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Adenovirus DNA Replication

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The adenovirus genome can be replicated in vitro by the combined action of three viral proteins, two cellular transcription factors, and topoisomerase I (Table 1). Early viral transcription results in expression of three viral genes, preterminal protein (pTP), DNA polymerase (Ad pol), and DNA-binding protein (DBP), that genetic evidence indicates are required for viral DNA replication. With the development of in vitro systems (Challberg and Kelly 1979), it was soon shown that cellular factors were required in addition to the three viral proteins. One of these proteins could be replaced by calf thymus topoisomerase and was required for complete elongation of the genome (Nagata et al. 1982). In contrast, the other factors were both sequence-specific DNA-binding proteins that recognized DNA within the inverted terminal repeats (ITRs) of the adenovirus genome (Nagata et al. 1983; Pruijn et al. 1986). Because all of these proteins have been cloned, overexpressed, and purified, the adenovirus genome represents one of the few eukaryotic systems in which all the components for DNA replication have been precisely defined.

Within virions, the adenovirus genome is maintained in a highly condensed form by small basic proteins, but after infection of susceptible cells, the virion is uncoated and viral DNA is released into the cell nucleus. Adenovirus genomes are linear double-stranded DNA molecules of 35–36 kb with ITRs of about 100 bp, the exact size depending on serotype. DNA replication initiates at either end of the linear 36-kbp viral genome by a protein-priming mechanism in which a covalent linkage is formed between the α-phosphoryl group of the terminal residue, dCMP, and the β-OH group of a serine residue in pTP. This reaction is catalyzed by Ad pol, which then utilizes the 3′-OH group of the pTP/dCMP complex as a primer for synthesis of the nascent strand. DNA synthesis is accompanied by displacement of the non-template strand, which can form a partial duplex by base-pairing of the ITRs on which a second round of DNA synthesis may be initiated (Hay et al. 1984; Stow 1982). Alterna-

DNA Replication in Eukaryotic Cells

Table 1 Properties of proteins involved in Ad DNA replication

Protein	Size (kD)	Domain required for DNA replication	Interacting protein(s)	Activities
Ad pol	140	all	Ad pTP, NFI, NFIII	DNA polymerase 3′→5′ proofreading exonuclease; as pTP/pol binds to the core of the origin; transfers dCMP onto pTP during initiation of DNA replication; catalyzes strand-displacement synthesis during elongation phase of DNA replication
Ad pTP	80	all	Ad pol	protein primer for initiation of DNA replication; as pTP/pol binds to the core of the origin; substrate for the adenovirus protease
DBP	59	DNA-binding domain aa 174–529	—	enhances initiation and elongation during DNA replication; binds single- and double-stranded DNA; stimulates binding of NFI to origin; reduces K_m for dCTP; increases processivity of Ad pol; acts as a helix-destabilizing protein
NFI/CTFI	55	DNA-binding domain aa 1–220	Ad pol	binding to auxiliary origin enhanced by DBP; enhances initiation of DNA replication
NFII	30	all	—	topoisomerase I; required for synthesis of genome-length molecules
NFIII/Oct-1	90	DNA-binding domain aa 270–440	Ad pol	binds to auxiliary origin; enhances initiation of DNA replication

tively, displaced complementary strands may simply reanneal to form double-stranded products. This proposal is based on the in vitro properties of DBP, which promotes intermolecular reannealing but inhibits intramolecular reannealing (Zijderveld et al. 1993). The accepted model, based on analysis of replication intermediates, for adenovirus DNA synthesis is discussed by Brush and Kelly (this volume).

The subject of adenovirus DNA replication has been reviewed extensively (Hay and Russell 1989; Stillman 1989; van der Vliet 1990; Salas 1991), but new developments in the field warrant further evaluation. In this chapter, I describe the components of the adenovirus replicon and analyze how these components assemble into a nucleoprotein complex that precedes initiation of viral DNA replication.

ADENOVIRUS ORIGINS OF DNA REPLICATION

The *cis*-acting DNA sequences that define *ori*, the origin of DNA replication, are located at the ends of the genome within the ITRs. Covalently bound to each 5′ end of the DNA is a terminal protein (TP), which may well be an additional *cis*-acting component of *ori*. Comparisons of *ori*s from a wide range of adenoviruses and extensive mutational analysis of *ori* DNA have defined four regions within the terminal 50 bp of the Ad2 genome that contribute to *ori* activity in vitro and in vivo. As with many other *ori*s, that of Ad2 consists of an essential core region and auxiliary regions that enhance the efficiency of DNA replication (Fig. 1). The terminal 18 bp of the viral genome represents the minimal replication origin and contains a 10-bp region (bp 9–18, Fig. 1) conserved in all human adenoviruses (Stillman et al. 1982). Although the integrity of this region is required for *ori* function, in isolation it supports only a limited, basal level of initiation of DNA replication (Tamanoi and Stillman 1983; Van Bergen et al. 1983; Challberg and Rawlins 1984; Guggenheimer et al. 1984; Lally et al. 1984; Hay 1985a; Wides et al. 1987). Separated from the minimal *ori* by a spacer region of defined length (Adyha et al. 1986; Wides et al. 1987; Bosher et al. 1990) is the DNA-binding site of the cellular protein nuclear factor I (NFI) or CAAT transcription factor (Nagata et al. 1982; Jones et al. 1987). Distal to the NFI-binding site is the DNA recognition site for another cellular protein, octamer-binding protein (Oct-1) or nuclear factor III (NFIII) (Pruijn et al. 1986; Rosenfeld et al. 1987; Wides et al. 1987; O'Neill and Kelly 1988; Sturm et al. 1988). In contrast to Ad2, Ad4 requires only the terminal 18 bp of the viral genome to efficiently initiate DNA replication in vivo (Hay 1985b) and in vitro (Harris and Hay 1988; Temperley and Hay

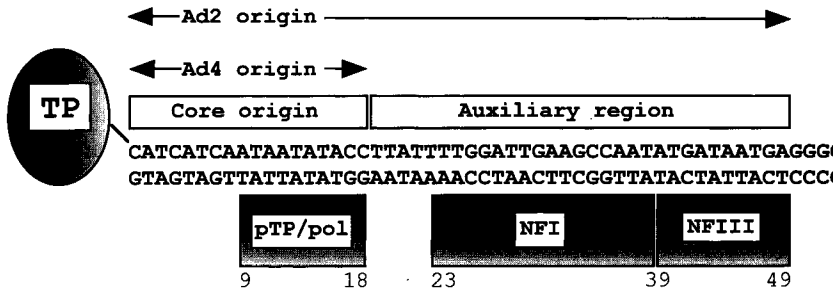

Figure 1 Structure and organization of the Ad2 and Ad4 origins of DNA replication. The binding sites for pTP/Ad pol, NFI, and NFIII/Oct-1 are indicated.

1991; Temperley et al. 1991). Ad4 therefore appears to have circumvented the need for the host factors NFI and NFIII/Oct-1. The ITR does not contain an NFI recognition site, and although it does have a binding site for NFIII/Oct-1, neither factor is required for DNA replication in vivo or is capable of stimulating DNA replication in vitro (Hay et al. 1988). The reasons for the different requirements of Ad2 and Ad4 DNA replication are not obvious, and DNA-binding assays have indicated that the affinity of baculovirus-expressed Ad4 pTP/Ad pol for the core origin is not substantially different from that of the Ad2 pTP/Ad pol (I.R. Leith and R.T. Hay, unpubl.)

ROLE OF pTP, TP, AND PROTEASE IN THE REPLICATION OF VIRAL DNA

Although mature adenovirus DNA contains a 55-kD protein covalently attached to both 5′termini (TP), early in vitro studies indicated the presence of an 80-kD precursor terminal protein (pTP) covalently attached to 5′ends of nascent DNA (Challberg et al. 1980). Ad2 pTP is cleaved to TP via an intermediate (iTP), and because the cleavage sites are conserved in all sequenced pTPs, it is likely that cleavage of pTP to TP, via iTP, plays an important role in the infectious cycle (Webster et al. 1989). Analysis of an Ad2 mutant (H2ts1) with a temperature-sensitive protease (Ad protease) that replicates efficiently at the restrictive temperature, but whose progeny virions are not infectious, revealed that in contrast to a wild-type virus infection, pTP was not processed to TP (Stillman et al. 1981). Thus, pTP is a substrate for Ad protease, which is essential for viral infectivity and accumulates to high levels late in the infectious cycle. Site-directed mutagenesis and sequence alignments of viral proteases indicate that the enzyme may represent a new subclass of

cysteine proteases (Rancourt et al. 1994). Ad protease is synthesized in an inactive form but is activated by a disulfide-linked peptide (Mangel et al. 1993; Webster et al. 1993) derived from the carboxyl terminus of the pVI virion protein (Webster et al. 1993). Although it was proposed that DNA was a cofactor in the protease reaction (Mangel et al. 1993), subsequent studies demonstrated that, although DNA can stimulate protease activity under certain defined conditions, it is not required for protease activity (Webster et al. 1993, 1994).

In adenovirus-infected cells, pTP exists in the form of a heterodimer in which it is tightly bound to Ad pol (Lichy et al. 1982; Stillman et al. 1982). Work on pTP has been facilitated by cloning of cDNA copies of the gene (Pettit et al. 1988) into vaccinia virus (Stunnenberg et al. 1988) or baculovirus (Bosher et al. 1990) for high-level expression in human or insect cells. Although a variety of approaches have been employed to define functional domains in pTP that are required for viral DNA replication, it has not proved possible to ascribe specific activities to domains within the protein. Genetic and biochemical experiments have indicated that the amino-terminal region, removed during processing by Ad protease, is required for specific DNA binding, interaction with Ad pol, and initiation of DNA replication (Freimuth and Ginsberg 1986; Pettit et al. 1989; Fredman et al. 1991; Roovers et al. 1993; Webster et al. 1994). The sequence-specific DNA-binding activity of pTP that allows it to recognize the origin of DNA replication (Temperley and Hay 1992) may be a target for cellular regulatory mechanisms, since it has been demonstrated that origin binding by pTP is dependent on the phosphorylation state of the protein (Kusukawa et al. 1994).

In considering the role of the pTP processing by Ad protease in DNA replication, free pTP and pTP/TP that is covalently attached to the 5′ end of DNA should be regarded as distinct functional entities. Whereas the role of free pTP is to act as the protein primer for DNA replication, the significance of the covalently attached pTP/TP remains to be fully elucidated. However, it has been known for some time that in transfection experiments, the infectivity of TP-DNA is orders of magnitude greater than that of naked DNA. Genome-bound pTP/TP may protect viral DNA from exonucleases, allow attachment of virus DNA to the nuclear matrix (Schaack et al. 1990), and participate in unwinding of the DNA duplex at the origin of replication. In addition, genome-bound TP serves to stabilize binding of the incoming pTP-pol heterodimer at the origin of DNA replication (Pronk and van der Vliet 1993) and induces a conformational change in origin DNA. However, the relationship between these two phenomena has yet to be established. It should be em-

phasized that in vivo the template for early transcription and the first round of DNA replication is TP-DNA, whereas the template for subsequent rounds of replication is pTP-DNA irrespective of whether infections are carried out with wild-type Ad2 or Ad2ts1 at the permissive or nonpermissive temperatures. It has been suggested that cleavage of pTP-DNA to TP-DNA is required to free TP-DNA from the nuclear matrix prior to assembly of virus particles (Fredman and Engler 1993). However, because pTP-DNA is packaged within virus particles during Ad2ts1 infections at nonpermissive temperatures, this would appear less likely (Weber 1990). It cannot be ruled out, however, that the nuclear matrix-binding properties of TP-DNA and pTP-DNA are distinct and that this has some bearing on the transcription/replication sites of viral DNA. Another possibility is that processing of pTP-DNA to TP-DNA is used to create different templates for either early transcription or the first round of DNA replication. Since pTP binds to DNA and Ad pol, and TP does not (Webster et al. 1994), it would not be surprising to find differences between the properties of TP-DNA and pTP-DNA as templates for DNA replication.

ADENOVIRUS DNA POLYMERASE

Purification of an activity required for viral replication from an Ad2-infected cytosolic extract yielded two polypeptide species: the previously described pTP and a 140-kD protein with a unique DNA polymerase activity (Enomoto et al. 1981). Complementation experiments with temperature-sensitive mutants of Ad5 (group N mutants) defective in both the initiation and elongation of DNA replication in vivo demonstrated the viral origin of the DNA polymerase (Stillman et al. 1982; Van Bergen et al. 1983). Extracts prepared from nuclei of cells infected with these mutant viruses were unable to support initiation of DNA replication, but activity was restored by addition of Ad pol (Stillman et al. 1982). More recent experiments (Chen et al. 1994) have defined the biochemical defects associated with the temperature-sensitive phenotype. Insertion of pol cDNAs (Shu et al. 1987) into recombinant vaccinia virus (Stunnenberg et al. 1988) and baculovirus (Watson and Hay 1990) has allowed high-level expression of the protein in human and insect cells.

Recognizable in Ad pol are the conserved domains that are characteristic of DNA polymerases from across the evolutionary spectrum (Coen, this volume) and like many DNA polymerases, Ad pol also possesses an intrinsic 3′→5′ proofreading exonuclease activity. However, Ad pol

also appears to contain additional domains that may contribute to the unique properties of the protein. pTP, NFI, and NFIII all independently contact Ad pol in the preinitiation complex. Although it is likely that these proteins bind to spatially distinct domains on the surface of Ad pol, their precise location has yet to be determined. In addition, two potential zinc-binding motifs have been identified which mutational analyses have indicated are important for DNA binding and initiation of DNA replication (Joung and Engler 1992). DBP was shown to have a profound effect on both DNA polymerase and exonuclease activities of Ad pol (Field et al. 1984). DNA synthesis on poly(dT):oligo(rA) was stimulated 10- to 100-fold by the presence of DBP due to an increase in processivity of Ad pol. Other single-stranded binding proteins (SSBs), such as *Escherichia coli* SSB, were unable to substitute for Ad DBP. Likewise, Ad DBP could not substitute for human SSB in stimulating the activity of human DNA polymerase-α. It is thought that DBP may act by stabilizing the interaction between Ad pol and the DNA template. In contrast to DNA polymerase activity, the proofreading exonuclease activity of Ad pol is inhibited by DBP. Inhibition by DBP is a direct effect on the exonuclease activity of Ad pol rather than binding to and protecting DNA ends (Lindenbaum et al. 1986). Although DBP does increase the thermostability of Ad pol (Lindenbaum et al. 1986), it has not yet been possible to demonstrate a direct physical association between the two proteins.

Intriguingly, a role for phosphorylation in the activity of Ad pol has been suggested (Ramachandra et al. 1993) by the observation that Ad pol is phosphorylated on S67 by a stably associated "cdc2-like" histone H1 kinase (Ramachandra and Padmanabhan 1993). Dephosphorylation of the protein altered its ability to participate in the initiation reaction.

ADENOVIRUS DNA-BINDING PROTEIN

The large amount of DBP that accumulates during the adenovirus infectious cycle (up to 5×10^6 molecules per cell) was responsible for its being the first of the viral nonstructural proteins to be identified and subsequently purified (van der Vliet and Levine 1973). An essential role of DBP in viral infectivity was demonstrated genetically by isolation of temperature-sensitive mutations H5ts125 and H5ts107 in the DBP gene. Nuclear extracts from both of these mutants are defective for full-length Ad DNA replication on both exogenous and endogenous templates (Friefeld et al. 1983). In both cases, activity is restored by the addition of purified wild-type DBP. DBP displays multiple biochemical activities that are required for efficient viral DNA synthesis (Brough et al. 1993)

and can be found in a large protein complex associated with cellular proteins (Ricigliano et al. 1994). However, the functional significance of this interaction has yet to be determined.

Although the role of DBP in the elongation phase of DNA replication, both in vivo and in vitro, has been well documented, its specific role in the initiation of Ad DNA replication has been more contentious. However, studies using purified components reveal a stimulatory effect on the level of initiation when DBP is present (Kenny and Hurwitz 1988; Cleat and Hay 1989; Mul and van der Vliet 1993). This may be a consequence of a functional interaction between DBP and NFI. The ability of NFI to stimulate initiation in vitro is dependent on the concentration of DBP (De Vries et al. 1985). DBP increases the affinity of NFI for its recognition site in the adenovirus origin of DNA replication (Cleat and Hay 1989; Stuiver and van der Vliet 1990). Because a direct interaction between the two proteins has not been detected, the observed effect is likely a consequence of DBP's ability to alter the structure of bound DNA. In fact, DBP can remove the tertiary structure of double-stranded DNA fragments upon binding (Stuiver et al. 1992). It therefore seems likely that in the DBP/DNA complex, the relative positions of hydrogen-bond donor and acceptor groups in the major grooves are altered, causing a fine-tuning of the contacts between NFI and DNA that leads to a higher-affinity interaction. DBP can also stimulate initiation of DNA replication by decreasing the K_m of the polymerase for the initiator nucleotide dCTP (Mul and van der Vliet 1993).

Partial chymotryptic digestion of purified DBP yields a carboxy-terminal fragment of around 40 kD and a highly phosphorylated 27-kD amino-terminal fragment (Klein et al. 1979; Cleghon et al. 1993). The amino-terminal fragment appears to be dispensable for DNA replication, because the carboxy-terminal domain of the molecule can fully substitute for the intact molecule during Ad DNA replication in vitro. Within the carboxy-terminal region are three highly conserved motifs (Kitchingman et al. 1985; Vos et al. 1988; Eagle and Klessig 1992) that mutational studies have identified as important for binding to single-stranded DNA (Neale and Kitchingman 1990). Photocrosslinking has revealed that the two residues, Met-299 and Phe-418 in the carboxy-terminal domain, play an important part in DBP's ability to bind single-stranded DNA (Cleghon and Klessig 1992). Determination of the three-dimensional structure of the carboxy-terminal DNA-binding domain of DBP has revealed that the conserved motifs described above are important elements in the architecture of the DBP molecule. Two zinc atoms stabilize the overall folding of the molecule, and although structural data are not yet available for the

DNA/protein complex, a positively charged region incorporating the cross-linked F418 has been suggested as the surface that mediates interactions with DNA (Tucker et al. 1994). Biochemical experiments have implicated the carboxy-terminal region of DBP as important for cooperative binding to DNA, and this is confirmed in the crystal structure. A carboxy-terminal extension of 17 amino acids from one molecule "hooks onto" a second molecule, thus forming a protein chain around which a DNA chain is proposed to wind (Tucker et al. 1994).

Ad DBP has been shown to possess the properties of a helix-destabilizing protein (Monaghan et al. 1994; Zijderveld and van der Vliet 1994). On DNA templates containing a large proportion of single-stranded DNA, the double-stranded portion is efficiently unwound in a highly cooperative reaction. Entirely double-stranded templates are also unwound, but this reaction is restricted by the length and G+C content of the DNA fragment (Monaghan et al. 1994). Like other helix-destabilizing proteins, DBP-catalyzed DNA-unwinding requires neither ATP nor $MgCl_2$. In fact, the latter is inhibitory to the process, as are other agents, such as NaCl, which increase the stability of duplex DNA molecules. These properties are very similar to those of the calf thymus and herpes simplex virus coded single-strand-specific DNA-binding proteins that appear to be involved in cellular and viral DNA replication (Georgaki et al. 1992; Boehmer and Lehman 1993; Georgaki and Hübscher 1993). Two alternatives could explain the ability of DBP to unwind completely double-stranded DNA. DBP could first bind to double-stranded DNA (Cleat and Hay 1989; Stuiver and van der Vliet 1990) then invade the DNA duplex and bind to the exposed single strands in a stable fashion. Alternatively, DBP could bind transiently to single-stranded regions of DNA exposed during "breathing" of short double-stranded DNA molecules. However, when double-stranded DNA fragments are tightly bound by either NFI or NFIII/Oct-1 at their cognate recognition sites, DBP is unable to unwind the DNA. This could be because the bound proteins stabilize the DNA duplex, or the bound proteins may interfere with the ability of DBP to form a continuous protein chain on the DNA. Unlike DNA helicases that translocate unidirectionally on DNA, there does not appear to be a strict directionality to the unwinding reaction catalyzed by DBP. The ability of DBP to impose a rigid structure on DNA is responsible for the ability of DBP to inhibit intramolecular renaturation but promote intermolecular renaturation (Zijderveld et al. 1993). The denaturation reaction described above is inhibited by high concentrations of monovalent and divalent cations, but the renaturation reaction is highly resistant to their presence (Zijderveld et al. 1993).

The ability of DBP to destabilize double-stranded DNA duplexes could be utilized at a number of points in viral DNA replication. As discussed above, DBP stimulates the initiation of Ad2 DNA replication by decreasing the K_m for transfer of dCMP onto pTP and by increasing the binding of NFI to the replication origin. However, it is likely that neither of these reactions involves melting duplex DNA, and DBP may stimulate initiation of DNA replication by additional activities. Ad4 DBP dramatically stimulates initiation of DNA replication, but in this case, the extent of stimulation is independent of the concentrations of NFI and dCTP (Temperley and Hay 1991). One possibility is that DBP may participate with other replication proteins in unwinding the DNA double helix at the termini of the genome prior to initiation. TP (Pronk et al. 1992; Pronk and van der Vliet 1993), NFI (De Vries et al. 1987; Mul and van der Vliet 1992), and NFIII/Oct-1 (Verrijzer et al. 1991) can all modify the structure of viral DNA. In combination with the ability of DBP to distort and unwind short, fully duplex DNA, this property could allow adenovirus replication proteins to destabilize and unwind *ori* DNA. The role of DBP in elongation is likely to be a consequence of DBP's stabilizing displaced single strands and altering the previously described properties of the Ad pol.

ROLE OF CELLULAR PROTEINS INVOLVED IN AD DNA REPLICATION

Limited initiation of DNA replication in vitro occurs in the presence of virally encoded pTP/Ad pol and *ori*. However, addition of a crude nuclear extract of uninfected HeLa stimulated initiation activity. This stimulatory effect was due to the presence of two cellular transcription factors NFI and NFIII/Oct-1. Since these factors are also discussed by van der Vliet (this volume), only their relevance to the formation of an Ad preinitiation complex is discussed here.

NFI

Uninfected HeLa cell nuclear extract can stimulate the efficiency of initiation and elongation obtained in vitro with infected cytosol or purified pTP/pol heterodimer and DBP (Ikeda et al. 1982; Lichy et al. 1982). This stimulatory activity, purified from the nuclear extract, contained a single major protein species with a size of 47 kD and was termed NFI (Nagata et al. 1982). Initial characterization of NFI concentrated on its ability to specifically bind the sequence 5′-TGGC (N_6) GCCAA-3′ present in a

number of adenovirus origins of DNA replication (Leegwater et al. 1985). Interaction of NFI with this DNA sequence of the genome increases the frequency of initiation of viral DNA replication both in vivo and in vitro (Nagata et al. 1983; Guggenheimer et al. 1984; Rawlins et al. 1984; De Vries et al. 1985; Hay 1985a,b). By a number of criteria, NFI proteins are indistinguishable from CTF transcription factors (Jones et al. 1987), a family of proteins involved in cellular gene transcription that are distinct from the CCAAT-binding proteins (Zorbas et al. 1992). Analysis of cDNAs from various human NFI/CTF mRNAs have shown that they all originate from a single gene, giving rise to multiple mRNA transcripts by differential splicing of a precursor molecule (Paonessa et al. 1988; Santoro et al. 1988; Meisterernst et al. 1989). Mutagenesis studies on the cDNA of the largest NFI protein (CTF-1) identified two functional domains: a highly conserved amino-terminal domain, which contains the functions for DNA binding, dimerization, and DNA replication (Santoro et al. 1988; Mermod et al. 1989; Gounari et al. 1990; Bosher et al. 1991; Bandyopadhyay and Gronostajski 1994), and a less highly conserved, carboxy-terminal domain, which contains a transcriptional activation function (Mermod et al. 1989; Altmann et al. 1994).

The mechanism of stimulation of Ad DNA replication by NFI in vitro is complex (Mul et al. 1990). Stimulation is strongly dependent on the concentration of pTP/Ad pol. At low pTP/Ad pol concentrations, NFI or the DNA-binding domain of NFI stimulated replication up to 50-fold, whereas at high concentrations of pTP/Ad pol, stimulation was less than 2-fold. Thus, in vitro, the need for NFI can be overcome by increased pTP/Ad pol concentrations. This result provides an explanation for the different levels of stimulation by NFI reported previously (De Vries et al. 1985; Adhya et al. 1986), as well as a strong indication that NFI and pTP/Ad pol interact directly. The orientation and spacing between the NFI site and the core origin sequence (Fig. 1) are critical in Ad DNA replication. Insertion of additional sequence between these two sequence regions abolished NFI-mediated stimulation of DNA replication in vitro (Adhya et al. 1986; Wides et al. 1987; Coenjaerts et al. 1991) and in vivo (Bosher et al. 1990). This suggested that a strict constraint on the spatial arrangement between the core origin sequence and the NFI site is necessary to allow specific protein-protein interactions between NFI and other replication proteins. A direct interaction between NFI and Ad pol was subsequently demonstrated (Bosher et al. 1990; Chen et al. 1990; Mul et al. 1990), the function of which is to stabilize the relatively weak interaction between the pTP/Ad pol heterodimer and its recognition site in the adenovirus origin of DNA replication. Further dissection of this interac-

tion by mutational analysis has demonstrated that the DNA-binding and Ad pol interaction activities of NFI can be separated, although both are required for stimulation of viral DNA replication (Armentero et al. 1994).

NFIII

The host-encoded cellular factor NFIII was initially identified in HeLa nuclear extracts through its ability to stimulate the initiation of Ad DNA replication in vitro in the presence of NFI (Pruijn et al. 1986). Purified NFIII has a size of 92 kD and, when bound to its recognition site in the adenovirus origin, stimulates the level of initiation in vitro 3- to 7-fold (O'Neill and Kelly 1988). A combination of DNase I footprinting and methylation protection studies on Ad2 identified the core binding site as 5′-TATGATAAT-3′, which is situated between nucleotides 39 and 48 in the Ad2 ITR. NFIII is indistinguishable from Oct-1 (O'Neill et al. 1988; Pruijn et al. 1989). As with other octamer-binding proteins, NFIII/Oct-1 contains a conserved DNA-binding region known as the POU domain, which, like the amino-terminal domain of NFI, is sufficient for the in vitro stimulation of Ad2 DNA replication (Verrijzer et al. 1990). In vivo, the situation is less clear, as mutant Ad2 viral genomes with a deleted NFIII/Oct-1 site are as infectious as wild-type virus with an intact NFIII/Oct-1 site (Hay and McDougall 1986), although the site may be functionally redundant with sequences adjacent to the ITR (Hatfield and Hearing 1993). However, transfection assays on plasmids containing only the core origin of replication linked to an NFIII/Oct-1 site (no NFI site) indicated that these templates could replicate more efficiently than plasmids containing only the core origin of replication (Hay et al. 1988). This confirmed in vitro replication studies by Mul et al. (1990), who found that both the NFIII/Oct-1 and NFI-binding sites were needed for optimal stimulation of replication. Since the binding sites of the two proteins overlap, they could interact in a cooperative manner to stimulate DNA replication. However, this does not appear to be the case. It seems that both proteins can, by binding to their respective recognition sites, stimulate initiation independently (Mul et al. 1990). NFIII/Oct-1 can induce bending of the DNA at the origin of DNA replication, which may stimulate DNA replication by promoting interactions between the various components in the preinitiation complex (Verrijzer et al. 1991). In fact, direct interactions between the POU homeodomain and the pTP/Ad pol heterodimer are responsible for stabilization of the preinitiation complex formed at *ori* (Coenjaerts et al. 1994), and it is likely that

both mechanisms contribute to NFIII/Oct-1-induced stimulation of Ad DNA replication.

A MODEL FOR THE INITIATION OF AD DNA REPLICATION

On the basis of the observations described above, a model outlined in Figure 2 has been formulated to describe events that lead to the initiation of Ad DNA replication. Ad2 DBP, originally classified as a single-stranded DNA-binding protein, but which also binds to double-stranded DNA, is produced in such large amounts that it is likely that in vivo all template molecules are coated with the protein. This has the effect of increasing the affinity of NFI for its recognition site in the Ad2 *ori* and thus ensuring that the NFI-binding site in *ori* is fully occupied (Cleat and Hay 1989; Stuiver and van der Vliet 1990). A direct protein-protein interaction between NFI and Ad pol then recruits the pTP/Ad pol heterodimer into the preinitiation complex (Bosher et al. 1990; Chen et al. 1990; Mul et al. 1990; Armentero et al. 1994). This interaction is further stabilized by direct interactions between pTP/Ad pol and the POU domain of NFIII (Coenjaerts et al. 1994). Correct positioning of the pTP/Ad pol heterodimer at the origin is accomplished by an interaction between pTP/Ad pol and the DNA sequence from bp 9–18 that is perfectly conserved in all human adenoviruses sequenced to date and is regarded as the core of the Ad2 *ori* (Mul and van der Vliet 1992; Temperley and Hay 1992). Further stabilization of this complex may be accomplished by interactions between the incoming pTP/Ad pol and genome-bound TP (Pronk and van der Vliet 1993). At this stage, the origin must unwind to expose the single-stranded DNA that is the template for DNA synthesis. The ATP-independent helix-destabilizing properties of DBP may be particularly important in catalyzing this reaction, and it is worth noting that under certain conditions, Ad DNA replication in vitro can proceed in the absence of ATP (Pronk et al. 1994). Unlike most other systems, this unwinding reaction does not appear to require the activity of a bona fide helicase. Chemical modification experiments have recently demonstrated that this unwinding takes place in the highly conserved region within the core of the viral origin of DNA replication (I.R. Leith and R.T. Hay, unpubl.). The pTP/Ad pol heterodimer must then be repositioned for initiation such that the acceptor serine in pTP is positioned opposite the GTA at positions 4–6 in the genome rather than the same sequence present at positions 1–3. A pTP-CAT intermediate is then synthesized which remarkably "jumps back" to occupy positions 1–3 before elongation proceeds (King and van der Vliet

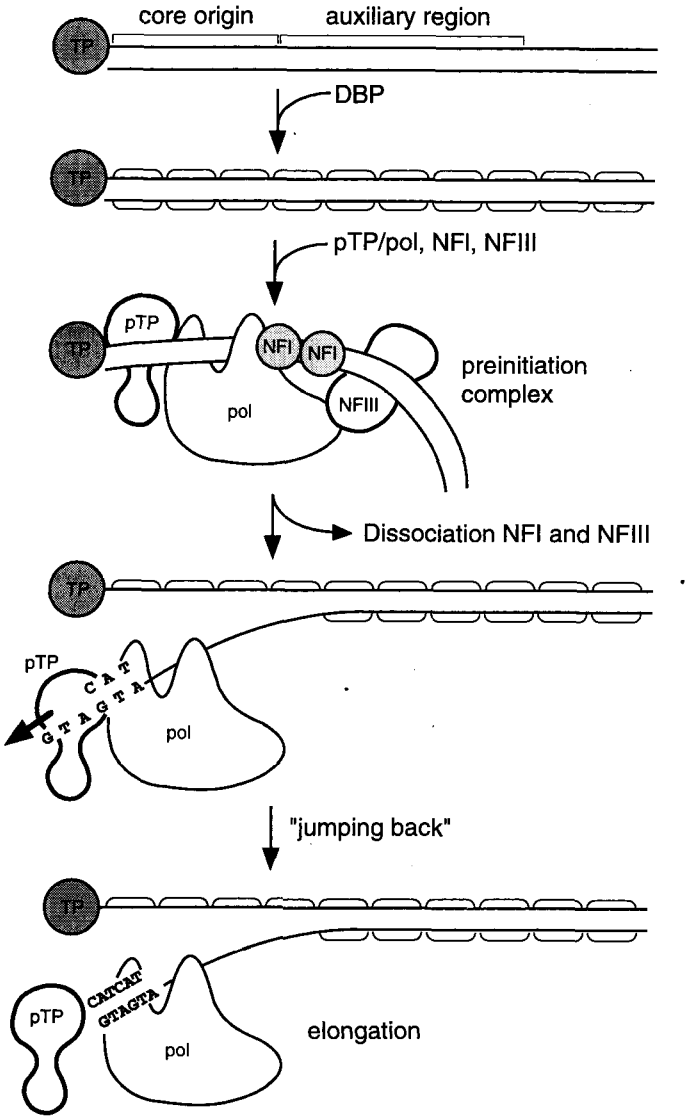

Figure 2 Model for the initiation of Ad2 DNA synthesis showing formation of the preinitiation complex, helix opening at the origin of DNA replication, and the "jumping back" mechanism of initiation.

1994). This type of mechanism appears to have been adopted as an evolutionary solution to the problem of initiation on linear protein-linked genomes and is discussed in detail elsewhere in this volume (Salas et al.). The events that follow the transition from initiation to elongation are not

well defined, although NFI dissociates from the origin at an early stage in the process (Coenjaerts and van der Vliet 1994).

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