114 and 2 additional amino acids (ala, leu). As a consequence the in-frame acceptor splice sites at positions 8784 for pol and 10,589 for pTP would be used and the corresponding polypeptides would be considerably longer, 142 amino acids for pol and 18 amino acids for pTP, respectively. Use of these splice sites would mean that the end of the pTP gene and the beginning of the pol gene overlap for 199 basepairs in different reading frames (12). Such a gene structure would be in better agreement with (i) the apparent 140 kDa molecular weight of pol in SDS gels (1,2), (ii) the presence of pTP mutations and pol mutations (13; D.J. Roovers and J.S. Sussenbach, unpublished observations) located outside the open reading frame mentioned in the first splicing model and (iii) the high sequence and amino acid conservation between Ad2, Ad5, Ad7 and Ad12 in the proposed N-terminal coding regions between the main bodies of the pol and pTP genes (12).

Based on these two models we have constructed two different pol genes (pol and truncated pol) and one pTP gene under the control of a vaccinia 11K late promoter (14,15). We show here that the products of the pol and pTP genes are functionally active in DNA replication while the truncated pol gene product is inactive.
MATERIALS AND METHODS

Construction of recombinant vaccinia viruses.

Construction of pATA-pol: a Bgl II-Pst I fragment (Ad 5 positions 8914-8407) was cloned into M13 mp19. The plasmid was digested with the restriction enzymes Apa I and Eco RI and ligated in the presence of two complementary synthetic oligonucleotides which provide an Eco RI adaptor followed by the sequence coding for the three amino acids present in the leader at coordinate 39 (14114-14105) and the sequence downstream the pol splice acceptor site (8784) up to the Apa I restriction site (8766). The EcoRI resulting Kpn I fragment of the M13 subclone containing the AUG and the splice junction was cloned into the pATA-18 vector containing a Kpn I/Sph I fragment of the Ad5 genome (8533 to 5137).

Construction of pATA-truncated pol: a Sac I/Sph I fragment (8374-5137) was isolated through partial digestion with Sac I and cloned into the polylinker of the pATA-18 recombination vector.

Construction of pATA-pTP: a Xba I/Pst I fragment of Ad5 DNA (10,589-8407) was subcloned into pUC-12. The plasmid was digested with Xba I and the protruding 5'-ends removed by nuclease S1 treatment. After inactivation of the enzyme the DNA was digested with Eco RI and ligated in the presence of two complementary synthetic oligonucleotides which provide and Eco RI adaptor followed by the sequence present in the leader at coordinate 39. The resulting Eco RI/Pst I fragment (2191 bp) containing the pTP gene was isolated and cloned into the polylinker of the pATA-18 recombination vector.

Homologous recombination and subsequent selection and plaque purification of recombinant vaccinia virus were performed as previously described (15) by infecting human 143 tk- cell monolayers with vaccinia virus temperature sensitive mutant ts7 (16) followed by co-transfection with 100 ng of the different recombination vectors and wild type vaccinia DNA.

Pulse labeling.

Cell lysates were prepared by infecting 3 cm dishes with confluent monolayers of RK13 cells with the different recombinants (2 PFU per cell) and incubating overnight at 37°C. The cells were washed with PBS and incubated in a serum and methionine free medium which was replaced after one hour by 0.75 ml of fresh serum and methionine free medium containing 75 μCi of 35S-methionine and the incubation was
continued for an additional hour. The cells were washed with PBS and resuspended in lysis buffer containing: 50 mM Tris-HCl pH 7.5 - 150 mM NaCl - 5 mM MgCl₂ - 1% Triton-X-100 and 4 μg/ml of PMSF added immediately before use. The lysate was incubated on ice for 10 min and centrifuged at 13 krpm. for 10 min. The supernatant was used for DNA polymerase activity assays. Aliquots of the supernatant (50 μl) were TCA precipitated. The precipitates were collected by centrifugation, washed with 50% acetone, dried and resuspended in 50 μl of sample buffer. A 10 μl aliquot was denatured and applied to a 7.5 % SDS polyacrylamide gel. Pulse-chase experiments were performed as described above with the exception that methionine starvation and ³⁵S-methionine labeling were performed 8 hrs after infection and the cells were collected after a 12 hrs chase in complete medium.  

**Immunoprecipitation**

200 μl of the cytoplasmic lysate was incubated on ice in the presence of 1-10 μl of anti-pol (10) or anti-pTP antiserum. After one hour 35 μl of pre-swollen protein A-Sepharose Cl 4B was added and the incubation was continued for one hour at 4°C with rotation. The Sepharose beads were collected by centrifugation and washed three times with 1 ml of 10 mM Tris-HCl pH 7.5 - 150 mM NaCl - 2 mM EDTA - 0.2% Triton-X-100, twice with the same amount of buffer containing 500 mM NaCl and once with 1 ml of 10 mM Tris-HCl pH 7.5. Sample buffer was applied to the beads and the proteins were denatured and applied to a SDS-polyacrylamide gel. The gel was fixed with 10 % TCA - 10 % acetic acid - 30 % ethanol, rinsed extensively with water, soaked in Enhance (DuPont) supplemented with 20 % ethanol, rinsed with water, dried and exposed at -70°C with an intensifying screen.  

**Ad DNA replication in vitro**

HeLa cells grown in suspension were infected at a concentration of 3 x 10⁶ per ml in phosphate buffered saline (PBS) at a multiplicity of 5 PFU/cell (17) and diluted 10-fold with fresh medium after 60 min at 37°C. After 16 hrs the infected cells were harvested, washed twice with cold PBS - 0.5 mM MgCl₂ and suspended in 20 mM Hepes pH 7.5 - 5 mM KCl, 0.5 mM dithiothreitol (DTT) at a density of 5 x 10⁷ cells per ml. All further procedures were at 4°C. After 10 min cells were Dounce homogenized and nuclei were spun down at low speed. The supernatant was centrifuged for 30 min in an Eppendorf centrifuge and supplemented with 20 % glycerol - 10 % sucrose - 2 mM DTT (final concentrations) and
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stored frozen at -80°C. Nuclear extracts were prepared by incubating nuclei for 60 min in 50 mM Hepes pH 7.5 -10 % sucrose - 2 mM DTT - 0.2 M NaCl followed by centrifugation for 10 min in an Eppendorf centrifuge. The cleared extracts were supplemented with 10 % glycerol and stored frozen at -80°C. Extracts were stable for one week at 4°C and for at least 3 months when frozen.

Replication of terminal fragments of Xho I digested DNA-TP isolated from virions was performed essentially as described before (18) with 0.4 µg Ad DNA binding protein per assay (15 µl), 1 µl crude nuclear extract from uninfected HeLa cells and supernatant from vaccinia infected HeLa cells to replace purified pTP-pol. In some cases purified nuclear factor I or nuclear factor III substituted for the crude nuclear extract. These proteins were purified as described before (18,19). Reaction mixtures were analyzed as described (18). Initiation was measured as the formation of a 32p-pTP-dCMP complex after incubation in the presence of 1µM [32p]-dCMP and 40 µM dideoxy ATP, omitting dATP, dTTP and dGTP. The products were analyzed by polyacrylamide gel electrophoresis (18).

RESULTS

Construction of recombinant vaccinia viruses.

In order to connect the large open reading frames of pTP and pol to the three amino acids from the leader at coordinate 39, synthetic oligonucleotides were used. The different gene constructs were cloned into the vaccinia pATA-18 recombination vector (Fig. 1B) which contains a mutated 11K late promoter (-100 to +8) followed by the multiple cloning site of pUC-18 and they are flanked by the non-essential sequences of the vaccinia virus thymidine kinase (tk) locus (20). These flanking sequences are necessary for the homologous recombination and are subsequently used for selection of the recombinant virus (17). The promoter mutations included a conversion of four bases into their complement thereby introducing a Cla I site at position -9 (the A-residue of the ATG start codon is referred to as +1). This Cla I restriction site was subsequently used to insert synthetic oligonucleotides which provided an A-residue at +3 within the promoter between the T- and G-residues of the translation start codon located within the essential and conserved TAAATG motif (21,22). This new promoter construct enables the synthesis of high amounts of foreign
Fig. 1. Construction of recombinant vaccinia virus
A. Schematic drawing of the E2B region of the adenovirus 5 genome. The position of the putative ORFs of pol and pTP and their start and stop codons are indicated. Note that the reading frames of pol and pTP are overlapping. The sequence coding for the putative three amino acids within the leader at coordinate 39 is indicated.

B. Schematic drawing of the vaccinia pATA-18 recombination vector. The gene proximal parts of the wild-type and mutated 11K promoter sequences are indicated. L-TK and R-TK represent the left and right hand side of the vaccinia tk locus consisting of a 1301 bp fragments (HindIII to HpalI) which is located in the HindIII-J fragment in the central part of vaccinia genome.

Polypeptides in the late phase of virus infection, initiating translation at their authentic start codon (de Magistris and Stunnenberg, unpublished observations). A detailed transcriptional and translational analysis will be presented elsewhere.

Characterization of recombinant products.
For the analysis of the new polypeptides synthesized in cells infected with the different recombinant viruses we used (i) pulse labeling and immunoprecipitation, (ii) Ad DNA polymerase activity measurements and (iii) in vitro Ad DNA replication assays. Rabbit Kidney RK13 cells were infected with plaque purified recombinant or wild type vaccinia virus and the newly synthesized polypeptides were pulse-labeled with $^{35}$S-methionine at 16-18 hrs post infection and analyzed by polyacrylamide gel electrophoresis (Fig. 2). Two major new polypeptides and a minor one were made in cells infected with the pTP-recombinant with molecular weights of 74 kDa, 76.5 kDa and 80 kDa (Fig. 2A, lane 9) which should be compared with a calculated molecular weight of 76,506.
Fig. 2. Pulse labeling and immunoprecipitation of newly synthesized Ad DNA polymerase and pTP polypeptides

A. Autoradiogram of cell lysates separated by SDS gel electrophoresis. The odd numbers indicate the crude extracts and the even numbers immune precipitates. MI = mock-infected. WR = wild type vaccinia virus. tr pol, pol and pTP indicate the recombinant viruses containing truncated DNA polymerase, DNA polymerase and precursor terminal protein, respectively. The arrows point to the newly synthesized recombinant products. Marker molecular weights and positions are indicated.

B. Pulse-chase experiment. For details see text.

Da. All three polypeptides could be immuno-precipitated with anti-pTP antibodies (lane 10). The presence of several different pTP polypeptides is either due to premature stops or degradations or, what seems more likely, to translation initiation on internal AUG start codons. The sequence surrounding the first AUG is not optimal according to the Kozak rule (23) i.e. a T-residue at -3 whereas e.g. the first in frame start codon (at position 10,544) is optimal in this respect and is probably also used for initiation of translation. We have observed that initiation of translation occurs in 5-10% of the cases on internal AUG start codons in vaccinia virus infected cells even if the first AUG is
Table I

<table>
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<td>3,120</td>
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(1) DNA polymerase activity* (units/10^6 cells)

(2) RK13 cell lysates were prepared as described in the legend of Fig. 2, omitting the 35S-methionine labeling procedure. DNA polymerase activity was measured using activated calf thymus DNA as template (25). One unit corresponds to one nmol dTMP incorporated per 20 min at 30°C.

supposed to be optimal according to the Kozak rules (de Magistris and Stunnenberg, unpublished observations). Several other vaccinia-specific late polypeptides were observed that also appear in wild type-infected cells and are difficult to remove by washing due to their high affinity for protein-A. Upon infections with the pol or truncated-pol (start codon at 8367) recombinants two newly synthesized polypeptides of 140 kDa and 125 kDa, respectively, were observed (Fig. 2A, lanes 7 and 5). Both polypeptides could be immuno-precipitated with anti-pol antibodies (lanes 8 and 6). These molecular weights agree well with the calculated molecular weight of 135,504 Da and 120,412 Da, respectively. Again, vaccinia-encoded late polypeptides are present as contaminants in the immune precipitates.

We investigated the stability of the foreign polypeptides by pulse-labeling at 8 hrs post infection followed by a chase of 12 hrs (Fig. 2B). The 80 kDa pTP polypeptide was stable whereas the amount of 74 kDa as well as the minor 76.5 kDa pTP polypeptide were drastically reduced during the chase period. The 140 kDa pol protein also appeared to be stable in a double (pTP + pol) infection while without the pTP we detected a 50% reduction of the pol protein in 16 hrs (not shown). The truncated 125 kDa protein was stable even in the absence of the pTP. Incubation of the infected cells in the presence of tunicamycin did not result in a change in mobility of pTP or pol indicating that they are not N-glycosylated although potential sites are present in the pTP. However,
Fig. 3. **Ad DNA replication in vitro with recombinant pTP and DNA polymerase**

Extracts supernatants were prepared as described below and replication of origin containing Xho I fragments B and C was studied (A, B) or formation of a pTP-dCMP initiation complex (C).

A. Lane 1 contains 1μl of an extract from adenovirus infected cells. Lanes 2-6: 1μl of vaccinia pol supernatant and 1, 0, 0.001, 0.01 and 0.1 μl pTP supernatant. Lanes 7-10: 1μl pTP supernatant and 0, 0.001, 0.01 and 0.1 μl pol supernatant. Lane 11: 1μl of supernatant from double infected (pTP + pol) cells.

B. Dependence of nuclear factors. Incubation was with 1μl of pTP supernatant, 1μl pol supernatant and either a crude nuclear extract from uninfected HeLa cells (NE) or purified nuclear I and III as indicated.

C. Formation of an initiation complex with 1μl each of Ad supernatant (lane 1), pol (lane 2), pTP (lane 3), pol + pTP (1μl each, lane 4), truncated pol (pol', lane 5), truncated pol + pTP (lane 6). Lanes 7 -11 contain 1, 0.3, 0.1, 0.03, 0.01 μl of supernatant from double infected (pol + pTP) cells.
O-glycosylation cannot be excluded. We analyzed whether the foreign proteins were functional by measuring DNA polymerase activity in crude extracts of cells infected with the two pol recombinant viruses. As shown in table I the pol recombinant overproduced a DNA polymerase activity which was largely aphidicolin resistant in agreement with authentic Ad DNA polymerase (2,24,25) while the truncated pol protein was inactive.

**Adenovirus DNA replication in vitro with recombinant proteins**

Adenovirus DNA replication in vitro is completely dependent upon pol and pTP. We compared crude cytoplasmic or nuclear extracts from adenovirus or recombinant vaccinia virus infected HeLa cells for their ability to sustain replication of the adenovirus genome. Ad5 DNA-terminal protein complex was digested with Xho I and incubated in the presence of purified Ad DNA Binding Protein (DBP) and a crude nuclear extract of uninfected HeLa cells supplying Nuclear Factor I and Nuclear Factor III which are origin-binding proteins required for initiation (26,27,28). As shown in Fig. 3A preferential labeling of the origin containing Xho I B and C fragments occurred while in addition in some cases labeled single stranded B and C fragments are observed, caused by multiple rounds of replication (29,30). High levels of replication were only observed when the extracts from recombinant pTP and pol vaccinia infected cells were mixed (e.g. lane 2) or when an extract from double infected cells was used (lane 11). Tritration of pTP extracts in the presence of excess pol (lanes 3-6) or pol extracts in the presence of excess pTP (lanes 7-10) indicate that neither pol nor pTP alone can sustain a significant level of replication. When comparing the recombinant vaccinia infected extracts with the extracts from the same cells infected with Ad5 (lane 1) it appears that the 100-fold diluted pTP extract (lane 5) is still at least as active as the extract from Adenovirus infected cells. The DNA polymerase extract can be diluted between 10 and 100-fold to reach the same level as Adenovirus infected cell extracts (lanes 9-10). The recombinant pol and pTP were stimulated to the same extent by purified nuclear factors I and III (Fig. 3B) as previously reported for the purified Ad pol-pTP complex (27). The same extracts were also used to study the formation of a pTP-dCMP initiation complex (Fig. 3C). Only the stable 80 kDa pTP form was able to bind dCMP whereas the smaller labile polypeptides were not. Again, initiation required both proteins (lane 4) while the truncated DNA
polymerase was inactive, even in the presence of excess pTP (lanes 5 and 6). Titration of the double infected extracts (lanes 7-11) showed that it was about 30-fold more active than extracts from Ad infected cells (lane 1). These experiments were performed with cytoplasmic extracts, but similar results were obtained with nuclear extracts, which were about half as active (not shown). We conclude that fully functional pTP and pol are produced in the recombinant virus infected cells.

**DISCUSSION**

Several previous attempts to express the pol gene in E.coli (10,11,31) or in vitro coupled transcription/translation system (32) have failed to produce enzymatically active proteins. We suspect that the main reason for this lack of success has been the absence of an essential, 138 amino acids long N-terminal part of the DNA polymerase, since all constructs used the second ATG codon at position 8367. This notion is supported by our results with the truncated DNA polymerase, starting at the same position, which also lacks enzymatic activity when expressed in a recombinant vaccinia virus. Recently evidence was obtained that functionally active DNA polymerase can be produced after transfection of COS cells provided that sequences encoding the exon at coordinate 39 are present (33). A similar result was obtained for pTP (34). The genomic constructs described here are based upon best fit computer analyses and, although the gene products are apparently fully functional, we can not formally exclude that the recombinant proteins differ from the authentic ones. However, we would like to stress that other theoretically possible constructs including the exon at coordinate 39 would use unlikely splice donor sites that are, moreover, not in frame. More importantly, analysis of mRNAs obtained from COS cells expressing functional pTP and pol has shown that they have the same composition as the genomic construct used in our study (M.S. Horwitz and J.A. Engler, personal communication). Therefore the model depicted in Fig. 1A is in all likelihood correct. In Ad infected cells the pTP and pol are present as an equimolar pTP-pol complex. Whether this complex formation requires other (viral) proteins is presently unknown. When the individually expressed recombinant proteins are mixed at 0°C, the activity is comparable to extracts from double infected (pTP+pol) cells. From the functional analysis of pTP and pol in DNA replication we can
only conclude that both proteins are active but we do not know whether a pTP-pol complex is formed upon mixing or whether the two proteins act separately during initiation of DNA replication. Preliminary studies favour the spontaneous formation of a pTP-pol complex at 0°C since upon further purification only one DNA-replication activity peak is observed which coincides with the position of authentic pTP-pol from Ad infected cells (R.J. van Miltenburg and P.C. Van der Vliet, unpublished). The amount of overexpression is difficult to establish exactly but is at least 30-fold based upon the results obtained for initiation (see Fig. 3C). Possibly the expression of pTP is even higher judged from the dilution experiment with pTP extracts (Fig. 3A, lane 5) as well as the presence of an additional free pTP-peak observed upon screening of columns in the presence of pol extract (R.J. Van Miltenburg) and P.C. Van der Vliet; unpublished). The level of expression may depend on the sequence between the promoter and the initiation codon as well as on the infection conditions and we are presently further optimizing the expression level.

The expression-system described here offers several interesting possibilities for further studies of eukaryotic DNA replication. The Ad DNA polymerase is unique among eukaryotic DNA polymerases in that it is capable to translocate through long duplex DNA regions in the absence of ATP hydrolysis. The only additional protein required for this efficient translocation and displacement reaction is the Adenovirus DNA binding protein (35,36). The availability of large amounts of pol protein enables a biochemical study of the possible physical interaction of these two proteins, which has not yet been demonstrated conclusively. In addition, since the Ad DBP has been crystallized (37) structural information about this protein will become available and might be combined with structural information on the pol protein.

Apart from its function in Ad DNA replication, Ad DNA polymerase has also been implicated in cellular transformation based upon the phenotype of the mutant H5ts36. This mutant contains a phenylalanine at position 249 instead of a leucine (38). This mutation is located outside the regions of short homology present between various eukaryotic and prokaryotic viral DNA polymerases (39-41). Mutagenesis could further demonstrate the role of these conserved regions. Also, in addition to direct mutagenesis studies, recombinant vaccinia viruses could provide a helper function to rescue lethal pol or pTP Adenovirus.
mutants constructed in vitro. This approach is presently under investigation.

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