Identification of Two Distinct Regions within the Adenovirus Minimal Origin of Replication That Are Required for Adenovirus Type 4 DNA Replication In Vitro

S. M. TEMPERLEY,^{1†} C. R. BURROW,² T. J. KELLY,² AND R. T. HAY^{1*}

Department of Biochemistry and Microbiology, University of St. Andrews, Irvine Building, North Street, St. Andrews, Fife KY16 9AL, Scotland,¹ and Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205²

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The adenovirus type 4 origins of replication are located at each end of the linear, protein-linked viral DNA molecule and consist of the terminal 18 bp of the viral genome. The sequence of the first 8 bp of the viral genome varies among different adenovirus serotypes, but the sequence from bp 9 to 18 is conserved in all human serotypes, suggesting that it may be of critical importance to origin function. Using an *in vitro* system in which purified fractions or crude extracts of adenovirus type 4-infected HeLa cells can support initiation and elongation on linearized plasmid templates containing cloned origin sequences, we examined the effect of single base changes in positions 9 to 18 of the adenovirus origin on DNA replication *in vitro*. Changes in positions 12 to 16 have little effect, whereas alterations at positions 9, 10, 11, 17, and 18 all reduce the efficiency of initiation of DNA replication by between 50 and 90%. Our results show that the region from bp 9 to 18 contains two sets of bases essential for DNA replication which are separated by 5 bp in which single base changes can be accommodated. The likely role of the region from bp 9 to 18 as containing the recognition sequence for a DNA-protein interaction essential for viral DNA replication is discussed.

The adenovirus genome is a linear double-stranded DNA molecule of 35 to 36 kb with inverted terminal repeats (ITRs) of about 100 bp, the exact size depending on serotype (3). A 55-kDa terminal protein is attached to each 5' end of the genome (34, 36). Replication of the genome is a highly efficient process giving rise to some 10⁵ to 10⁶ progeny molecules 24 h postinfection. DNA synthesis is initiated at either of the termini by transfer of dCMP, the terminal nucleotide, onto an 80-kDa precursor of terminal protein (pTP) in a template-dependent reaction. The 3' OH of the pTP-dCMP complex serves as a primer for synthesis of the nascent strand by the viral DNA polymerase. Concomitant displacement of the nontemplate strand generates a single-stranded molecule (25) which then acts as a template for a second round of DNA synthesis (for recent reviews, see references 7, 19, and 40).

The development of a cell-free system in which DNA synthesis can be initiated *in vitro* by using the viral genome or plasmids containing the origin of replication as template (6) has led to the identification of the sequences important for origin function and the isolation and purification of the proteins required for viral DNA replication. *In vitro* studies on adenovirus types 2 and 5 (Ad2 and Ad5, respectively) have shown that three domains within the terminal 51 bp of the viral genome are important for efficient replication. The terminal 18 bp are absolutely required but in isolation are capable only of supporting very limited initiation (8, 42). Two additional sequences from nucleotides 19 to 39 and from 40 to 51 can each independently contribute to the efficiency of replication. The first contains the recognition sequence for the group of cellular proteins NFI/CTF which, on binding, stimulates the frequency of initiation (11, 13, 26,

29, 33, 39), and the second is the binding site for the cellular factor NFIII/oct-1, which results in a further stimulation (30-32, 37, 45). Ad2 origin requirements have also been defined *in vivo* (20), and deletion analysis has shown that the terminal 45 bp were sufficient for efficient replication (15). In addition, virus genomes containing deletions which removed the NFI/CTF site were noninfectious, whereas genomes containing 45 bp were fully infectious (18). Transfection assays carried out with Ad4, however, showed that in contrast to results with Ad2 and Ad5, only the terminal 18 bp of the viral genome were required for fully efficient replication *in vivo* (16). Similarly, it has been demonstrated by using an Ad4-infected HeLa cell cytoplasmic extract that linearized plasmid containing only the terminal 18 bp of an adenovirus ITR could support initiation of DNA replication *in vitro* as effectively as a template containing a complete Ad4 ITR (14). The proteins required for DNA replication differ markedly between Ad2 and Ad5 and Ad4 in that Ad2 and Ad5 are dependent on the host factors NFI/CTF and NFIII/oct-1 for efficient DNA replication. The precise roles played by these proteins in DNA replication have yet to be elucidated, but in each case the DNA-binding domain of the protein is sufficient to stimulate adenovirus DNA replication (12, 27, 38, 44). Ad4, however, appears to have circumvented the need for auxiliary factors for DNA replication. It does not possess an NFI/CTF recognition site, and while it does have an NFIII/oct-1 binding site, it is not required for origin function and neither factor stimulates Ad4 DNA replication *in vitro* even in the presence of its cognate recognition site (17). It is likely that host factors play a general mechanistic role such as stabilization of a multiprotein complex at the origin, or facilitation of strand opening to allow initiation. It may be that Ad4 is able to replicate its DNA without the help of host factors because its own replication proteins alone can perform these functions.

To investigate the sequence requirements for Ad4 DNA

* Corresponding author.

† Present address: Pharmaceutical Proteins, Edinburgh EH9 3JQ, Scotland.

replication *in vitro*, a series of plasmid templates, each containing the origin of replication with single base changes at position 9 to 18, were assayed for their ability to support replication by crude extracts or a purified fraction from Ad4-infected HeLa cells. Both initiation and elongation assays revealed that the region from bp 9 to 18 contains two regions which are essential for initiation separated by 5 bp in which limited sequence alteration can be tolerated without loss of function. These data are surprising in view of the complete conservation of these sequences among the 41 different human adenovirus serotypes but may be explicable on the basis of a model in which the mutationally sensitive base pairs represent points at which replication proteins approach the DNA template.

MATERIALS AND METHODS

Cells and virus. HeLa S3 Spinner cells were used to prepare virus stock and as a source of virus-infected cell extract. Cells were grown in suspension in Earle's minimal essential medium containing 50 U of penicillin per ml, 50 µg of streptomycin per ml, and 5% newborn calf serum. A549 cells were used to titrate virus by the method of Williams (46). Cells were grown in monolayers in Glasgow modified minimal essential medium containing penicillin, streptomycin, and sodium bicarbonate plus 10% newborn calf serum.

Templates. Plasmid pHR18 contains the terminal 18 bp of the Ad2 ITR, which is identical in Ad2 and Ad4, and has been previously described (15). p4A2 contains the Ad4 ITR and is identical to p4A1 (15). pUDI67 pm9 to 16 (pM9 to pM16) all contain the terminal 67 bp of the Ad5 ITR carrying a single base transition in each base pair from positions 9 to 16 from the terminus. pMDC10 pm17 and 18 (pM17 and pM18) contain the terminal 69 bp of the Ad5 ITR and carry single base transitions at positions 17 and 18 (33). Plasmid DNA was prepared by two rounds of CsCl-ethidium bromide centrifugation. The ethidium bromide was removed by repeated extraction with cesium chloride-saturated isobutanol, and the DNA was desalted by ultrafiltration in a Centri-con-30 apparatus.

Preparation of cell extracts. One liter of HeLa S3 cells was infected with 100 PFU of Ad4 per cell. After 90 min, medium containing 2% calf serum plus 10 mM hydroxyurea was added. Cells were incubated for 22 h at 37°C, and extract was prepared as described previously (6). Extract was depleted of nucleic acids by passage over DEAE-Sephacel in 0.2 M NaCl and concentrated by precipitation with ammonium sulfate as described previously (21). The precipitate was resuspended in 2 ml of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.5)–5 mM KCl–0.5 mM MgCl₂–0.5 mM dithiothreitol (DTT) per 3×10^8 cells, dialyzed against the same buffer, and stored in small aliquots at –70°C. For the preparation of purified fraction (43), crude cytoplasmic extract was prepared from Ad4-infected HeLa S3 Spinner cells as described previously (6). The NaCl concentration was then adjusted to 50 mM prior to being loaded on a DEAE-Sephacel column equilibrated in buffer containing 20 mM HEPES (pH 8.0), 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM DTT, and 50 mM NaCl. The column was washed with 2 volumes of buffer containing 50 mM NaCl and then with 2 volumes of buffer containing 0.2 M NaCl. Fractions were collected and assayed for *in vitro* DNA replication activity, and their protein concentrations were determined (5). Activity was recovered in the 0.2 M eluate. This was applied to a denatured calf thymus DNA-Sephacel column equilibrated in buffer containing 0.2 M NaCl.

The column was washed with 2 volumes of buffer containing 0.2 M NaCl and 5 volumes of a concentration gradient from 0.2 M to 2 M NaCl, collected in 2-ml fractions. The protein concentration of each fraction was determined, and peak fractions were dialyzed against 20 mM HEPES (pH 8.0)–5 mM KCl–0.5 mM MgCl₂–5 mM DTT–1 mM phenylmethylsulfonyl fluoride and stored at –70°C in small volumes.

Assay for the transfer of dCMP to pTP. One to twelve microliters of Ad4-infected HeLa cell cytoplasmic extract or purified replication proteins was incubated in a 30-µl reaction mixture containing 25 mM Tris-HCl (pH 8), 3 mM MgCl₂, 2 mM DTT, 3 mM ATP, and 3 µCi of [α -³²P]dCTP (specific activity, 3,000 Ci/mmol) and various amounts of template DNA for 90 min at 37°C. Reactions were stopped by heating to 70°C for 5 min, and mixtures were treated with 1 U of micrococcal nuclease in the presence of 2 mM CaCl₂ for 30 min at 37°C. Gel loading buffer (12 µl) containing 20% (vol/vol) glycerol, 5% (wt/vol) sodium dodecyl sulfate (SDS), 570 mM 2-mercaptoethanol, 33 mM Tris-HCl (pH 6.7), and 0.2% (wt/vol) bromophenol blue was added, and the samples were denatured by heating to 100°C for 2 min. Reaction products were resolved at 35 mA for 4 h on a 10% SDS-polyacrylamide gel which was dried and then subjected to fluorography at –70°C in the presence of an intensifying screen.

Assay for elongation of DNA initiated *in vitro*. Restriction-enzyme-cleaved template DNA was preincubated in a total volume of 7 µl for 30 min at 30°C with 1 to 3 µl of Ad4-infected HeLa cell cytoplasmic extract or purified viral replication proteins in a buffer containing 25 mM HEPES-KOH (pH 7.5), 4 mM MgCl₂, 1 mM DTT, 0.1 mg of bovine serum albumin (BSA) per ml, and 0.2 mM aphidicolin. Elongation proceeded by addition of 7 µl of a buffer containing 80 µM each of dTTP, dATP, and dGTP; 5 µM dCTP; 0.3 µCi of [α -³²P]dCTP (specific activity, 3,000 Ci/mmol); 4 mM ATP; 10 mM creatine phosphate; 10 µg of creatine phosphokinase per ml; 0.1 mg of BSA per ml; 25 mM HEPES-KOH; 4 mM MgCl₂; and 1 mM DTT to give a total reaction mixture volume of 14 µl. The total reaction mixture was incubated at 30°C for 90 min. Reactions were stopped by the addition of 6 µl of buffer containing 5% SDS, 50% glycerol, 100 mM EDTA, and bromophenol blue and heated at 70°C for 5 min. Reaction products were resolved at 35 mA for 4 h on a 2% agarose gel containing 0.1% SDS, which was fixed in 10% acetic acid, dried, and subjected to fluorography at –70°C in the presence of an intensifying screen.

Quantitation of *in vitro* initiation and elongation data was carried out by excising bands from the gel and measuring the amount of radioactivity incorporated by scintillation counting by using an Intertechnique SL 30 liquid scintillation spectrometer or, for elongation, precipitating nascent DNA incorporating ³²P by adding 5 ml of 10% trichloroacetic acid per reaction mixture, filtering under vacuum through a Whatman GF/C filter, and measuring the acid-precipitable radioactivity by scintillation counting.

RESULTS

Effect of point mutations in the adenovirus origin of replication on initiation of Ad4 DNA replication *in vitro*. Previous studies have demonstrated that the terminal 18 bp of the ITR, which are identical in Ad2, Ad5, and Ad4, are sufficient to serve as an origin for Ad4 DNA replication (14, 16, 17). Within this region, bp 9 to 18 from the terminus are perfectly conserved in all human serotypes so far examined (2, 22),

FIG. 1. Structure of plasmids containing an adenovirus ITR. A single copy of the ITR is inserted between the *EcoRI* and *BamHI* sites. Cleavage with *EcoRI* linearizes the plasmid, exposing the origin sequence at one of the molecular ends. The single point mutations at positions 9 to 18 from the terminus and the plasmid in which they are contained are indicated. The nontemplate strand is shown.

suggesting that the specific sequence in this region plays an important functional role in DNA replication.

To examine the sequence requirement for initiation of DNA replication in Ad4, we determined the consequence of single base changes from positions 9 to 18 on initiation of DNA replication in vitro. The relative efficiency of initiation was assayed quantitatively by measuring the transfer of [32 P]dCMP to pTP, this being the first synthetic step of DNA replication. A series of 10 plasmids, pM9 to pM18, each of which contains an Ad5 ITR with a single base change in positions 9 to 18, were cleaved with *EcoRI* to expose the ITR sequence at a molecular end (Fig. 1). Incubation of the template with an Ad4-infected HeLa cell cytoplasmic extract, [α - 32 P]dCTP, and optimal levels of $MgCl_2$ and ATP results in the transfer of dCMP to pTP. Reaction products were denatured, and the polypeptides were resolved by electrophoresis on an SDS-containing polyacrylamide gel.

Labelled species were detected by autoradiography and excised from the gel, and the radioactivity was determined by liquid scintillation counting.

Plasmid p4A2, which contains one complete copy of an Ad4 ITR, and pHR18, which contains the terminal 18 bp of Ad2 and Ad5 ITRs, both supported initiation with approximately equal efficiency, consistent with previous reports establishing that Ad4 requires only the terminal 18 bp for efficient initiation (Fig. 2A, lanes 1 and 2). Similarly, single base substitutions from positions 12 to 15 from the terminus had no deleterious effect on the efficiency of initiation (Fig. 2A, lanes 6 to 10). However, alterations at positions 9, 10, 11, 17, and 18 all resulted in a reduction in efficiency of initiation by between 50 and 90% (Fig. 2A, lanes 3, 4, 5, 11, and 12).

To test the effect of varying template concentration on initiation of DNA replication in vitro, we incubated increas-

FIG. 2. (A) Initiation with mutated templates. Plasmids (50 ng) containing the Ad4 ITR (p4A2), the terminal 18 bp of the Ad2 ITR (pHR18), and the Ad5 ITR with point mutations in each position from 9 to 18 (pM9 to pM18, respectively) were cleaved with *EcoRI* and incubated with cytoplasmic extract (3 μ g) from Ad4-infected HeLa cells in a standard initiation reaction mixture (see Materials and Methods) containing [α - 32 P]dCTP (3,000 Ci/mmol). After incubation at 32°C for 90 min and micrococcal nuclease digestion, polypeptides were resolved by SDS-PAGE and labelled species were detected by autoradiography. (B) Titration of template. *EcoRI*-cleaved p4A2 and pM18 were supplied to standard initiation reaction mixtures containing 3 μ g of Ad4-infected cell extract and [α - 32 P]dCTP in the quantities shown. Samples were processed for autoradiography, and the region of the gel containing the pTP-dCMP complex was excised and the radioactivity was determined by scintillation counting. The information was then used to quantify incorporated dCMP.

FIG. 3. (A) Elongation of initiated template. Plasmid templates (100 ng) p4A2, pHR18, and pM9 to pM18 were cleaved with *EcoRI* and *AvaII* and incubated with 3 μ g of cytoplasmic extract from Ad4-infected HeLa cells in a standard elongation reaction mixture containing [α - 32 P]dCTP (see Materials and Methods). After incubation at 30°C for 90 min, reaction products were resolved by electrophoresis on a 2% agarose gel containing 0.1% SDS. Labelled species were detected by autoradiography. Nascent DNA incorporates [32 P]dCMP and has pTP attached (black arrowhead) and so is resolved from uninitiated input template (white arrowhead), which is labelled at a low level because of repair synthesis. The 0.9-kb fragment containing only plasmid sequences, under these conditions, ran out of the gel. (B) Titration of template. *EcoRI*- and *AvaII*-cleaved plasmids p4A2 and pM18 were supplied to standard elongation reaction mixtures containing 3 μ g of Ad4-infected cell extract in the amounts shown. Reactions were stopped by addition of 10% trichloroacetic acid and then filtered under vacuum through a glass fiber disc. The acid-precipitable radioactivity was then determined by scintillation counting, and the information was used to calculate the total incorporated nucleotide.

ing concentrations of *EcoRI*-cleaved p4A2 and pM18 as templates in a series of standard initiation reactions and quantitated the amount of dCMP transferred to pTP by excising the bands as described above. The efficiency of initiation on both p4A2 and pM18 increased with concentration to a maximum at 150 ng per reaction mixture, followed by a marked decrease (Fig. 2B). Increasing the concentration of template pM18 could not compensate for the deleterious effect of the mutation.

Elongation of initiated molecules in vitro. To further examine the effect of the single point mutations, we tested the relative efficiency with which wild-type sequences 1 to 18 and plasmids pM9 to pM18 could support elongation from initiated products in vitro. Templates were cleaved with *EcoRI* and *AvaII* to give a 1.6-kb fragment with the origin sequence at the *EcoRI* terminus (Fig. 1) and a 0.9-kb fragment containing only plasmid sequences and incubated with Ad4-infected HeLa cell extract, dNTPs (including [α - 32 P]dCTP), optimal $MgCl_2$, and an ATP regeneration system. Reaction products were fractionated by electrophoresis in an agarose gel containing SDS, and replicated templates which had incorporated [32 P]dCMP were detectable by autoradiography. Correctly initiated products have pTP linked at the 5' end, resulting in a lower electrophoretic mobility (Fig. 3A, black arrowhead) than input template molecules which incorporated ^{32}P at a low level as a result of repair synthesis (Fig. 3A, white arrowhead). p4A2 and pHR18 both supported elongation with equal efficiency, as did pM12 to pM16 (Fig. 3A, lanes 1, 2, and 6 to 10). pM9, -10, -11, -17, and -18, however, all replicated at a reduced efficiency, corresponding to the observed reduction for initiation (Fig. 3A, lanes 3, 4, 5, 11, and 12).

To determine the effect of template concentration on elon-

gation, we incubated increasing concentrations of *EcoRI*- and *AvaII*-cleaved p4A2 and pM18 under standard elongation conditions. Both templates gave an increase in elongation levels in response to template concentration being increased to a maximum of 100 ng. Above this level, a marked decrease in the total amount of elongated product was observed, particularly with p4A2 (Fig. 3B). As was the case for initiation, the reduced level of elongated product obtained with the mutant template could not be compensated for by increasing the concentration of template added to the reaction.

Initiation and elongation in the presence of $MnCl_2$. It has been previously observed that initiation of replication can proceed in the absence of $MgCl_2$ if manganese chloride is provided (13a) and, furthermore, that a marked stimulation of initiation is obtained in the presence of $MnCl_2$. A stimulatory effect of $MnCl_2$ has also been observed for certain DNA-modifying enzymes, although this is often accompanied by a decrease in substrate specificity. To accurately determine the optimal $MnCl_2$ concentration for Ad4 DNA replication in vitro, we used a $MgCl_2$ -free cytoplasmic extract from Ad4-infected HeLa cells incubated with *EcoRI*- and *AvaII*-cleaved p4A2 and assayed for initiation and elongation at various $MnCl_2$ concentrations, holding the ATP concentration constant. A similar titration with $MgCl_2$ was carried out for comparison (Fig. 4A). $MgCl_2$ had to be present at 2 mM before any detectable level of initiation was attained. When the concentration of $MgCl_2$ was increased to 3 mM, an optimal level of initiation was attained; above 3 mM, a gradual decrease in initiation was seen. In contrast, the maximum level of initiation in the presence of $MnCl_2$ was attained at 0.75 mM and was nearly threefold higher than at the optimal $MgCl_2$ concentration. When the concentration of

FIG. 4. (A) Initiation in the presence of MnCl_2 . *Eco*RI-cleaved p4A2 (50 ng) was incubated under standard initiation conditions with 3 μg of Ad4-infected cell extract in MgCl_2 -free buffer in the presence of [^{32}P]dCTP and a series of increasing MnCl_2 concentrations. A separate set of reactions containing a series of increasing MgCl_2 concentrations was also carried out for comparison. In all reactions, the ATP concentration was held constant at 3 mM. Reaction mixtures were processed for autoradiography, and the region of the gel containing the pTP-dCMP complex was excised from the gel and the radioactivity was determined by scintillation counting. This information was then used to quantify the transfer of dCMP to pTP. (B) Initiation with mutant templates in the presence of MnCl_2 . *Eco*RI-cleaved pHR18, p4A2, and pM9 to pM18 (50 ng each) were used as templates for initiation under MgCl_2 -free conditions in the presence of 0.75 mM MnCl_2 . All other conditions were standard.

MnCl_2 was increased above 0.75 mM, a rapid decrease in the level of initiation was observed.

To test whether the requirement for sequence specificity of the template was maintained in the presence of MnCl_2 , we incubated 50 ng of *Eco*RI-cleaved p4A2, pHR18, and pM9 to pM18 under standard initiation conditions with 0.75 mM MnCl_2 . p4A2 and pHR18 both supported initiation efficiently, as did pM12 to pM16 (Fig. 4B, compare lanes 1, 2, and 6 to 10). pM9, -10, -11, -17, and -18 all gave reduced levels of initiation, which corresponded to the reduction in efficiency obtained in the presence of an optimal MgCl_2 concentration. These data demonstrate that initiation of DNA replication *in vitro* in the presence of MnCl_2 was as sensitive to changes in the template sequence as in the presence of MgCl_2 .

Initiation and elongation by a purified fraction of Ad4-infected cell extract. Although it may be desirable to employ a crude cell extract for initial replication studies, as all the components required for replication should be present, it is also conceivable that contaminating activities may complicate the interpretation of data. For instance, it has been shown that partially single-stranded templates generated as a result of exonuclease action behave differently than double-stranded templates (24). Additionally, the presence of cellular DNA polymerases in a crude extract could potentially interfere with the levels of DNA replication observed. To exclude these possibilities, a fraction of Ad4-infected HeLa extract which was shown to contain four protein species detectable by SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining was purified. The fraction was used for *in vitro* DNA replication and contained pTP, polymerase, and DNA-binding protein, the latter representing 95% of the total protein (43).

Standard initiation reactions in which crude Ad4-infected extract was replaced with the purified fraction by using p4A2 and pM9 to pM18 as templates were carried out. Initiation was supported by p4A2 and pM12 to pM16 with equal

efficiency (Fig. 5A, lanes 1, 5, 6, 7, 8, and 9). pM9, -10, -11, -17, and -18 all gave very-much-reduced levels of initiation (Fig. 5A, lanes 2, 3, 4, 10, and 11) which corresponded to those obtained when a crude extract was used for initiation.

To test the ability of the purified fraction to elongate initiated molecules, we incubated *Eco*RI- and *Ava*II-cleaved p4A2 and pM18 with the purified fraction under standard elongation conditions (Fig. 5B). A greatly reduced amount of elongated product was obtained when pM18 was used as template, consistent with its compromised ability to support initiation in the crude extracts. These data indicated that the potential effects of contaminating factors in the infected crude extract were negligible.

Mutations which were defective in initiation were found to be similarly defective in elongation (Fig. 6). These data indicate that, as predicted, the conserved sequence from bp 9 to 18 in the adenovirus ITR plays a vital functional role in viral DNA replication even though certain limited sequence changes appear to be tolerable for *in vitro* replication. The corresponding decrease in the efficiency of elongation which is observed for mutants exhibiting impaired initiation suggests that it is only initiation of DNA replication that is being affected by changes in the origin sequence and that elongation can proceed unimpaired from those molecules which have initiated.

DISCUSSION

Much of the information which has led to our current understanding of the mechanism of adenovirus DNA replication has been derived from work carried out with Ad2 and Ad5. Ad2 and Ad5 rely on the interaction of host factors NF1/CTF and NFIII/oct-1, with their respective binding sites at the origin of replication for efficient DNA replication. Ad4 has apparently dispensed with the need to employ these host DNA-binding proteins in the replication of its DNA and as a result has a remarkably simple origin construction which

FIG. 5. (A) Initiation with a purified fraction from Ad4-infected HeLa cytoplasmic extract. *Eco*RI-cleaved templates pHR18, p4A2, and pM9 to pM18 (50 ng each) were incubated under standard initiation conditions with 3 μ g of protein and [α - 32 P]dCTP. The templates used for each reaction are indicated. (B) Elongation with a purified fraction from Ad4-infected HeLa cytoplasmic extract. *Eco*RI- and *Ava*II-cleaved templates p4A2 and pM18 (100 ng each) were incubated under standard elongation conditions with a purified fraction of Ad4-infected cell extract. Reaction products were resolved by electrophoresis on a 2% agarose gel containing 0.1% SDS, and labelled species were detected by autoradiography. The positions of correctly initiated (black arrowhead) and input molecules (white arrowhead) are indicated.

potentially makes it an ideal system for studying the mechanism of adenovirus DNA replication. The data presented in this article identify DNA sequences within the conserved domain of the minimal origin of replication between bp 9 and 18 that are essential for Ad4 DNA replication. Two critical regions separated by a region in which single base changes could be tolerated were defined (Fig. 6); the latter spans bases 12 to 16, and any single base transition in this region had no effect on DNA replication and could support a level of initiation comparable to the wild-type sequence. Any base change in the two critical flanking regions had a serious deleterious effect on DNA replication in vitro. The sequence between bp 9 and 18 may well play an important part in

FIG. 6. The relative activities of each template shown as a percentage of wild-type activity by initiation assay and elongation assay. The data were obtained by excising the part of the gel containing the pTP-dCMP complex and measuring the radioactivity by scintillation counting. The wild-type sequence of the nontemplate strand between bases 9 and 18 is shown beneath the graph. The bases which are critical for initiation of DNA replication are in uppercase and those which can be altered without an effect on DNA replication in vitro are in lowercase.

recognition by and positioning of the replication proteins at the origin prior to initiation of DNA replication. By employing an indirect binding assay, it has been suggested that the pTP/polymerase heterodimer can recognize the region between bp 1 and 18 (35), but confirmation by direct binding studies has been inconclusive (1, 29). Clearly, a route by which pTP and/or polymerase is able to specifically recognize a specific sequence or sequences in the origin would be the simplest way of positioning the replication apparatus on the template. Recent evidence demonstrating a direct interaction between polymerase and NFI suggests that Ad2 and Ad5 utilize the binding of NFI/CTF adjacent to the origin to stabilize a preinitiation complex between the viral replication proteins and the template prior to initiation (4, 9, 28). The importance of precise spacing between these functionally important sequences has been demonstrated by the observation that mutations at either position 19 or 20 do not affect levels of Ad5 DNA replication in vitro but that an insertion of more than 2 bp at this position which moves the NFI site relative to the 1- to 18-bp sequence reduces the efficiency of initiation in vitro (1, 45) and in vivo (4). The affinity of NFI for its binding site is in turn further increased by the presence of viral DNA-binding protein, although it has not been demonstrated that there is any direct protein-protein interaction involved in this functional cooperation (10, 41). It has been demonstrated that mutations in positions 17 and 18, while being adjacent to the NFI/CTF binding site, do not affect the binding of NFI (37). Because mutations in either of these positions result in a substantial drop in the level of DNA replication in vitro in Ad2 and Ad5 (33) and Ad4, which has no requirement for NFI/CTF, it is apparent that a decrease in NFI/CTF binding is not responsible for the observed effect on DNA replication in Ad2 and Ad5. However, the three-domain structure of the 9- to 18-bp region suggests that it may be part of a protein recognition site since the boundaries of the two domains in which mutations have the profoundest effect on DNA replication lie approximately one turn of the helix apart and thus could be contact points in a DNA-protein interaction on one side of the DNA helix. A recent observation shows that the Ad2 pTP/polymerase heterodimer recognizes and binds to a sequence in the terminal 18 bp (43a). Each protein in the heterodimer may

contact distinct points on the template such that a mutation in one site would reduce the stability of the complex formed between the template and the heterodimer, thereby reducing the efficiency of initiation. Our data are consistent with such a model since a single mutation in one of the regions, which results in a drop in the efficiency of initiation, fails to abolish initiation completely, suggesting that while one protein is able to bind to the template, a preinitiation complex still forms by virtue of the protein-protein interaction, albeit with a reduced stability. Clearly, analysis with templates with mutations in both sites will be required to test this hypothesis.

Although single point mutations in positions 12 to 16 do not affect Ad4 DNA replication *in vitro*, it is likely that this region plays an essential role in maintaining the conformation of the DNA. It has been shown that a template carrying a double mutation at positions 13 and 14 gave a marked reduction in initiation of Ad5 DNA replication (42). The double mutation, unlike a single base change, may prevent effective interaction of replication proteins with the origin or alternatively may alter the conformation of the origin template such that the concerted structural changes that are essential for origin function do not take place. The length of this region may also be critical if it serves to separate two regions where interactions with proteins occur. Clearly, a functional analysis with mutants with insertions at positions 12 to 16 will be required to comprehensively characterize this region.

The template titration data demonstrated that the dramatic drop in the level of initiation seen with mutant template pM18 could not be compensated for by increasing the template concentration, suggesting that the mutation may be exerting its effect by way of severely destabilizing an interaction between replication proteins and the origin of replication. Our data clearly show that mutations at positions 9, 10, 11, 17, and 18, all of which reduce the efficiency of DNA replication *in vitro*, affect only initiation. If this inhibition is due to an effect on a DNA-protein interaction which is in turn critical for initiation of replication, then clearly a way of directly assaying for binding of these proteins to the mutant templates will be necessary. Some evidence does exist from comparison of various mutant oligonucleotides that formation of a complex on an ITR sequence roughly correlates to the ability of the same oligonucleotides to support initiation *in vitro* (24). It should be noted that the data presented in this study have all been obtained by using plasmids as templates for *in vitro* DNA replication. It has been previously observed that viral DNA with terminal protein attached (DNA-protein complex) serves as a more efficient template than plasmid for Ad4 DNA replication *in vitro* (14). A possible explanation for this is that terminal protein plays an important part in the positioning of the replication apparatus in relation to the template prior to initiation. It has also been demonstrated with Ad2 and Ad5 that the presence of a host protein, pL, which has 5'-to-3' exonuclease activity, is required to expose a single-stranded region at the 3' end of the template strand as a prerequisite for DNA replication on a plasmid template (13, 23). Under reaction conditions similar to those used for *in vitro* DNA replication, we observed no decrease in the specific activity of ³²P 5' end-labelled origin DNA incubated with Ad4-purified fraction, indicating that there was no significant 5'-to-3' exonuclease activity present. It was therefore concluded that a 5'-to-3' exonuclease activity is not important for Ad4 DNA replication on plasmid templates *in vitro*.

We have been able to demonstrate that a purified fraction

from Ad4-infected HeLa cells containing four major species has all the components required for replication of Ad4 DNA and has the same template recognition characteristics as crude extract. Further purification and characterization of these components should lead to a better understanding of the mechanisms underlying adenovirus DNA replication.

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