REVIEW ARTICLE

Recognition mechanisms in the synthesis of animal virus DNA

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INTRODUCTION

In eukaryotic cells, initiation of DNA replication is tightly controlled and chromosomes are replicated once and only once per cell cycle. However, DNA viruses replicating in the nuclei of host cells must have evolved mechanisms to escape stringent copy number control, since each infected nucleus may contain as many as 10^5 viral genomes during a lytic infection. Viruses appear to accomplish this by replicating via a mechanism that is distinct from that of the host, e.g. adenoviruses, or by synthesizing large amounts of a particular protein that preferentially directs the initiation of replication, e.g. simian virus 40. Nevertheless, many viruses persist in a latent state and utilize the cell copy number control mechanisms to distribute the viral genome to daughter cells without high level replication.

The universality of DNA replication suggests that many of the basic mechanisms will have been conserved throughout evolution. Accordingly, the details of the biochemistry and genetics of DNA replication deduced from prokaryotes (reviewed by Kornberg, 1988) should serve as excellent models for the studies that we shall describe on eukaryotic viruses. In general terms initiation of DNA replication at prokaryotic origins of replication (e.g. *ori C* and *ori λ*) appears to be preceded by the formation of a large nucleoprotein assembly which is directed by specific recognition events between protein and DNA. In the presence of ATP this complex catalyses opening of the DNA helix. Replication is then accomplished by a multienzyme complex or 'replisome' held together by specific molecular interactions. In this Review we will focus on the molecular mechanisms involved in replication of eukaryotic virus chromosomes. Most information has been gained from studies on papovaviruses and adenoviruses, where systems *in vitro* which faithfully initiate DNA replication have been developed.

Papovavirus DNA replication

The papovaviruses, simian virus 40 (SV40) and polyomavirus (PyV), contain small (about 5 kb), covalently closed, double-stranded circular DNA genomes which are organized into mini-chromosomes containing histones derived from the host cell. These mini-chromosomes replicate in the nuclei of permissive cells and, apart from the virally encoded T-antigen (T-ag), all other factors required for replication and segregation of the viral chromosomes appear to be derived from the host cell. For this reason these viruses have long been regarded

as excellent models for the replication of mammalian chromosomes. The replication and structure of these viruses have been reviewed in detail (DePamphilis & Bradley, 1986) and only the more recent data are discussed below.

SV40 and PyV DNA replication is initiated at the genetically defined origin of replication and proceeds bidirectionally from this point. At the replication fork DNA synthesis is continuous on the forward arm and discontinuous on the retrograde arm. Discontinuous synthesis is repeatedly primed by short (approx. 10 bp) oligoribonucleotides which are extended by DNA polymerase to form Okazaki fragments of about 200 bp in length. After RNA excision and gap filling the DNA chains are eventually joined by the action of DNA ligase. DNA synthesis continues and replication forks accumulate at a point 180° from the origin, whereupon the sibling molecules separate and replication is terminated. Superhelical molecules are generated as a result of nucleosome assembly which occurs on both sides of the replication fork throughout the replicative cycle. The major advances made in recent years in our understanding of this process stem largely from its reproduction *in vitro* (Li & Kelly, 1984) and the subsequent dissection of the replication cycle into a series of steps catalysed by purified proteins.

T-antigen

Initiation of SV40 DNA replication requires interaction of T-Ag with the origin of DNA replication (Shortle *et al.*, 1979). In the origin region three binding sites (I, II and III) are recognized by T-Ag, with each binding site containing pentamer recognition sequences that are contacted by the protein (Jones & Tjian, 1984). Although T-Ag binds to site I with the highest affinity, this site is not within the genetically defined origin of DNA replication, and it is thought that interaction of T-Ag with this site is important for autoregulation of early virus gene expression rather than for the initiation of DNA replication.

A variety of approaches have been used to localize the specific DNA-binding domain of T-Ag, which appears to reside in a relatively small region of the protein, between Lys-131 and Pro-259 (Simmons, 1986; Paucha *et al.*, 1986; Strauss *et al.*, 1987; Mole *et al.*, 1987). However, specific DNA binding of T-Ag can be masked by additional regions of the protein (Strauss *et al.*, 1987). These negatively acting domains are phosphorylated and located at the N- and C-termini of the protein (Scheidtmann *et al.*, 1982). Dephosphorylation of serine

Abbreviations used: SV40, simian virus 40; PyV, polyoma virus; BPV, bovine papillomavirus; HSV, herpes simplex virus; VZV, varicella-zoster virus; EBV, Epstein-Barr virus; SFV, Shope fibroma virus; AAV, adeno-associated virus; T-Ag, T-antigen; DBP, DNA-binding protein; TP, terminal protein; pTP, preterminal protein; NF nuclear factor; OBR, origin of bidirectional replication; SSB, single-strand-specific DNA-binding protein; pol, DNA polymerase; PCNA, proliferating cell nuclear antigen; ITR, inverted terminal repeats; PMS, plasmid maintenance sequences; OTF, octamer transcription factor.

Fig. 1. Model for the initiation of SV40 DNA replication

The components of the replication origin (*ori-core*, *aux-1* and *aux-2*), the origin of bidirectional replication (OBR), purine/pyrimidine stretch (R/Y), the A + T-rich region (AT) and the pentamers recognized by a tetramer of T-antigen (T-Ag) are described in the text. The single-strand-specific DNA-binding protein (SSB), DNA polymerase α -primase and DNA polymerase δ -PCNA are described in the text. Binding sites for the transcription factors SI and AP2 are also indicated ('SpI/AP2'). Short thick lines represent RNA primers and narrow lines represent DNA with the direction of synthesis indicated by the arrow. DNA with an altered helical structure is indicated by the zig-zag lines. Early transcription is to left and late transcription to the right.

residues in T-Ag increased its ability to support DNA replication *in vitro* (Grässer *et al.*, 1987; Mohr *et al.*, 1987) and activated specific DNA binding to site II, located within the origin of replication (Mohr *et al.*,

1987) (Fig. 1). The role of phosphorylation in regulating DNA binding and DNA replication has been examined by mutating sites of phosphorylation and determining the properties of viable mutant viruses. A wide range of

phenotypes was obtained with respect to DNA replication and DNA-binding activity, indicating that phosphorylation may define functionally distinct classes of T-Ag (Schneider & Fanning, 1988).

Under standard DNA binding conditions, T-Ag binding to site I is preferred over binding to site II, which is located within the origin region. However, in the presence of ATP, under conditions which are optimal for DNA replication *in vitro*, T-Ag binds to site II with high affinity (Deb & Tegtmeyer, 1987). Presumably ATP induces a conformational change in T-Ag, since the enhancement of binding is also evident with non-hydrolysable analogues of ATP (Borowiec & Hurwitz, 1988a).

High-affinity binding of T-Ag to site II is accompanied by structural changes in the origin of replication. Chemical accessibility experiments with dimethyl sulphate and potassium permanganate indicate that on the 'early' side of the origin 8 base pairs are melted by T-Ag. On the 'late' side of the origin, in the A + T-rich region, extensive structural changes were detected, although the DNA helix was not unwound (Fig. 1). Thus, in the presence of ATP, but in the absence of ATP hydrolysis, T-Ag alone can induce major conformational changes in the structure of the origin DNA (Borowiec & Hurwitz, 1988b).

Hydrolysis of ATP by origin-bound T-Ag in the presence of a single-stranded DNA-binding protein (SSB), and a topoisomerase that can remove positive supercoils, results in formation of a unique nucleoprotein structure at the origin of replication. Visualization of this nucleoprotein complex by electron microscopy indicates that the DNA is unwound bidirectionally from the origin of replication. Analysis of the DNA products by gel electrophoresis revealed an anomalous, rapidly migrating species that was extensively underwound (Dean *et al.*, 1987a,c; Dodson *et al.*, 1987; Wold *et al.*, 1987). Formation of this complex appeared to be important for DNA replication since there is a distinct lag before DNA synthesis *in vitro* begins. This lag can be abolished by preincubation of the template under conditions which permit the formation of the highly underwound template (Wold *et al.*, 1987; Wobbe *et al.*, 1987; Fairman & Stillman, 1988). ATP-dependent unwinding of the origin is catalysed by the helicase activity that is associated with T-Ag (Stahl *et al.*, 1986).

Characterization of this activity has indicated that at low ionic strength the helicase will unwind DNA in the 3' to 5' direction in a DNA-sequence-independent fashion, the preferred template being a duplex DNA with a single-stranded extension at the 3' end of greater than 5 bp (Goetz *et al.*, 1988; Wiekoski *et al.*, 1988). At physiological ionic strength, however, T-Ag is transformed into a DNA-sequence-specific helicase and unwinding is dependent on the presence of a functional T-Ag-binding site (Goetz *et al.*, 1988). As would be expected, mutations in T-Ag that abolish specific DNA binding or helicase activity also block replication activity of T-Ag. An additional class of T-Ag mutants retain origin binding and helicase activity but are not competent for initiation of replication (Mohr *et al.*, 1987), thus suggesting that T-Ag may directly interact with other components of the replication machinery.

The ability of T-Ag to form complexes with other cellular proteins has recently been shown to be important for DNA replication. T-Ag binds DNA polymerase α *in vitro* (Smale & Tjian, 1986) and is complexed to DNA polymerase α *in vivo* (Mole *et al.*, 1987). The oncogene

product p53 also binds to T-Ag and competes with DNA polymerase α for T-Ag (Gannon & Lane, 1987). A consequence of this competition is that murine p53 inhibits SV40 origin-dependent DNA replication *in vivo* (Braithwaite *et al.*, 1987).

DNA sequences required for the initiation of SV40 and PyV DNA synthesis

Extensive mutational analysis of the SV40 origin of replication has identified four *cis*-acting DNA sequence elements that can contribute to the efficiency of DNA replication *in vivo*. A 64 bp sequence, located between nucleotides 5209 and 29, is absolutely required for replication and is regarded as the *ori*-core (Deb *et al.*, 1986a). Flanking *ori*-core are two auxiliary regions, *aux*-1 (5164–5208) and *aux*-2 (30–72), that can increase the efficiency of replication 2–5-fold but cannot substitute for each other. *aux*-1 encompasses T-Ag-binding site I whereas *aux*-2 contains three copies of the G-boxes that are binding sites for transcription factors Sp1 and AP2 (Mitchell *et al.*, 1987). In the presence of *aux*-1 and *aux*-2, deletion of the SV40 transcriptional enhancer (72 bp repeats) is without consequence for replication. However in the absence of *aux*-2, which alone reduces replication 2-fold, deletion of the enhancer can result in a 100-fold decrease in replication efficiency (Hertz & Mertz, 1986).

The influence of these transcriptional elements on DNA replication directed by *ori*-core is independent of their orientation but is dependent on their position in that they must be adjacent to the 'late' side of *ori*-core (Innis & Scott, 1984; Lee-Chen & Woodworth-Gutai, 1986; Chandrasekharappa & Subramanian, 1987). Although SV40 DNA replication *in vitro* requires both *ori*-core and *aux*-1 for optimal DNA synthesis, *in vitro* systems do not respond to the presence of the G-boxes or the enhancer (Li *et al.*, 1986; Stillman *et al.*, 1985).

In contrast to SV40, where *ori*-core can direct initiation of DNA replication at a basal level, PyV *ori*-core requires the presence of a transcriptional enhancer for its activity in mouse embryonic and differentiated cells (DePamphilis *et al.*, 1988). While the complete PyV enhancer is most efficient in stimulating replication, various subfragments of the enhancer are also active (Veldman *et al.*, 1985). Heterologous enhancers can also substitute for the PyV enhancer in the stimulation of *ori*-core and their cell-type specificity for transcriptional activation is reflected in their cell-type-specific activation of DNA replication (Campbell & Villarreal, 1986; de Villiers *et al.*, 1984). Like SV40, activation by the transcriptional control element is position-dependent and orientation-independent (de Villiers *et al.*, 1984).

The size and structural organization of the SV40 and PyV *ori*-core regions are very similar. Each contain an A + T-rich sequence on the late side of *ori*-core, a central palindrome that is located within the T-Ag-binding region and, on the early side of *ori*-core, a domain in which purine and pyrimidine bases are confined to opposite strands (Fig. 1). The importance of these individual domains and the DNA sequences which separate them in viral replication has been determined from an analysis of single base pair mutations. Base pair changes within the aforementioned domains tend to have a severe deleterious effect on replication *in vivo* and *in vitro*. In the central domain, mutation in any of the T-Ag recognition pentamers simultaneously reduces T-Ag

binding and DNA replication *in vivo* and *in vitro*, while mutation of the bases that separate the pentamers is not seriously inhibitory (Deb *et al.*, 1986a,b, 1987). The point mutations also show the importance of the T-Ag-catalysed unwinding reaction at the origin: templates with mutations that reduce the ability of T-Ag to unwind the DNA *in vitro* drastically reduce DNA synthesis *in vivo* and *in vitro* (Dean *et al.*, 1987b).

Base pair changes in the regions separating the domains tend not to reduce initiation, but insertions or deletions in these regions reduce initiation drastically (Deb *et al.*, 1987). Thus the SV40 origin consists of three sequence-specific domains separated by two sequence-independent spacer regions.

Although there is a strong correlation in the effect of mutations on DNA replication *in vivo* and DNA unwinding and replication *in vitro*, replication *in vivo* is particularly stringent in its sequence requirement. This may result from the absence of other cellular proteins in the reactions *in vitro*. Two cellular factors which bind to the origin have been identified (Yamaguchi & DePamphilis, 1986; Traut & Fanning, 1988) although their role in replication will only be clarified once they have been purified and assayed *in vitro*.

Protein components required for replication

Fractionation of the original extracts which support SV40 and PyV replication *in vitro* (Li & Kelly, 1984, 1985; Stillman & Gluzman, 1985; Wobbe *et al.*, 1985; Murakami *et al.*, 1986b; Decker *et al.*, 1987) has identified a number of factors that are required, in addition to T-Ag, for viral DNA synthesis. As expected, the complex of DNA polymerase α and primase is required for DNA synthesis *in vitro*. With both SV40 and PyV there is a requirement that the DNA polymerase α -primase be derived from a cell type permissive for viral replication. Thus mouse cell extracts supplemented by SV40 T-antigen do not support SV40 replication *in vitro*. Immunodepletion of the endogenous DNA polymerase α -primase and its replacement by human or simian-derived DNA polymerase α -primase allowed the extracts to replicate the SV40 template (Murakami *et al.*, 1986a). Conversely, PyV DNA is not replicated in human cell extracts, but addition of DNA polymerase α -primase from permissive mouse cells enabled these extracts to utilize efficiently a PyV origin containing template (Murakami *et al.*, 1986b). Although DNA polymerase α -primase is clearly involved in the initiation and elongation of DNA chains at the origin of replication and on the retrograde arm of replication forks (Decker *et al.*, 1986), it may not be the only DNA polymerase involved in the replication of SV40 and PyV DNA *in vivo*. Fractionation of human cell extracts has identified a 36000- M_r protein that is required for efficient synthesis *in vitro* of SV40 DNA (Prelich *et al.*, 1987a). This protein has been identified as the cell-cycle-regulated proliferating cell nuclear antigen (PCNA) (Celis *et al.*, 1987), which is also recognized as being an auxiliary protein of DNA polymerase δ (Bravo *et al.*, 1987; Prelich *et al.*, 1987b). On templates containing long single-stranded stretches of DNA, PCNA increases the activity and processivity of DNA polymerase δ (Tan *et al.*, 1986) but has no effect on DNA polymerase α (Tan *et al.*, 1987). The effect of PCNA on synthesis of SV40 DNA *in vitro* is confined to elongation of DNA chains. Analysis of the products of DNA synthesis in the absence of PCNA reveals that

early replicating intermediates accumulate and long nascent DNA chains are absent, suggesting that only lagging strand DNA synthesis is taking place (Prelich & Stillman, 1988; Wold *et al.*, 1987). A role for DNA polymerase δ is also suggested by the finding that p-n-butylphenyl-dGTP, which inhibits DNA polymerase α but not δ , failed to inhibit the replication *in vitro* of SV40 chromatin under conditions that inhibited the replication of plasmid DNA containing the SV40 origin of replication (Decker *et al.*, 1987). Thus DNA polymerase α -primase complex may initiate and extend DNA on the retrograde arms of replicate forks, while the processive DNA polymerase δ -PCNA complex may be responsible for DNA synthesis on the forward arms of replication forks.

An additional protein that appears to be required for SV40 and PyV DNA replication is a single-strand-specific DNA binding protein (SSB). SSB purified from HeLa cells is composed of a 70000- M_r polypeptide (Wobbe *et al.*, 1987) and two tightly associated polypeptides of M_r 34000 and 11000 (Fairman & Stillman, 1988; Wold & Kelly, 1988). This eukaryotic SSB differs from its bacterial counterparts in having a relatively high affinity for double-stranded DNA. It is the single-stranded DNA-binding activity of the HeLa SSB that is required for formation of the prepriming complex, since it can be substituted by the *Escherichia coli* SSB, which has a very low affinity for double-stranded DNA (Wobbe *et al.*, 1987). However, the HeLa SSB may play additional roles in SV40 replication, since *E. coli* SSB is unable to substitute for HeLa SSB (RP-A) in the complete reconstituted reaction.

Topoisomerases I and II from HeLa cells are required for the complete synthesis of covalently closed circular product DNA molecules. It is also likely that activities such as DNA ligase, RNAase H and a 5' $\rightarrow$ 3' exonuclease will be required for the completion of DNA synthesis.

Origin of bidirectional DNA replication

The origin of bidirectional DNA replication (OBR) is defined as the transition point between discontinuous (initiation sites) and continuous (no initiation sites) DNA synthesis. This information can be obtained by analysing template locations of RNA-primed nascent DNA chains present on replicating intermediates isolated from infected cells (Hay & DePamphilis, 1982). Analysis of RNA-primed initiation events at the SV40 (Hay & DePamphilis, 1982; Hay *et al.*, 1984a) and PyV (Hendrickson *et al.*, 1987a,b) origins of replication revealed that the OBR was located on the early side of *ori*-core (Fig. 1). In both cases initiation events occurred on only one side of *ori*-core using the same template as that used for early mRNA synthesis. Examination of initiation of nascent DNA chains at points distant from the replication origin indicate that at PyV and SV40 replication forks DNA synthesis proceeds semi-discontinuously: DNA synthesis on forward arms of replication forks is continuous; DNA synthesis on retrograde arms of replication forks is discontinuous, being repeatedly primed by the synthesis of oligoribonucleotides, 6–9 residues long, which begin with either A (80%) or G (20%). Comparison of initiation events at *ori* with other regions of the genome reveal that the size, composition, frequency of initiation and template location are the same in both cases, suggesting that the mechanism used to initiate Okazaki fragments at replication forks is also utilized at

the origin of DNA replication (Hay *et al.*, 1984a; Hendrickson *et al.*, 1987a). The factors which determine the precise positioning of the OBR in SV40 and PyV are not known, but a comparison of *ori* structure and organization in the two viruses permits a number of proposals to be forwarded. Transition points on the late mRNA side of SV40 and PyV *ori* were identical, and a similar analysis of mutant templates indicated that the position of this transition was determined by the binding of T-Ag within *ori* core. In contrast, transition points on the early mRNA side were located in different positions within *ori* core and appear to be determined by the binding of transcription factors which may interact with the initiation machinery located at *ori* (DePamphilis *et al.*, 1988).

Model for initiation of SV40 and PyV DNA replication

The available data on SV40 and polyoma are consistent with the 'initiation zone model' originally proposed by Hay & DePamphilis (1982) (Fig. 1). In the present model, T-Ag binds to the origin of replication and initiates unwinding within the region of the asymmetrically disposed purines and pyrimidines. This unwound region is stabilized by SSB and, driven by the hydrolysis of ATP, the helicase activity of T-Ag unwinds the entire *ori*-core region. Presumably at this stage a dimer of T-Ag is distributed to each strand. T-Ag on the templates for early mRNA synthesis then migrates in the 3' to 5' direction, creating a replication bubble which is stabilized by SSB. The T-Ag molecule left at *ori* interacts with DNA polymerase α -primase and transfers it to the opposite strand, where primase synthesizes a short oligoribonucleotide which is extended by DNA polymerase α . During this process DNA polymerase α -primase dissociates from T-Ag and the two proteins migrate in opposite directions. DNA synthesis is initiated on the template for late mRNA synthesis when DNA polymerase α -primase associates with T-Ag which is migrating on the early mRNA template. Again the polymerase α -primase is transferred to the opposite strand, where DNA synthesis is initiated. After an Okazaki-fragment-sized piece of DNA is synthesized, polymerase α -primase dissociates from the template, reassociates with T-Ag, which has continued to unwind the template, and initiates another Okazaki fragment. The Okazaki fragments synthesized at the origin may then be extended by DNA polymerase δ -PCNA, thus forming the continuously growing nascent DNA chains on the forward arms of replication forks.

This model implies that T-Ag is involved not only in initiation of DNA replication but also during the elongation phase, serving as a 'mobile promoter' for the synthesis of RNA-primed Okazaki fragments by DNA polymerase α -primase on the retrograde arms of replication forks. In fact, T-Ag has been shown to be present on replicating intermediates where it is associated with synchronous fork movement (Tack & Proctor, 1987). A direct role for T-Ag in elongation has been previously suggested based on its intrinsic ATPase activity and the ability of anti-T-Ag monoclonal antibodies to inhibit SV40 replication in an elongation assay (Stahl *et al.*, 1985; Wiekowski *et al.*, 1987).

Papillomavirus DNA replication

Papillomaviruses, although members of the Papo-

vaviridae, demonstrate other aspects of DNA replication control. In transformed rodent cells the bovine papillomavirus (BPV) genome exists as a nuclear, stably inherited, multicopy plasmid. Analysis of the viral replicon *in vivo* has demonstrated that a number of viral complementation groups are involved in DNA replication. Positive and negative viral *trans*-acting factors have been identified which play a role in the maintenance of the copy number of the viral genome (Berg *et al.*, 1986; Lusky & Botchan, 1986a; Roberts & Weintraub, 1986).

The *cis*-acting BPV DNA sequences that are required to maintain plasmids (plasmid maintenance sequences, PMS) in transformed cells *in vivo* have been identified and shown to be separable into two domains (Lusky & Botchan, 1986b). One domain, which represents the core element for DNA replication, overlaps a promoter (Stenlund *et al.*, 1987) and a *cis*-acting site involved in the negative control of replication (Roberts & Weintraub, 1986). The latter domain appears to operate like an enhancer *in vivo*, in that it is functional in a position and orientation-independent manner and can be replaced by other known enhancers.

Recently the replication *in vitro* of BPV DNA containing PMS-1 has been demonstrated in extracts of mouse cells. Surprisingly, efficient replication was detected in both virus-infected and uninfected mouse cell extracts, provided that a functional PMS-1 element was present. Mutations which abolish DNA replication *in vivo* also abolished DNA replication *in vitro*, indicating that both the core element and the enhancer are required *in vitro* (M. Lusky & J. Hurwitz, unpublished work). This is in contrast to the situation in SV40 and PyV, where the *in vitro* systems do not respond to the presence of enhancer DNA sequences on the template (Li *et al.*, 1986; Prives *et al.*, 1987).

Whereas SV40 and polyoma can be described as 'runaway replicons' that are not subject to copy number control in permissive cells, the replication of BPV DNA is tightly controlled *in vivo* and each BPV genome replicates once and only once per cell cycle (Botchan *et al.*, 1986). Construction of SV40-BPV hybrid replicons and analysis of their replication properties *in vivo* have demonstrated that, even in the presence of excess initiator protein (T-Ag), the *cis*-acting negative controls are dominant and the hybrid replicon is replicated only once per cell cycle (Roberts & Weintraub, 1988). The development of an *in vitro* system derived from uninfected cells opens up a number of exciting possibilities, ranging from the isolation of cellular origins of replication to the reconstruction of the copy number control phenomena *in vitro*.

Adenovirus DNA replication

Adenovirus has a linear double-stranded DNA genome of about 35 kbp, with inverted terminal repeats (ITRs) and a covalently linked terminal protein (TP) attached to the 5' termini (Rekosh *et al.*, 1977). The mechanism of replication has been analysed extensively *in vivo* and *in vitro* and a number of reviews have been published which provide detailed information on earlier studies in this area (Kelly, 1984; Friefeld *et al.*, 1984; Tamanoi, 1986).

The adenovirus origin of DNA replication is located at either end of the linear DNA molecule, and a number of specific recognition events are necessary for replication

to proceed. Initiation of replication takes place with the formation of an ester linkage between the phosphate of dCMP and the β -OH of a serine residue in the virus-coded pre-terminal protein (pTP). The 3'-OH group present in this pTP-dCMP complex can then function as a primer to extend a new chain of DNA using one strand of the virus DNA as template, the other strand with attached TP being displaced forming a 'panhandle' structure and utilized as template apparently in a similar fashion (Stow, 1982; Hay *et al.*, 1984b).

Comparison of DNA sequences which constitute origins of replication from many different adenoviruses, and extensive mutagenesis of the adenovirus type 2 origin of replication, have identified three sequence domains within the origin that can contribute to replication efficiency. Each of these domains appears to represent the binding site for proteins which may assemble into a nucleoprotein complex at the origin of replication. Replication *in vitro* requires three viral proteins: the single-strand-specific DNA-binding protein (DBP) and a DNA polymerase (pol) that forms a heterodimer with the preterminal protein (pTP). Two cellular, sequence-specific DNA-binding proteins (NFI and NFIII) and a cellular topoisomerase (NFII) can contribute to replication efficiency. There are also indications that additional, as yet unidentified, cellular proteins may participate in viral replication.

Cis-acting DNA sequences required for replication

The adenovirus genome has terminal proteins attached to each 5' end of the DNA molecule and it is this template that is utilized most efficiently for the initiation of DNA replication. However, replication is also observed with protein-free templates both *in vitro* (Tamanai & Stillman, 1982) and *in vivo* (Hay *et al.*, 1984b) albeit with reduced efficiency. It is therefore possible to introduce mutations into these cloned templates and determine the effect of the mutation on replication efficiency both *in vivo* and *in vitro*.

Utilization of the *in vitro* system developed to study adenovirus type 2 replication (Challberg & Kelly, 1979) has led to the recognition of three domains within the terminal 51 bp of the viral genome that contribute to the efficiency of replication. The terminal 18 bp of the genome appears to be necessary, but in isolation can support only limited initiation (Tamanai & Stillman, 1983; van Bergen *et al.*, 1983; Challberg & Rawlins, 1984; Lally *et al.*, 1984). The additional presence of DNA sequences between nucleotides 19 and 39 stimulates DNA replication in the presence of the cellular protein NFI, which binds to this sequence (Nagata *et al.*, 1983a; Guggenheimer *et al.*, 1984; Rawlins *et al.*, 1984; de Vries *et al.*, 1985; Leegwater *et al.*, 1985; Schneider *et al.*, 1986). DNA sequences between nucleotides 40 and 51 appear to constitute a third domain which can increase the efficiency of replication and is the recognition site for the cellular protein NFIII (Prujn *et al.*, 1986), or ORP c (Wides *et al.*, 1987; Rosenfeld *et al.*, 1987).

The adenovirus type 2 origin of replication has been defined *in vivo* using a transfection assay in which linear plasmid molecules containing inverted copies of the adenovirus ITR are introduced into cells in the presence of helper virus (Hay *et al.*, 1984b). Using this assay, deletion analysis demonstrated that the terminal 45 bp of adenovirus type 2 genome could fulfil the requirements

of an origin of DNA replication (Hay, 1985a). This conclusion was reinforced by the construction of viable virus genomes with deletions in the left ITR. Whereas viral genomes containing the terminal 45 bp of the left ITR were fully infectious, genomes containing the terminal 36 bp of this ITR were non-infectious (Hay & McDougall, 1986). Although the three domains all contribute to replication efficiency *in vivo*, the NFIII domain, when linked to the essential 1-18 domain, can stimulate replication in the absence of the NFI domain. Similarly the NFI domain can stimulate replication in the absence of the NFIII site and can do so in either orientation (Hay *et al.*, 1988).

When transfection assays were carried out with the subgroup E adenovirus type 4 it was shown that, in contrast to adenovirus type 2, only the terminal 18 bp of the viral genome were required for efficient replication *in vivo* (Hay, 1985b) and *in vitro* (Harris & Hay, 1988). Initiation was not enhanced by the presence of either the NFI site, the NFIII site, or both sites together (Harris & Hay, 1988).

Protein factors required for adenovirus DNA replication

Three viral proteins are required for adenovirus DNA replication *in vitro* and this reaction is stimulated to varying degrees by the addition of cellular proteins. It is notable that all of the virus-coded replication proteins map within the E2 region of the genome and their transcripts are initiated early at a single promoter at coordinate 75 on the conventional adenovirus map (Kruijer *et al.*, 1981; Stillman *et al.*, 1981). There are two groups of spliced primary transcripts, one (E2A) being related to the expression of the DBP. The complete protein contains 529 amino acid residues with a molecular mass of 59 kDa. At late times of infection another promoter appears to function, allowing transcription of E2A to be independently regulated. The other primary transcription unit (E2B), encoding an mRNA of some 22 kb, allows the expression of both the pTP and pol proteins following a complex splicing mechanism. Recent investigations have suggested that functional pol and pTP require sequences at map position 39 (in a minor exon) as well as those in the main body of the transcripts (Shu *et al.*, 1987; Pettit *et al.*, 1988; Stunnenberg *et al.*, 1988). Indeed, earlier attempts to express functional pol appeared to have been unsuccessful because constructs were used with an ATG codon at base 8367 rather than an ATG at base 14114. Thus it appears that the pol protein initiates at 14114 and after three codons the mRNA is spliced to an acceptor site at 8784 to the main body of the reading frame. The pTP seems to initiate at the same ATG as pol and the mRNA is spliced at the same donor site to an acceptor site at 10589 in a different reading frame to the main body of the gene. This gives an overlap of some 199 bp in the pTP and pol genes. It thus seems very likely that these two gene products are co-ordinately regulated and it is not surprising therefore that they seem to function at some stages of the replication process in close concert with one another (see below).

The adenovirus TP has attributes in terms of protein priming of replication which have, as yet, only been described in two bacteriophage systems ϕ 29 (Watabe *et al.*, 1984) and PRD1 (Bamford & Mindich, 1984). In all these cases the TP is closely associated with the virus-coded DNA polymerase. In the presence of the appro-

appropriate DNA template it is the associated DNA polymerase which transfers dNMP onto the TP.

Little is known of the functional characteristics of the virus-coded polymerase, although earlier studies had clearly shown that it could be distinguished from cellular enzymes by its relative resistance to aphidocolin and by its template requirements. The availability of sequence data from a range of DNA polymerases (Jing *et al.*, 1987; Wong *et al.*, 1988) indicates some limited degree of sequence conservation consistent with the presence of a cleft region possibly involving dNTP binding. Indeed it has been suggested that polymerases can be classified into at least three groups, the adenovirus-coded enzyme being incorporated into one group along with PRD1/ ϕ 29, T4 DNA polymerase and other virus-coded enzymes. The adenovirus *pol* protein, like other members of this group, seems to contain an intrinsic 3' $\rightarrow$ 5' single-strand exonuclease activity presumably functioning as a proof-reading mechanism.

The adenovirus DBP has been the subject of intensive study since this gene product plays a role in transcriptional control and assembly as well as replication (for review see Chase & Williams, 1986). The protein has an apparent M_r of 72000 but in fact contains 529 amino acids with a combined M_r of 59000 (Kruijer *et al.*, 1981). This difference seems to be mainly the result of post-translational modifications such as phosphorylation.

Mild chymotrypsin treatment cleaves the protein into a phosphorylated *N*-terminal domain of 174 amino acid residues and a *C*-terminal domain of 355 residues, which appears to be just as effective as DBP in an elongation reaction *in vitro* (Ariga *et al.*, 1980; Friefeld *et al.*, 1983). It has been assumed therefore that phosphorylation of the DBP does not play a significant role in the replication process, although the *N*-terminal domain may influence the DNA-binding properties of the *C*-terminal domain (Krevolin & Horwitz, 1987). Indeed it is interesting to note that replication-deficient DBP mutants seem to fall into two groups, one being in the domain around residues 410–420 and the other at 270–290. The latter domain, which is conserved in a variety of serotypes (Vos *et al.*, 1988), includes a region relatively rich in His and Cys residues. In the light of data indicating that a number of

DNA-binding proteins contain Cys/His-rich regions associated with zinc binding, it is feasible that one of the domains is essential for DNA binding and the other for association with the *pol* protein (discussed below).

The *C*-terminal domain of DBP has been crystallized (Tsernoglou *et al.*, 1984) and the three-dimensional structure of this protein, once available, will shed light on many of these problems.

In the presence of a template containing the adenovirus origin of replication, the three viral proteins are unable to initiate DNA replication efficiently unless the reaction is supplemented by extracts from uninfected cells. Fractionation of these extracts has identified a number of cellular proteins which can increase replication efficiency. Nuclear factor I (NFI) is a sequence-specific DNA-binding protein which interacts with a site located between 19 and 39 on the viral genome and in doing so stimulates the initiation of DNA replication (Nagata *et al.*, 1982). Extensive mutational analysis of the NFI binding site has identified base pairs that are important in the NFI–DNA interaction (de Vries *et al.*, 1985; Gronostajski, 1986; Wides *et al.*, 1987). A detailed contact point analysis (de Vries *et al.*, 1987) has indicated that NFI binds symmetrically to one side of the DNA helix (probably as a homodimer), making major groove contacts with the bases and phosphate backbone between bases 22 and 39 (Fig. 2). NFI has been extensively purified using recognition site affinity chromatography (Rosenfeld & Kelly, 1986; Diffley & Stillman, 1986; Jones *et al.*, 1987), but there is still debate as to the exact polypeptide species that constitute the functional (in DNA replication) NFI protein. Adjacent to the NFI binding site is the recognition site for NFIII (or ORP c), another sequence-specific DNA-binding protein from HeLa cells that can stimulate adenovirus DNA replication *in vitro* (Pruijn *et al.*, 1986). NFIII binds to a sequence present at 39–50 in the adenovirus origin of replication (Pruijn *et al.*, 1986; Rosenfeld *et al.*, 1987) and consists of a 92000- M_r polypeptide (O'Neill & Kelly, 1988) that makes major and minor groove contacts with one side of DNA helix (Pruijn *et al.*, 1987).

Recently it has become apparent that both NFI and NFIII are almost indistinguishable from factors that

Fig. 2. Potential DNA-protein and protein-protein interactions at the adenovirus origin of replication

The sequence of the terminal 50 bases of the strand attached to terminal protein (TP) is shown with the *ori*-core, nuclear factor I (NFI) and nuclear factor III (NFIII) binding site indicated by brackets. Sites where the viral DNA-binding protein (DBP), DNA polymerase (pol) and preterminal protein (pTP) may interact are also suggested.

play important roles in enhancing transcription from a variety of key genes. Thus NFI appears to be identical to CTF, a transcription factor that binds to the CCAAT motifs found in many eukaryotic promoters (Jones *et al.*, 1987). Similarly, NFIII (Prujn *et al.*, 1987; O'Neill & Kelly, 1988) binds to the 'octamer' sequence (ATGCAAT) present in a number of enhancer and promoter elements (Prujn *et al.*, 1987). Moreover, a recent study using purified NFIII and purified octamer transcription factor (OTF-1) has shown that they are physically and biologically indistinguishable (O'Neill *et al.*, 1988). The dual role of these proteins in transcription and replication may indicate that they activate both processes by a similar mechanism. Additional factors which stimulate initiation of replication have been identified in partially purified fractions. BR-70 stimulates initiation of adenovirus type 2 DNA replication in the presence of NFI (Rosenfeld *et al.*, 1987), while initiation of adenovirus type 4 DNA replication is stimulated by a nuclear factor that is distinct from NFI, NFIII or BR-70 (Harris & Hay, 1988). Elucidation of the precise role of these proteins in viral replication requires their complete purification and biochemical analysis.

An additional cellular protein (NFII) is required for complete elongation of the viral genome *in vitro*. In the absence of NFII, nascent DNA chains 25% of full genome length accumulate. Full-length nascent DNA chains are only observed after addition of NFII. Topoisomerase I from HeLa cells can substitute for NFII but prokaryotic topoisomerases are unable to do so (Nagata *et al.*, 1983a).

Functional interactions between proteins

An obvious example of protein-protein interactions during initiation of adenovirus DNA replication is the tight association of pTP and pol, which can only be separated in the presence of denaturing agents such as urea (Enomoto *et al.*, 1981; Stillman *et al.*, 1982). The multifunctional DBP is also thought to engage in a number of interactions with other proteins that are required for DNA replication.

A study of the elongation reaction catalysed by pol using poly(dT)-oligo(dA) as template-primer demonstrated that DBP functioned not only as a DNA-binding protein but also as a specific factor co-operating with pol in the elongation process (Lindenbaum *et al.*, 1986). The presence of DBP proteins increased the processivity of pol by at least two orders of magnitude and permitted the translocation of the polymerase through duplex DNA. Although this reaction was stimulated by ATP, no hydrolysis of ATP was observed, and indeed no ATPase activity has been detected in any of the components of the replication systems. Thus the nature of the interaction between DBP and pol is obscure and the molecular mechanisms involved have still to be unravelled. In the presence of NFI, DBP also appears to be capable of stimulating the initiation reaction (Nagata *et al.*, 1982; de Vries *et al.*, 1985). Although the biochemical basis for this stimulation has not been elucidated, it has recently been demonstrated that DBP specifically increases the affinity of NFI for its recognition site within the replication origin (P. H. Cleat & R. T. Hay, unpublished work). Specific interactions between DNA-bound proteins are also suggested by the strict spatial arrangement of the different domains within the origin. Insertion or deletion of sequences located between the 1-18 domain and the

NFI binding site have a severe deleterious effect on replication (Adhya *et al.*, 1986; Wides *et al.*, 1987).

Virion DNA appears to be closely associated with a number of basic virus-coded proteins, and viral cores can support limited initiation *in vitro* (Goding & Russell, 1983). However, addition of purified core proteins to the system *in vitro* inhibits replication (Korn & Horwitz, 1986) and it is possible that the inhibitory action of these proteins correlates with the packaging of the DNA and assembly into virions.

Mechanism of initiation

Although the mechanism of initiation of replication at the adenovirus origin of replication has not been established it is possible to put forward a plausible sequence of events based on the available data. One requirement seems to be the generation of a nucleoprotein complex containing NFI, NFIII and DBP at the origin of replication (Fig. 2). To date, specific recognition of the 1-18 domain has not been unequivocally demonstrated, but there are indications that the pTP-pol complex has an increased affinity for this region (Rijnders *et al.*, 1983) and it may be that the role of this domain is to facilitate the helix-opening reaction that is thought to precede initiation. A requirement for single-stranded DNA at the origin is suggested by the finding that protein-free templates require the action of a host protein factor pL (Guggenheimer *et al.*, 1984) before they can function as templates in the initiation reaction. It transpires that factor pL is a 5' → 3' exonuclease which degrades the non-template strand at the origin of replication and in doing so creates a partially single stranded template that is efficiently utilized in the initiation reaction (Kenny *et al.*, 1988). Factor pL has no effect on templates that are linked to TP and it may be that TP is involved in creating a single-stranded region that can be utilized by the pTP-pol. However, merely exposing a single-stranded region of DNA is not sufficient for initiation and it appears that a particular sequence must be present, or a particular conformation adopted, before initiation can take place. Extracts from adenovirus type 4 infected cells demonstrate a surprising specificity for initiation with single-stranded synthetic oligonucleotides: whereas the strand used as template during initiation *in vivo* was active as a template *in vitro* the complementary strand was inactive (Harris & Hay, 1988). It has been recently demonstrated that purified adenovirus type 2 pTP-pol was 5-20-fold more active in pTP-dCMP complex formation with template strand oligonucleotides than with other single-stranded DNAs (Kenny & Hurwitz, 1988). Thus helix opening, catalysed by the nucleoprotein complex located at the origin, is probably followed by recognition of the template strand by the pTP-pol complex and subsequent pTP-dCMP complex formation. During elongation the pol may dissociate from pTP and associate with DBP, although this remains to be demonstrated.

Herpesvirus DNA replication

The genome of herpes simplex virus (HSV) is double-stranded DNA (Russell, 1962) containing 150 kbp (see Roizman & Batterson, 1985). It consists of a long (U_L) and a short (U_S) segment of unique DNA each flanked by inverted repeat sequences (Sheldrick & Bertholet, 1975). Having a much larger genome than the adeno-

viruses it seems likely that the replication process will be less dependent on cellular factors, and it can be anticipated that a substantial number of virus gene products will be involved. Evidence to date has indicated that origins of replication can be located in three separate regions. Two of these (ORI_s) are found within the inverted repeats flanking the U_s segment (Vlazny & Frenkel, 1981; Stow, 1982) and the other, ORI_L , is located in the middle of the U_L sequence (Spaete & Frenkel, 1982; Weller *et al.*, 1985). However, the mode of replication of the DNA has not yet been unravelled, although the observation that replicating DNA exists as head-to-tail concatamers suggests that replication proceeds via some kind of rolling circle model.

Sequence analyses (Stow, 1982; Weller *et al.*, 1985) of the origins have been carried out and have shown A + T-rich, palindromic sequences in all four origins that have been examined. A number of approaches have been used to identify the gene products essential for viral DNA replication. Standard genetic analyses using *ts* and null mutants have, in general, yielded results (Weller *et al.*, 1988) not dissimilar to other more direct approaches using co-transfection with HSV origin containing plasmids and plasmids containing subclones of HSV DNA with the putative replication genes (Challberg, 1986). These techniques have identified seven virus genes required for replication, including HSV DNA polymerase (136 kDa), a major DNA-binding protein (126 kDa) (Weller *et al.*, 1983) and five others also mapping in the U_L sequence i.e. U_{L5} (99 kDa), U_{L8} (80 kDa), U_{L9} (94 kDa), U_{L42} (51 kDa) and U_{L52} (114 kDa) (Wu *et al.*, 1988; McGeoch *et al.*, 1988). Other virus gene products are related to various aspects of nucleotide metabolism and appear to be only essential for DNA replication in situations where the appropriate cellular pathways are quiescent, e.g. in resting cells. In this category are thymidine kinase (Dubbs & Kit, 1964), ribonucleotide reductase (Dutia, 1983; Weller *et al.*, 1988), dUTPase (Preston & Fisher, 1984), alkaline nuclease (Moss *et al.*, 1979), dCMP deaminase and uracil-DNA glycosylase (Cardonna *et al.*, 1987). The HSV polymerase appears to be closely related to the human DNA polymerase α (Wong *et al.*, 1988) and six regions of sequence similarity have been assigned. Further studies (Larder *et al.*, 1987; Gibbs *et al.*, 1988) have indicated that interacting regions involved in drug sensitivity and substrate recognition can also be defined. It may also be of significance that expression of the polymerase appears to be partially controlled at the translation level (Yager & Coen, 1988). The major DNA-binding protein binds to single-stranded DNA, and the U_{L5} gene has a sequence domain consistent with an ATP-binding site that may be indicative of a helicase activity similar to that noted in SV40 T-Ag (Wu *et al.*, 1988). Another of these gene products, U_{L42} , is a DNA-binding protein that appears to be tightly associated with the polymerase (Vaughan *et al.*, 1985), a situation similar to that observed in adenovirus. It is evident that knowledge of the functions of these proteins is still rather ambiguous and, although many of these proteins have now been overexpressed in baculoviruses, further progress in relation to functional attributes must await the development of the appropriate *in vitro* systems. Recent results (Elias & Lehman, 1988) indicate that HSV-1 encodes a protein of apparent M_r 83000 that binds to ORI_s and appears to recognize a sequence CGTTCG that occurs as a nearly perfect direct repeat in

a number of origin sites. Another recent investigation (Holmes *et al.*, 1988) has characterized DNA primase activities in HSV-infected cells and concluded that a virus-specific primase activity was present. This was purified and shown to be associated with a 40 kDa polypeptide. Neither of these two proteins has as yet been related to any of the replication genes described above.

It is interesting that varicella-zoster virus (VZV), another herpesvirus, contains an origin of replication in a position equivalent to that of the herpes simplex ORI_s origin (Davison & Scott, 1986) that can be utilized by HSV when transfected into HSV-infected cells (Stow & Davidson, 1986). One 11 bp sequence is common to VZV and HSV and includes the sequence CGTTCG already referred to. VZV does not appear to contain an origin of replication equivalent to that of HSV ORI_L . Epstein-Barr virus (EBV) is another human herpesvirus but with a different lifestyle in that it infects and transforms B-lymphocytes. In this situation the viral genome is maintained episomally as a supercoiled DNA plasmid of 172000 bp (Baer *et al.*, 1984). A genetic element consisting of two non-contiguous EBV DNA sequences, designated ORI_p , allows plasmids containing these sequences to replicate. One of these regions consists of approx. 20 tandem imperfect copies of a 30 bp sequence. The other region is 114 bp long and contains a 65 bp region of dyad symmetry (Reisman *et al.*, 1985). Surprisingly, neither the orientation of the two components relative to each other nor the distance between them seems to be critical for the ORI_p activity. It is interesting, in view of the relationship to transcriptional enhancers noted in both adeno- and papovaviruses, that one of these components can also function as a transcriptional enhancer in the presence of EBNA, a virus encoded nuclear antigen required for EBV DNA replication (Reisman *et al.*, 1985; Reisman & Sugden, 1986). Moreover, mutations in EBNA which inactivate the ORI_p also affect the promoter activation. This relationship to *cis*-acting transcriptional elements seen with the EBV replication origin does not appear to operate within the two HSV origin regions (Stow *et al.*, 1986).

Poxvirus DNA replication

Poxviruses replicate their double-stranded linear genomes in 'factories' in the cytoplasm of infected cells (for a review see McFadden & Dales, 1982). The genomes are the largest that have been considered so far, being of the order of 200 kbp, and consequently it seems likely that they can code for all the proteins required for their replication, a supposition borne out by their ability to replicate in enucleated cells (Pennington & Follett, 1974). All poxviruses examined to date appear to have covalently closed hairpin termini (telomeres) with terminal repeats of about 10 kbp. In addition the terminal 104 nucleotides of these viral 'hairpins' are present in two orientations and include a number of bases which participate in non-complementary base pairing (Baroudy *et al.*, 1982). Examination of replicative intermediates (DeLange & McFadden, 1986) indicates that replication proceeds via a dimeric 'head-to-head' repeat with the original hairpin at the axis of symmetry.

Comparative analysis of the leipropoxvirus Shope fibroma virus (SFV) indicates that the virus has terminal inverted repeats of 12.4 kbp (Upton *et al.*, 1987). An *in vitro* transfection system (DeLange *et al.*, 1986) has

been developed using the telomeres of both vaccinia and SFV cloned into plasmids as imperfect inverted repeats with the original hairpin ends at the axis of symmetry. When these constructs were transfected into cells that had been previously infected with the homologous virus, replication and resolution of these inverted repeats generated linear monomeric and multimeric minichromosomes with viral hairpins at both ends. The minimum substrates for the action of the *trans*-acting SFV-induced proteins was studied by deletion analysis and indicated that several sequence features are shared with the analogous region in vaccinia virus. Thus, four conserved regions in the telomeres have been demonstrated in these two viruses and the minimal region for resolution of the telomeres in the *in vivo* transfection assay has been defined as two identical inverted copies of a DNA region 58 and 76 bp long, which includes a 5 bp sequence 5'-TAAAT distal to one region of the conserved sequences in both viruses (DeLange & McFadden, 1987). As a result of these investigations it has been suggested that the termini of poxvirus genomes can be subdivided into two domains: a terminal A-rich domain containing the extra helical bases, and an adjacent 76 bp domain related to the resolution of the inverted repeat and to the hairpin conformation. To date no information is available on the nature of proteins interacting with these sequences, although recently an enzyme capable of cross-linking substrate DNA *in vitro* has been purified from vaccinia virus (Merchinsky *et al.*, 1988). The cross-linking observed, coupled with specific cleavage near the border of the hairpin loop, suggested that the nuclease may be a nicking-closing enzyme. This enzyme seems to be multifunctional and behaves in many respects in a similar manner to ϕ X174 A* protein. Similar eukaryotic proteins may be involved in the replication of cross-linked telomeres occurring in *Paramecium* mitochondrial DNA, in *Saccharomyces cerevisiae* and in *Tetrahymena* extra-chromosomal rDNA elements.

Parvovirus replication

The parvoviridae family comprise the smallest of the DNA-containing viruses and a study of their replication should illuminate the features of the replication process which are absolutely essential for their propagation (for a review see Berns & Labow, 1987). One genus of this family, the adeno-associated viruses (AAVs), appears to be even less independent in that they seem to require functions supplied by a coinfecting helper virus (either adeno or herpes viruses), although recent discoveries have cast some doubt on this concept of defectiveness.

Studies on the physical and genetic structure of the AAV genome have shown that it contains a linear single-stranded genome of 4.7 kb. The genome is flanked by 145-base terminal repeats which function as replication origins by hairpin priming. Genetic and mutational analyses of the AAV genome has indicated that it contains three major transcripts (p5, p19 and p40). Mutations within the p5 or *rep* gene abolishes DNA replication (Hermonat *et al.*, 1984; Tratschin *et al.*, 1984). Deletion analyses (Labow & Berns, 1988) have further suggested that the p5 gene product negatively regulates its own expression and in many respects appears to have properties akin to SV40 T antigen in that they are both multifunctional, being required for replication and transcription of structural genes. Thus, despite its structural simplicity, the AAV genome appears to be capable of

autoregulation of its DNA replication through *cis*- and *trans*-acting regulatory elements. The nature of the *trans*-activating elements has still to be unravelled.

Recent data have shown that treatment of cells with hydroxyurea, certain toxins or carcinogens (Schlehofer *et al.*, 1986; Jacobson *et al.*, 1987) can render them permissive for AAV replication in the absence of helper and there is also evidence to suggest that an AAV gene function requiring an AAV target sequence can prevent DNA replication from a non-AAV *ori* under non-permissive conditions. These observations have led to the hypothesis (Labow & Berns, 1988) that the AAV genome may respond to the intracellular environment. In a favourable environment the *rep* gene functions to allow replication, whereas in an unfavourable environment the gene actively represses replication, thereby facilitating stable integration into cellular DNA, forming a provirus. It is further suggested that a similar negative regulation of DNA synthesis may operate for cellular DNA to ensure that cellular *oris* are only utilized during S phase.

The other major genus of the parvoviridae family contains viruses which are autonomous in their replication. A significant difference has been found between these two genera in the organization of their terminal sequences. As described above, AAV DNA has ITRs of 145 bases, the terminal 125 bases being palindromic, but the overall palindrome is interrupted by two shorter palindromes symmetrically disposed on either side of the larger palindrome. Thus, when the terminal 125 bases are folded to optimize base pairing, a structure is formed which can facilitate priming of DNA replication. The short internal palindromes can be substituted and as long as the overall conformation is maintained replication can proceed (Lefebvre *et al.*, 1984). In the case of the autonomous viruses both termini are palindromic but the sequences are unrelated to one another (Rhode & Klaasson, 1982). At one end there is some similarity to the AAV genome structure but the other is normally longer and different in sequence. It is not at all clear in what way, if any, these differences relate to the autonomy of the viruses.

Indeed many studies have been carried out in an effort to define the factors supplied both by the host cell and by the helper viruses in promoting virus DNA replication. It may be significant that the autonomous viruses have an absolute dependence on the cell cycle, some functions occurring in the S phase being necessary for the conversion of the double-stranded replication intermediate to mature single-stranded virion genomes. With AAV it has become apparent that there are many different aspects of the AAV metabolism which are affected by helper virus gene products, and extensive use of adenovirus mutants has indicated the complex nature of the phenomenon. Moreover, both the autonomous and dependent parvoviruses appear to utilize the cellular DNA polymerase α for their DNA replication, although a role for DNA polymerase δ -PCNA should not be ruled out.

Significant progress in characterization of these factors will depend on the development of appropriate *in vitro* replication systems.

CONCLUSIONS

As our understanding of eukaryotic viral DNA replication increases it is becoming apparent that the funda-

mental mechanisms elucidated for prokaryotic DNA replication also apply to eukaryotes. This is exemplified by the sequence of events that has been shown to lead to the initiation of SV40 DNA replication. The formation of a large nucleoprotein complex containing T-Ag at the SV40 origin and subsequent unwinding of the DNA duplex is reminiscent of the situation in *E. coli* and bacteriophage λ . In both cases a sequence-specific DNA-binding protein recognizes and binds to multiple sites in the origin of replication (Dna A for *E. coli* or O protein for λ) and creates a region of single-stranded DNA. Dna B is then loaded onto the single-stranded DNA (by Dna C for *E. coli* or P protein for λ) and unwinds DNA by virtue of its helicase activity (LeBowitz & McMacken, 1986; Baker *et al.*, 1986). In SV40 all of these activities are incorporated into the multifunctional T-Ag, although it should be noted that T-Ag and Dna B migrate on the DNA in opposite directions. Recently protein N, a component of the *E. coli* primosome, has been shown to possess helicase activity and migrate on DNA with the same polarity as T-Ag (Lasken & Kornberg, 1988). As suggested in the model for SV40 DNA replication, T-Ag may advance with the replication fork and may be an integral part of a replisome engaged in concurrent replication of both strands of the DNA duplex. In this model a loop of single-stranded DNA from the lagging strand is twisted through 180° such that the direction of polymerization is the same as the leading strand. Chain elongation on the lagging strand is accompanied by passage of the template strand through the replisome (Sinha *et al.*, 1980).

A feature of eukaryotic virus replication is the role of transcriptional elements as components of origins of replication (reviewed by DePamphilis, 1988). In many cases the complete replication origin consists of a core region that is absolutely required for DNA replication and an auxilliary region that contains DNA sequences that are also components of the transcriptional control process. Viral replication origins appear to fall into two groups depending on whether or not the transcriptional elements can activate DNA replication in a manner that is independent of their position with respect to the core region. Since many of these elements are cell- and tissue-specific or inducible enhancers, their incorporation into viral replication origins may allow viruses to target particular cells in a host for high-level replication, or to remain dormant and replicate only in response to a particular stimulus.

For a detailed biochemical understanding of the control of eukaryotic DNA replication we will have to journey down the same path as that travelled by those investigating prokaryotic DNA replication: development of *in vitro* systems, purification of individual components and reconstitution of the replication process.

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