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The Initiation of Simian Virus 40 DNA Replication *In Vitro*

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ABSTRACT: DNA replication is a complicated process that is largely regulated during stages of initiation. The Siman Virus 40 *in vitro* replication system has served as an excellent model for studies of the initiation of DNA replication, and its regulation, in eukaryotes. Initiation of SV40 replication requires a single viral protein termed T-antigen, all other proteins are supplied by the host. The recent determination of the solution structure of the T-antigen domain that recognizes the SV40 origin has provided significant insights into the initiation process. For example, it has afforded a clearer understanding of origin recognition, T-antigen oligomerization, and DNA unwinding. Furthermore, the Simian virus 40 *in vitro* replication system has been used to study nascent DNA formation in the vicinity of the viral origin of replication. Among the conclusions drawn from these experiments is that nascent DNA synthesis does not initiate in the core origin *in vitro* and that Okazaki fragment formation is complex. These and related studies demonstrate that significant progress has been made in understanding the initiation of DNA synthesis at the molecular level.

KEY WORDS: T-antigen, Simian Virus 40, DNA replication, eukaryotes.

SECTION 1: INTRODUCTION TO THE SIMIAN VIRUS 40 REPLICATION SYSTEM

In any organism, initiation of DNA replication is a complex, highly regulated biochemical process.[204] Establishing the fundamental features of the initiation process in higher eukaryotic organisms has been difficult as basic components of the initiation process, such as replication origins, have yet to be isolated and characterized.[47] Moreover, only preliminary studies have been made of the origin recognition complex (ORC), the assemblage of proteins that is likely to help recognize eukaryotic origins.[15,52,98,112,137,145]

These limitations have been overcome, in part, by using viral-based assays to study initiation of replication. The Simian Virus 40 (SV40) *in vitro* replication system[220,377,434] has proven to be particularly useful for these studies. SV40 has a well-defined origin of replication (Ref. 83 and references therein) and depends on a single viral protein, termed T-antigen (T-ag), for initiation of DNA replication.[388] With the exception of T-ag, all of the proteins required for initiation of DNA replication are supplied by cells that are

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permissive for viral replication.[396] This dependence on cellular factors for SV40 replication has enabled an extensive analysis of the enzymology, and attendant mechanisms,[38] that operate during DNA replication in higher eukaryotes.

DNA replication in eukaryotic organisms, and the regulation of this process, has been the focus of a number of recent reviews. The topics covered by these reviews have included the cell cycle control of DNA replication,[71,370] initiation of chromosomal DNA replication,[50,323] the DNA polymerases that catalyze DNA replication,[10,39,422,423] and a summary of the mechanisms that ensure that DNA replication occurs only once in a given cell cycle.[29] Furthermore, a wide range of topics related to DNA replication in eukaryotic cells were covered in a recent book[93] and an additional text reviewed many of the techniques used to study replication.[49] SV40 DNA replication, and the role of T-ag in this process, has also been reviewed extensively.[55,95,124,125,158,171,186,187,317,376]

This review provides a detailed account of the initiation of SV40 DNA replication. A major recent advance in this field was the determination of the solution structure of the T-ag domain that recognizes the SV40 origin.[239] Indeed, having solved the structure of this domain, it is now possible to reevaluate many previous studies of T-ag in terms of the structure of this key domain. Results from these analyses, summarized herein, are helping to refine our understanding of how T-ag recognizes the SV40 origin. Furthermore, the SV40 *in vitro* replication system has been used to examine many other fundamental aspects of DNA replication, such as the proteins required for DNA synthesis and the mechanism of formation of daughter or "nascent" DNA strands. These and related topics are also summarized in the following sections.

Although initiation of SV40 replication is the focus of this review, the studies described are relevant to a broader arena. For example, they are likely to provide insights into human JC and BK viruses. Under immunosuppressed conditions, the JC virus can cause the fatal demyelinating disorder progressive multifocal leukoencephalopathy (PML), and it induces tumors in tissues of neural origin (reviewed in Ref. 356). BK virus is associated with[356] brain tumors of ventricular surfaces and non-brain tumors, including insulinomas and osteosarcomas.[175,356] The nucleotide homology between the SV40, JC, and BK virus T-ags is ~70 to 80%.[133,298] Therefore, studies of SV40 replication are relevant to the replication of these viruses and the pathogenesis of these diseases.

SECTION 2. THE SV40 ORIGIN AND T-ANTIGEN

I. INTRODUCTION

When SV40 virus infects monkey or human cells, it orchestrates a series of events that alter the normal regulation of the cell cycle.[125,396] In a preliminary step, transcription initiates at the viral early promoter and leads to the synthesis of both large and small T-ags.[396] Large T-ag is present mainly in the nucleus, where it commandeers cellular control mechanisms in order to drive the cell cycle into S phase.[33,97] This is a necessary process, because SV40 replicates efficiently only in S phase.[60,73,108,321,410] In a subsequent step, large T-ag down-regulates its own transcription[2,155,194,278,390] and initiates viral DNA replication.[388] These events are followed by late viral gene expression[2,184,194] and assembly of virions. In cells nonpermissive for replication, such as rodent

cells, SV40 T-ag is responsible for the initiation and maintenance of transformation.[125,396]

Small t-antigen is not required for viral replication,[125] although under certain conditions it can stimulate viral replication *in vivo*.[61] It has also been reported that SV40 encodes a third early protein, the 17-kDa protein.[454] However, the biological function(s) of the 17-kDa protein during lytic infections are not known. As they are not essential for replication, small t-antigen and the 17-kDa protein are not considered in this review.

A critical event during the initiation of SV40 DNA replication, either *in vivo* or *in vitro,* is the binding of T-ag to the SV40 origin. Binding of T-ag to the SV40 origin is a complicated process that has been the subject of numerous studies (reviewed in Ref. 25, 125). Prior to considering recent progress in understanding the T-ag/origin interaction, the SV40 origin and T-ag are introduced as separate entities.

II. THE SV40 ORIGIN OF REPLICATION

The SV40 core origin of replication (Figure 1) extends between nucleotides 5211 and 31 (Ref. 83; references therein); these 64-bp of DNA are both necessary and sufficient for initiation of SV40 replication *in vivo* and *in vitro* (Refs. 90, 99, 222, 279, 375); references therein).

The core origin contains three functional regions;[77,83–85,291] the early palindrome (EP), a cluster of four GAGGC pentanucleotides, collectively referred to as the pentanucleotide palindrome (PEN) (also termed binding site II), and a 17-bp adenine-thymine (AT)-rich domain. These three domains coordinate the binding, melting, and unwinding activities of T-ag[25] (see below). The arrangement of the SV40 core origin is very similar to the core origin domains of human (JC and BK) and murine (polyoma) papovaviruses.[223]

The GAGGC pentanucleotides in the PEN are arranged as two pairs that are inverted relative to each other; as a result, this region is centered on a 27-bp perfect inverted repeat.[83,85] The individual pentanucleotides serve as T-ag binding sites,[91,389,393,394] and binding to all four pentanucleotides has been reported to be required for initiation of DNA synthesis.[85,99,279,346] Base substitutions in the single base pairs separating the pentanucleotides were associated with wild-type levels of DNA synthesis; however, duplication of the same base pairs drastically reduced

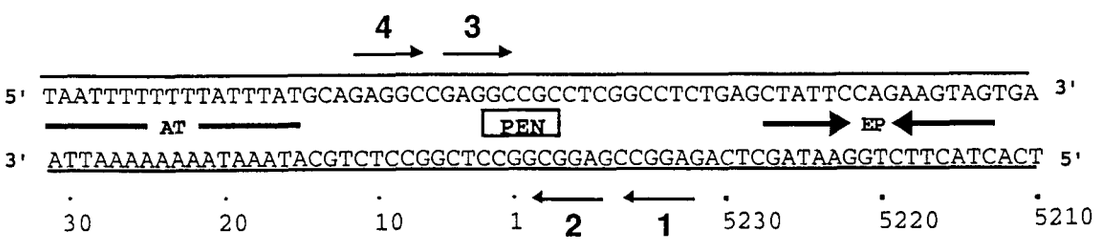

FIGURE 1. The SV40 core origin, a region of DNA that is both necessary and sufficient for SV40 replication.[83,222,375] The positions of four GAGGC pentanucleotides within site II that serve as binding sites for T-ag, are indicated by the arrows numbered 1 to 4. Also shown are the locations of the early palindrome (EP) and the adenine/thymine-rich (AT) region. Sequences are numbered according to Fiers.[396]

replication.[85,292] These and related studies[27,65,86,218,292,293] demonstrated that the relative position of each pentanucleotide is crucial to the initiation of replication.

The EP is a two-part domain that extends between 5213 and 5227 and includes most of an 8-bp region (nts 5210 to 5217) that is melted after T-ag binding to the SV40 core origin.[26,28,291,293] This 8-bp region is highly conserved among papovaviruses of primates, and mutations in this region result in a significant loss of function.[77,83] This region of the EP has also been termed a DNA unwinding element (DUE).[231] The second arm of the EP, the segment proximal to the PEN, can be mutated with only a small decrease in replication activity, and may function as a spacer.[83] Finally, in the presence of relatively high protein concentrations, the EP is capable of independent interactions with T-ag.[291]

The AT-rich region is essential for DNA replication,[138,375] and it is known to undergo protein independent DNA bending.[84,163] As with similar regions in other origins, the relatively weak base pairing of the AT tract may play a role in strand separation during T-ag-dependent DNA unwinding. Consistent with this hypothesis, the AT region is structurally distorted after binding of T-ag to site II,[28,291] and sequences from this region help to activate T-ag's unwinding activity.[223,291] Furthermore, the AT tract is the binding site for cellular factors, such as the transcription factor Oct 1,[198] which may negatively regulate viral replication. Finally, residues 16 to 21 serve as the TATA box for SV40 immediate-early transcription.[125]

Proof that all three domains are required for initiation of SV40 replication was provided by experiments demonstrating that single base substitutions in any domain resulted in a significant decrease in replication.[77,83,85] As with the spacing in the PEN domain, it is not possible to alter the spacing between the three core origin domains.[292] This is an additional indication that the precise structure of the origin is critical for efficient replication.

III. LARGE T-ANTIGEN

Large T-ag is composed of 708 amino acids; the predicted molecular weight of T-ag is ~82 kDa.[40] To initiate SV40 DNA replication *in vivo*, most of the T-ag molecule is required.[95] Moreover, numerous posttranslational modifications of T-ag have been described, including phosphorylation, O-glycosylation, adenylyation, ADP ribosylation, and acylation (reviewed in Refs. 95, 125, 368). With the exception of the phosphorylation events, little is known about the functional consequences of the posttranslational modifications. Studies of T-ag's functions during initiation of DNA replication have benefited tremendously from the baculovirus expression system for producing large amounts of this protein[209,277,286] and from immunoaffinity isolation techniques.[102,348] Finally, it is noted that a database has been compiled of mutations that alter the various activities of T-ag.[196]

A. The Structural and Functional Organization of T-Antigen

Studies of T-ag, including partial proteolysis,[338,349,350,443] expression of subfragments of T-ag as recombinant proteins,[5,62,380] and genetic studies,[5,66,294,380] have demonstrated that T-ag is composed of several interrelated functional, and in certain instances structural, domains (reviewed in Refs. 124, 125, 298, 313).

A map of T-ag depicting the locations of domains necessary, and in many cases,

sufficient, for a given activity is presented in Figure 2. What is known about the role(s) of the individual domains in the initiation of SV40 DNA replication is discussed in the following sections.

1. The N-Terminal Region of T-ag

The N-terminal region is frequently defined as the region extending between the initiation codon and the nuclear localization signal ([NLS]; the minimal signal sufficient for nuclear transport[181]). This assignment is based, in part, on the fragments generated by mild proteolysis of T-ag.[338,443]

The N-terminal region contributes to the transforming function of T-ag (reviewed in Ref. 125). It also interacts with a number of host cell proteins that are involved in regulating cell cycle progression and gene expression (e.g., Refs. 97, 103). Furthermore, this region contains at least two sites necessary for realization of the full mitogenic activity of T-ag. One site forms part of the binding site for Rb family members.[87,121,266] The interaction of Rb family members with T-ag is necessary for stimulating the transcription of E2F-dependent genes (reviewed in Refs. 125, 426).

What constitutes the second mitogenic activity present in the N-terminal region has not been completely established. However,

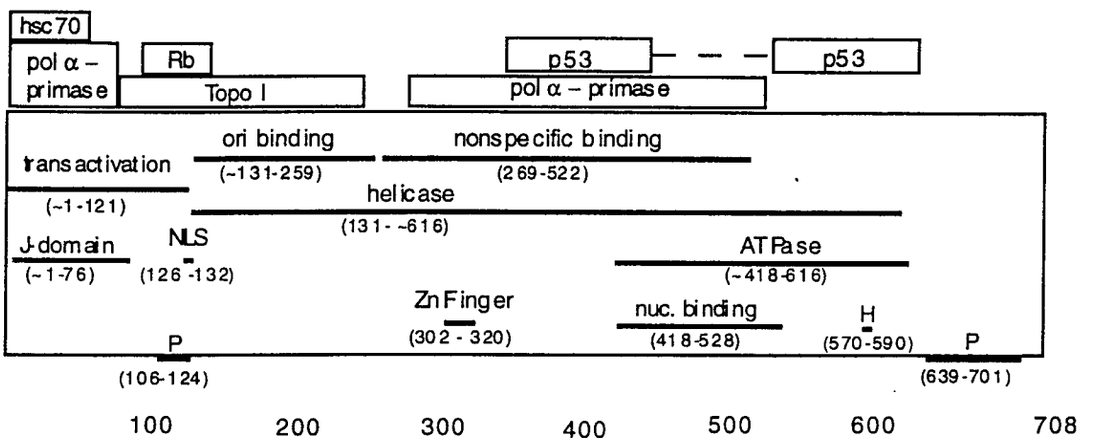

FIGURE 2. A map of T-ag depicting domains involved in SV40 DNA replication (within the rectangle), positions at which particular cellular proteins bind T-ag (upper surface of rectangle) and the locations of two clusters of serine and threonine residues at which regulatory phosphorylation events take place[331,409] (lower surface of rectangle). The numbers within the parenthesis provide the limits of a given domain or activity. Regions required for DNA replication include the "ori binding" domain (T-ag-obd)[125] and a segment of T-ag that interacts with the origin-binding domain to enable optimal "non-specific binding" to double-stranded DNA.[229] Also presented are the locations of residues required for T-ag's "helicase" activity,[354] a region containing the "ATPase" domain[62,139,443] and a region with an amino acid sequence similar to known "nucleotide binding" sites.[31,64] Moreover, the positions of the nuclear localization signal "NLS",[181] the putative "Zn finger" region,[234] Dna J domain,[51,188,362] a region required for low levels of "trans-activation" of cellular genes[457] and a region rich in hydrophobic amino acids (H)[296,384] are also indicated. It has also been noted that the region between 372 and 648 shares limited homology with the *recA* protein[339] and a slightly smaller region (400 to 600) is involved in translational regulation.[311] The significance of T-ag's interactions with individual cellular proteins, stippled rectangles, is discussed in the text.

it has been observed that the amino terminal ~75 residues of T-ag are strikingly homologous to the consensus "J-domain" motif,[51,185,188,362] and that this region functions as a J-domain in a bacterial assay system.[185] Furthermore, this region is known to interact with a member of the 70-kDa heat shock protein family, hsc 70[51,329] and with a yeast hsc 70 homolog.[362] In view of the sequence homology between the N-terminus of T-ag and other J-domains, it is interesting that the solution structure of the *E. coli* DNA J[164,297,383] and human HDJ–1 J-domains[310] have been determined. Both domains consist of four α-helices in which a highly conserved His-Pro-Asp motif, which is necessary for specific association with hsc 70,[51] is in a loop connecting helix II and helix III. Computer modeling experiments indicate that the J-domain of T-ag will have a very similar fold.[51,362] In view of these observations, it was proposed that the second mitogenic activity of T-ag may be related to its ability to regulate the assembly/disassembly of nucleoprotein complexes.[51] Srinivasan et al.[362] also proposed that the J domain may chaperone the rearrangement of multiprotein complexes.

The ability of the N-terminus to stimulate S phase entry may also be related to its capacity to activate gene expression in trans (the amino-terminal 121 or 138 amino acid residues of T-ag are sufficient for weak transactivation while full transactivation, requires the amino-terminal 249 or 383 residues) (Refs. 148, 17, 304, 363, 457 and references therein). According to this hypothesis, N-terminal-dependent induction of gene expression results in an increase in the activity of limiting cellular replication factors.[250] In view of this theory, it is interesting that mutations that inactivate the J domain for binding to hsc 70 also abolish the ability of T-ag to transactivate E2F-containing promoters.[345] Thus, the ability of this region to transactivate gene expression may be directly related to its ability to interact with heat shock protein family members.

While the exact mechanism(s) by which the N-terminal end of T-ag promotes S-phase entry has not been determined, it is apparent that other viral proteins rely on similar domains, and perhaps similar mechanisms, to control cell cycle progression. For example, it has been demonstrated that the C-terminus of polyoma virus large T-ag is able to replicate *in vivo* provided it is complemented in trans by a protein domain that is capable of inducing entry into S phase.[142]

This complicated domain of T-ag is also necessary for viral replication (e.g., Refs. 67, 143, 250, 300, 363). However, rather than supplying an activity directly required for DNA replication, the previously described mitogenic properties of this region are thought to contribute the essential replication function. Consistent with this hypothesis, the J-domain of T-ag is required for efficient viral DNA replication *in vivo*.[51,300] Indeed, it was suggested that T-ag might utilize its J-domain in a manner analogous to the way *E. coli* Dna J is used in bacteriophage λ replication.[51]

Whether the N-terminal region of T-ag promotes SV40 replication *in vitro* is less certain. For example, the "D2" T-ag lacks the amino-terminal 114 amino acids[12,395] and this molecule was only 20 to 30% as active as wild-type T-ag when used in *in vitro* replication reactions.[201] In addition, a T-ag fragment extending between amino acids 83 to 708 catalyzed SV40 replication at levels two to fivefold lower than wild type.[427] However, T-ag molecules containing a deletion in the N-terminal end, missing residues 67 to 82, catalyze replication better *in vitro* than wild-type T-ag.[250] These studies demonstrate that while there is some uncertainty regarding the ability of this region to stimulate DNA synthesis, the N-terminal end of T-ag is not required for SV40 DNA replication *in vitro*.

2. The T-Antigen Origin Binding Domain

Among the most extensively studied regions of T-ag is the origin binding domain (T-ag-obd) (reviewed in Refs. 124, 125). This domain has been mapped to amino acids 131 to ~259;[5,62,125,294,349,380] others have mapped the N-terminus of the binding domain to amino acid 132 and the C-terminus to approximately amino acid 246.[253] It has been suggested that the T-ag-obd is the central domain through which all interactions with DNA take place.[229] Consistent with this proposal, the T-ag-obd is sufficient for sequence specific binding to the SV40 origin region.[5,169,180,253,294,295,351,354] This domain is also required for nonspecific binding to double stranded DNA[229,443] and may be necessary for binding to single-stranded DNA.[253,443] Moreover, in the context of the whole protein, this domain is known to function in many of T-ag's related biological roles. For instance, mutagenesis studies suggest a role for this domain in oligomerization events and DNA structural distortions, including melting and untwisting the SV40 origin: it is also essential for DNA unwinding and for T-ag's helicase function.[5,253,294,349,380,443] Finally, it has an activity specifically related to DNA replication *in vivo* and it may even function in virus assembly.[353,444]

a. Solution Structure of the T-Antigen Origin Binding Domain

A major obstacle to understanding T-ag's functions, such as origin-specific binding, has been the total lack of structural information about this molecule. In part, this situation reflects the failure to obtain diffractable crystals of T-ag.[125] Therefore, it was decided to obtain structural information about individual T-ag domains. In an initial series of experiments, the solution structure of the T-ag origin binding domain (T-ag-obd$_{131-260}$) was determined using NMR techniques.[239] These studies revealed that the T-ag-obd$_{131-260}$ is a five-stranded antiparallel β-sheet, flanked on one side by two α-helices and on the second side by one α-helix and one 3_{10}-helix. It was concluded that the overall tertiary structure represents a new fold.[239]

A topological diagram of the secondary structure elements of the T-ag-obd$_{131-260}$ is presented in Figure 3. As shown in the diagram, helices connect parallel, non-adjacent, β-strands. Interpretation of the T-ag-obd$_{131-260}$ structure, in terms of its diverse biological functions, was greatly enhanced by previous mutagenesis studies (Refs. 353, 444; and references therein). Preliminary insights into the functions of the T-ag-obd, based on its solution structure, is presented in the following sections.

3. Domains C-Terminal of the T-ag-obd

Distal to the T-ag-obd are several additional domains essential for DNA replication (Figure 2). These include a region that cooperates with the origin-binding domain to give rise to wild-type levels of non-specific binding to duplex DNA.[229] Furthermore, a region overlapping the non-specific DNA binding domain is required for T-ag's helicase activity; as with non-specific DNA binding, the T-ag-obd is essential for T-ag's helicase activity.[443] Additional regions required for T-ag's helicase activity include the ATPase[62,139,443] and nucleotide binding domains.[31] Experiments have demonstrated that T-ag's ATPase activity is required for the initiation of DNA replication;[62] for

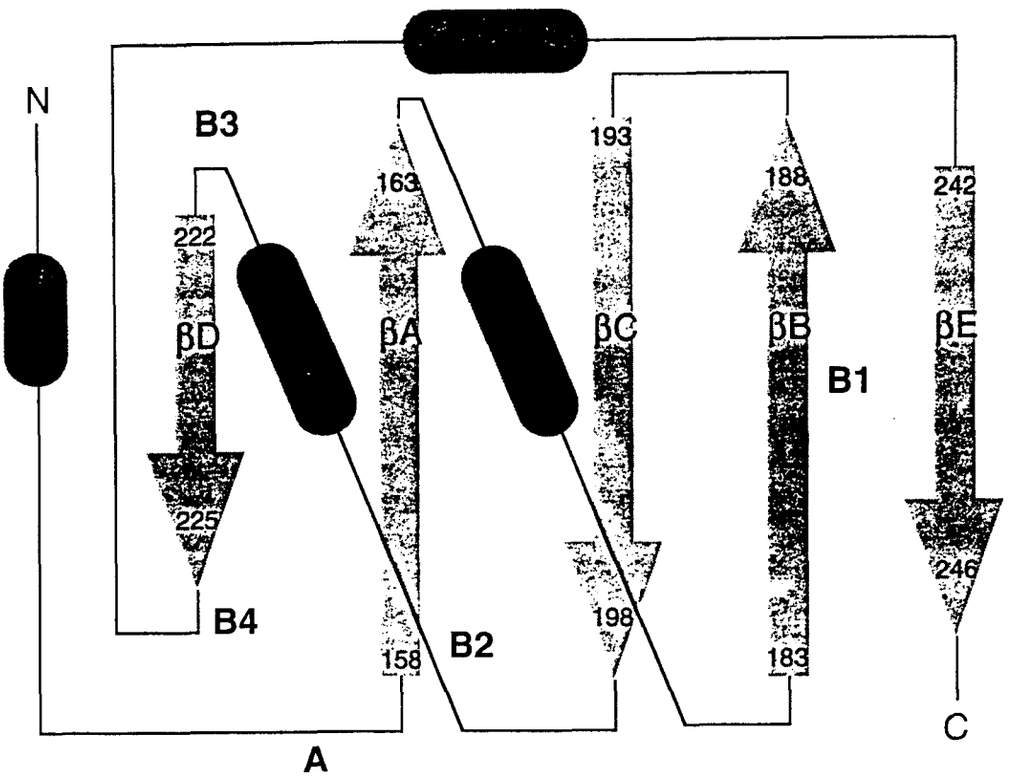

FIGURE 3. Topology diagram of the T-ag-obd$_{131-260}$ adapted from Luo et al.[239] Helices are shown as ovals and strands as arrows. The first and last residues of individual secondary structural element are numbered. Helices αC and αB are on one side of the central five-stranded antiparallel β sheet, while the 3_{10}A and αD helices are located on the opposite side. Also presented are the relative positions of five functional subdomains (A (147–159), B1 (183–187), B2 (203–207), B3 (213–220), and B4 (226–227)) located in the T-ag-obd$_{131-260}$.[351,444]

example, T-ag molecules containing the C11 mutations (Pro522→Ser and Pro 549→His) bind to the SV40 origin like wild-type T-ag, but are replication defective and lack any detectable ATPase activity.[243,375] A topological model for the ATP binding site (residues 418–528) has aided attempts at understanding this region in T-ag.[31]

The location of the putative C_2H_2 Zn finger region is also indicated in Figure 2. Although it is not clear whether this region functions as an independent domain, the zinc-binding motif is conserved among the Polyomavirus group,[298] and this region is known to play a role in T-ag/origin and T-ag/T-ag interactions.[169,235] Moreover, the region between residues 570 to 590 contains the largest stretch of hydrophobic amino acids in T-ag; studies indicate that these residues play a role in regulating T-ag's higher-order structural and functional relationships (e.g., oligomerization).[296,384] The extreme carboxy-terminal 38 amino acids of T-ag are not required for DNA replication but encode an activity that is thought to play a role in viral assembly.[193,299,364] This activity has been termed "helper function". Through an unknown mechanism, helper function also enables adenoviruses to replicate in normally nonpermissive monkey cells.[129,303] Finally, relative to the reference SV40 strain 776,

different strains of SV40 exhibit significant divergence in the C-terminus (~amino acids 623 to 708) of T-ag.[369]

It is noted that in addition to promoting viral DNA synthesis, T-ag also stimulates cellular DNA synthesis.[60,97,103,166,167,271] Relatively efficient stimulation of cellular DNA synthesis can be induced by a fragment of T-ag containing only the amino-terminal 147,[359] 259,[97,103] or 272 amino acids.[360]

B. Association of Cellular Proteins with T-Antigen

To carry out its multiple biological functions, T-ag must associate with many cellular proteins. The positions at which certain cellular proteins, particularly those that are directly required for DNA replication, bind to T-ag are indicated in Figure 2. The pol α–primase complex has two binding sites on T-ag: a strong binding site located between ~272 and 517 and a secondary site located within the first 82 residues.[105,107,136,334,355,427] Topoisomerase I (Topo I) interacts with two regions of T-ag; one region maps between residues 82 to 246 and a second weaker site is present after residue 246.[352] The human single-stranded binding protein (HSSB)[123,436,438] is known to interact with T-ag, but the site of interaction has yet to be established.[106,352]

T-ag also interacts with a group of proteins that play key roles in controlling the cell cycle, including p53,[208,233] Rb, and Rb family members p107 and p130.[87,109,121,453] The ability of T-ag to transform cells is thought to be mainly due to its ability to bind and sequester p53 and Rb (for a review see Ref. 217); however, the J domain is also necessary for transformation (e.g., Ref. 362). The p53 binding site on T-ag is complex; *in vitro* studies suggested it is located between residues 272 to 517,[263,334] while *in vivo* studies indicated that p53 binds to a bipartite region, including amino acids 351 to 450 and 533 to 626.[197] Related studies demonstrated that p53 competes with the pol α-primase complex for the same C-terminal site(s) on T-ag.[32,135,136,419] However, while relevant to T-ag-dependent transformation, it is clear that an interaction between T-ag and p53 is not required for SV40 replication.[230] Finally, the interaction of Rb and Rb family members with T-ag requires a domain extending between residues 102 to 114 that contains the LXCXE motif.[87,109,121]

Proteins involved in modulating aspects of transcription, such as CBP family members (CREB-binding protein (CBP), p300, and p400), also bind to T-ag. Experiments indicate that the carboxy-terminus of T-ag is sufficent for binding of CBP family members,[227] although the LXCXE motif may contribute to the stability of the complex.[110,227] Furthermore, T-ag associates with transcription factors, including AP2[258] and the TATA-binding protein (TBP) in the TFIID complex.[74,97,148,176] The TBP has been reported to interact with T-ag residues 5 to 172,[148] 101 to 249,[17] and both an amino terminal 138 fragment and a fragment spanning residues 133 to 249.[176] It has also been reported that a binding site for the Transcription Enhancing Factor 1 (TEF–1) is present in the region of T-ag spanning residues 133 to 249.[17,148] Indeed, the Ser 189 → Asn mutation disrupted the T-ag/TEF–1 interaction[17] and generated a T-ag defective for transformation.[97,182] Based on these observations, it was suggested that TEF–1 may play an important role in the regulation of genes important for cellular growth control.[17,97,103,148] Furthermore, the region of T-ag spanning amino acids 133 to 249 was reported to interact with the general transcription factor TFIIB,[97,176] the transcription activator Sp1 and the 140-kDa subunit of RNA polymerase II.[176] Additional studies indicated that Lysines 173 and 174 play

crucial roles in these protein-protein interactions;[176] however, the binding of TFIIB, Sp1, and RNA polymerase II to this region are mutually exclusive events.[176] The locations within the T-ag-obd$_{131-260}$ of Lysines 173 and 174 and Ser 189 (the residue implicated in TEF I binding) are shown in Plate 2.

T-ag also interacts with several other cellular and viral proteins that are not easily classified as replication, regulatory, or transcription factors. For instance, T-ag residues 116 to 126 are necessary for interaction with hepatitis B virus X protein.[342] Moreover, T-ag interacts with the MDM2 protein[35] and, as previously mentioned, with a member of the 70-kDa heat shock protein family, hsc70.[51,329] In mouse cells, the N terminus of T-ag interacts with a 185-kDa protein of unknown function.[203] It has been suggested that the NLS interacts with nucleolin.[446] It is likely that additional proteins will be discovered that interact with T-ag.

SECTION 3. BINDING OF T-ag (OR THE T-ag-obd) TO DNA

I. INTRODUCTION

A critical function of T-ag during the initiation of SV40 replication is site specific binding to the SV40 core origin.[244,314,393,394,433] Recognition and subsequent binding of T-ag to the SV40 origin is a rapid process[80,274,393] T-ag (and T-ag fragments) isolated from *E. coli* bind to the SV40 origin with activities similar to those of T-ag isolated from insect, monkey, or human cells (e.g., Refs. 5, 180). Because T-ag produced in bacterial cells does not undergo specific posttranslational modifications, these modifications are not essential for T-ag's origin DNA-binding activity. However, posttranslational modifications, particularly phosphorylations, play an important role in subsequent replication events[124,125,308] (see below).

II. BINDING OF T-ANTIGEN TO THE SV40 CORE ORIGIN

A subclass of phosphorylated T-ag monomers binds to individual GAGGC sequences within the SV40 core origin.[91,127,140,178,218,324,333,343,389,393,442] Methylation and ethylation interference assays were used to map essential nucleotide contacts between bound T-ag molecules and the SV40 origin.[28,91,178] These studies revealed that T-ag generally binds DNA via the major groove.[178] Because T-ag does not bind the four GAGGC sequences with equal stability, adjacent sequences must contribute to binding affinity.[218,442] The equilibrium dissociation constant (K_d) values for the T-ag/SV40 origin interaction was determined to be 4×10^{-9} to 10×10^{-10} *M*.[102,165,178]

A. Insights Into GAGGC Binding Based on Studies of the T-Antigen Origin Binding Domain

It was demonstrated that a T-ag-obd species purified from *E. coli* (T-ag-obd$_{1-249}$) preferentially bound the SV40 origin;[253,380] similar results were obtained using fragments extending between residues 1 to 260[5] and 131 to 260.[180] Studies also demonstrated that the T-ag-obd$_{131-260}$ selectively interacted with oligonucleotides containing GAGGC sequences.[239] Furthermore, Joo et al.[180] reported that on a molar basis, T-ag is only slightly better (2- to 3-fold) than the T-ag-

$obd_{131-260}$ in its ability to interact with DNA from the SV40 origin.[180] Thus, T-ag-obd containing polypeptides are necessary and sufficient for high-affinity binding to the SV40 origin and serve as excellent models for studies of T-ag's initial interactions with the origin.[180] Additional regions of T-ag may slightly stimulate binding,[5,62,169,253,254,261,313,316,337,350] while other regions may destabilize the T-ag-obd/origin interaction.[253,261,380] Collectively, these interactions may account for the slight reduction in the ability of the T-ag-$obd_{131-260}$ to interact with the SV40 origin relative to that of T-ag.

1. Mapping T-ag Residues Required for GAGGC Binding

Mutagenesis studies of the T-ag-obd, in the context of T-ag, have provided a wealth of genetic information regarding the organization of this domain. For example, it was observed that the T-ag-obd is itself subdivided into functional elements.[351,354] Two elements, termed A (residues 147 to 159) and B2 (residues 203 to 207), were demonstrated to be essential for sequence specific recognition of the GAGGC pentanucleotides within the SV40 origin.[351,444] The importance of these elements is underscored by the observation that the A and B2 regions are nearly identical in all known papovavirus T-ag's.[351] Within element A, Asn–153 and Thr–155 were reported to be the most important for origin-specific binding. Within element B2, Arg–204 was demonstrated to be the most important for sequence-specific binding, although residues Val 205 and Ala 207 are also important.[351,354] Moreover, the A and B2 elements contain the only residues where a mutation leads to a class I phenotype (origin binding negative but positive for nonspecific DNA binding and oligomerization[353]). In view of these studies, it was proposed that elements A and B2 make direct contact with the GAGGC pentanucleotides.[351]

The hypothesis that the A and B2 regions interact with the GAGGC pentanucleotides is supported by additional studies. For example, Paucha et al. also reported that the domain stretching between amino acids 147 and 166 (~element A) is involved in binding to site II and to site I.[294] (Site I is a 17-bp region flanking the core origin[325] that is discussed in a following section.) Furthermore, T-ag from the SV80 cell line contains a single point mutation that converted Ser 147 to Asn, a substitution that resulted in a replication-defective T-ag molecule.[141] Moreover, it is noted that the C6–2 mutation (Asn 153 → Thr)[143,309] also maps to the A region and that a deletion of four amino acids close to this region (Δ143 to 146) inactivated T-ag for replication.[397] Additional experiments with the T22 T-ag mutant (His 203 → Gln) demonstrated that His 203 is also important in site-specific binding.[242,261,375] Finally, unlike wild-type T-ag, T-ag molecules containing mutations in codons 157 and 166 can recognize an altered sequence at the SV40 origin.[244,347]

2. The Solution Structure of the T-ag-$obd_{131-260}$

The structure of the T-ag-$obd_{131-260}$[239] is shown in Plate 1*. The relative positions of the A (147–159) and B2 (203–207) elements within the T-ag-$obd_{131-260}$ are indicated by the "non-yellow" residues (the "red residue" Phe 197 is an exception [see Plate 1

* Plate 1 appears following page 504.

caption]). It is apparent from this figure that although the A and B2 elements are separated in the primary sequence (Figure 3), they are close to each other in the tertiary structure. Element A forms a loop between the 3_{10}-helix and strand βA. Element B2 resides partially in the loop between βC and αC and extends into αC.[239] In view of these observations, and the extensive mutagenesis studies, it was concluded that binding to individual GAGGC pentanculeotides is largely mediated by this pair of loops. The solution structure of the T-ag-obd$_{131-260}$ confirmed the prediction that these two elements would be located together in space.[351]

There is increasing evidence that polypeptide loops are a common binding motif for proteins that interact with DNA. For example, the HhaI methyltransferase binds to DNA via loops[202] and it is thought that the PerA DNA helicase[382] and RecA,[379] also interact with DNA via polypeptide loops. Loops also play an important role in autoantibody recognition of single-stranded DNA,[162] and the p53 protein utilizes polypeptide loops as part of a loop-sheet-helix motif when binding DNA.[59] Several other examples of proteins that interact with DNA via flexible loops are cited in Kwon et al.[207] Thus, the A and B2 loops of the T-ag-obd are additional examples of an important motif frequently employed by proteins that interact with DNA.

One concern regarding the mutagenesis studies used to suggest functional regions within the T-ag-obd was that they were all conducted in the context of full-length T-ag. Therefore, experiments were performed to determine whether purified T-ag-obd$_{131-260}$ molecules, containing mutations of interest, had the same phenotype as they do in full-length T-ag. For example, a T-ag-obd$_{131-260}$ variant containing the Thr 155 → Ser substitution[354] was constructed and subsequently purified.[177] HSQC experiments[13] demonstrated that this mutant T-ag-obd$_{131-260}$ molecule was properly folded. Nevertheless, as predicted by the mutagenesis studies employing full-length T-ag,[354] the T-ag-obd$_{131-260}$ molecule containing the Thr 155 → Ser substitution was no longer capable of site-specific binding to the SV40 origin.[177] These studies indicate that mutated forms of the T-ag-obd$_{131-260}$ share the same phenotypes as T-ag molecules containing the same mutations.

3. T-ag-obd$_{131-260}$ Structural Changes upon Binding to Double-Stranded DNA

Two-dimensional $^{1}H/^{15}N$ HSQC experiments were used to determine whether conformational changes take place in the T-ag-obd$_{131-260}$ after binding to oligonucleotides containing GAGGC pentanucleotides.[239] As shown in Figure 4, amide backbone resonances that exhibit the largest chemical shift changes after GAGGC binding were limited to four regions. One region spans region A (residues 147 to 159), a second region spans region B2 (residues 203 to 207), a third region spans region B4 (residues 226 to 227),[444] and the fourth region is in the extreme carboxy terminal region of the T-ag-obd$_{131-260}$ molecule. Inspection of Plates 4 and 5, and data in Luo et al.[239] demonstrate that these four regions constitute a continuous surface on the protein. Similar experiments were conducted with a control oligonucleotide lacking the GAGGC pentanucleotide. It was observed that the complex formed with the control oligonucleotide differed in several ways from the complex formed with the GAGGC containing oligonucleotide.[239] For instance, evidence for a low-barrier hydrogen bond[63] was detected with the GAGGC containing oligonucleotide but not the control oligonucleotide.[239] However, the largest chemical shifts induced by binding to the control oligonucleotide mapped to the same region of the

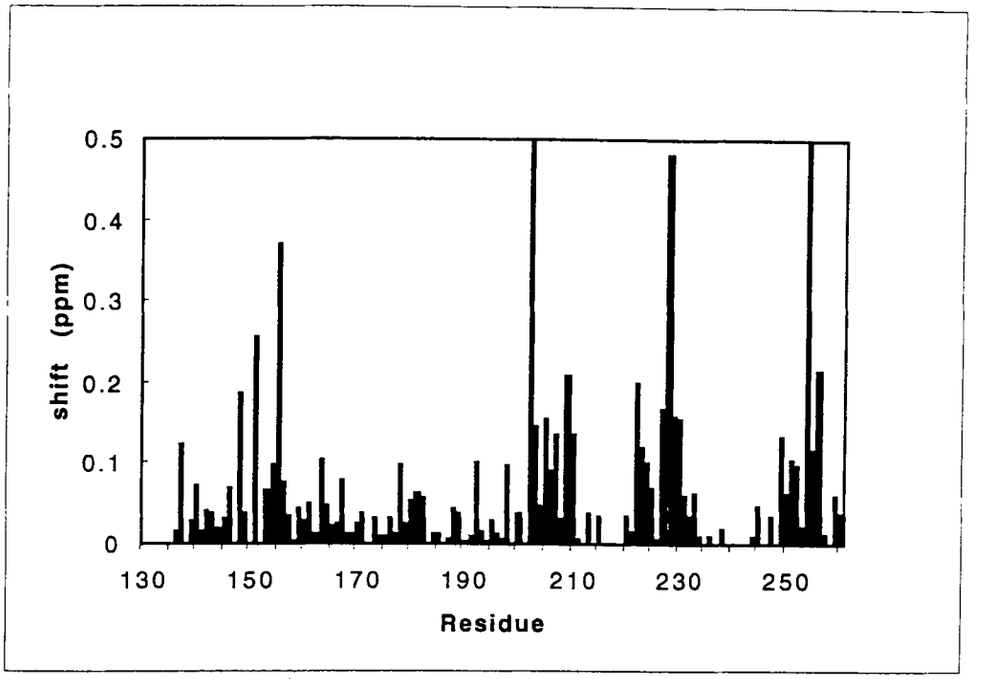

FIGURE 4. Regions of the T-ag-obd$_{131-260}$ that exhibit significant (>0.1 ppm in ^{1}H dimension) chemical shift changes in their amide backbone resonances after binding to a GAGGC containing double-stranded oligonucleotide. The A (147–159), B2 (203–207), B4 (226–227), and carboxy termini are among the regions that exhibit large chemical shifts after binding the GAGGC containing oligonucleotide. The B1 (183–187) and B3 (213–220) regions do not exhibit similar chemical shifts. Analogous chemical shift changes were induced after binding of a control double-stranded oligonucleotide.

T-ag-obd$_{131-260}$ surface as those induced by the GAGGC containing oligonucleotide.[239] These observations suggest that the same region of the molecule is responsible for both sequence specific and non-sequence specific DNA interactions.

4. Groups within GAGGC Pentanucleotides Necessary for the T-ag-obd$_{131-260}$/Origin Interaction

Mutagenesis studies of individual pentanucleotides have provided additional insights into the interactions of T-ag (and presumably the T-ag-obd$_{131-260}$) monomers and GAGGC sequences. For example, it was demonstrated that T-ag binds to pentanucleotides with alternative bases at positions 1, 2, and 5. However, alternative bases were not tolerated at positions 3 and 4.[91,442] In related experiments, Deb et al.[85] created single transversions of each base pair in pentanucleotide 1 (Figure 1), the strongest binding site in the origin palindrome,[218,389] and tested the ability of these mutants to replicate *in vivo*. They concluded that at the first position of the GAGGC sequence, T-ag makes contacts with molecular sites common to G-C and A-T base pairs (e.g., N7 atoms of either the guanine or adenine bases). Their studies also revealed that at the fourth position, T-ag is likely to make contacts with molecular sites

unique to the G-C base pair. Results from these experiments, combined with the previously described T-ag mutagenesis studies,[351,353,354,444] should serve as useful guides for determining the fidelity of T-ag/GAGGC complex structure(s) when they become available.

B. The T-ag-obd B3 Region: An Element That Promotes Interactions with the Core Origin and DNA Unwinding

A T-ag mutant (Lys 214 → Glu) was isolated and shown to be defective for viral DNA synthesis.[381] This mutation is now known to reside within an element termed B3 (residues 213 to 220), a region that promotes binding of T-ag to site II but not to site I.[351] The B3 region is also known to be involved in unwinding origin-containing closed circular DNA.[444] Inspection of Figure 4 reveals that unlike the A and B2 regions, the B3 region does not undergo structural distortions after binding to DNA.

The position of the B3 element within the T-ag-obd$_{131-260}$ (the region colored pink) is shown in Plate 2*. Additional residues known to be important for DNA replication 167–168 (the D element)[353,444] are positioned in space opposite the B3 element.

It is not clear how the B3 element promotes binding to the core origin or DNA unwinding, although studies demonstrating that phosphorylation of Thr 124 enhances the interaction of T-ag fragments 1 to 259 and 89 to 259 with the core origin may be relevant to this issue.[254] The phosphorylation-dependent activation of core origin binding was mediated by amino acids in element B3 and Lys 167.[254] It was proposed that phosphorylation of Thr-124 induces conformational changes in the T-ag-obd and that B3 residues may be involved in this conformational change.[254] These conformational changes may promote the interaction of the B3 element with sequences unique to the core origin, such as the EP, the site of initial DNA unwinding.[28,291]

C. Comparison of the T-ag-obd$_{131-260}$ and Related Folds

Having determined the solution structure of the T-ag-obd$_{131-260}$,[239] it was of interest to determine the extent to which it resembled previously described molecules. Therefore, the Cα coordinates were submitted to the Dali server for comparison to the structures in the Brookhaven Protein Data Bank.[168] The Dali server identified 15 structures with statistically significant homology to the T-ag-obd$_{131-260}$ (Z score greater than two). The two most significant matches were to the RNA binding domain of the U1A protein[8] (Z score of 4.6) and the DNA binding domain of the E2 protein of Bovine papilloma virus[161] (Z score of 4.2).

1. Homology with Bovine Papilloma Virus E2

The structural homology between the T-ag-obd$_{131-260}$ and the E2 protein (and therefore the structurally homologous DNA-binding domain of the Epstein-Barr virus origin-binding protein[21]) was of considerable interest given the limited structural information about proteins that interact with origins of replication. Indeed, structural information is limited to this set of three virally encoded DNA binding domains; two of

* Plate 2 appears after page 504.

which are identical (E2 and EBNA1) and one, the T-ag-obd$_{131-260}$, that contains regions that are partially homologous to the other two.

The topological resemblance between the T-ag-obd$_{131-260}$ and E2 DNA-binding domain of the bovine papillomavirus–1 extends between T-ag-obd$_{131-260}$ regions βA through βD (including α-helices B and C [Figure 3]) and E2 DNA-binding domain regions β1 to β5.[161] To further examine this homology, the structures of these two domains were superimposed using the optimal alignment suggested by the Dali server and the program INSIGHT (Biosym, San Diego, California). The Cα coordinates for the E2 protein were obtained from the Brookhaven Protein Data Bank (file 2BOP); the Cα coordinates for the T-ag-obd$_{131-260}$ are also present in the Brookhaven Protein Data Bank[239] (file 1TBD). Inspection of Plate 3* demonstrates that these molecules can be aligned to a significant extent. Not only are the β sheets aligned, but there is considerable overlap between T-ag-obd$_{131-260}$ helices αB and αC and E2 helices α1 and α2 (Plate 3).

While the origin DNA binding domains of E2, EBNA 1, and the T-ag-obd$_{131-260}$ are clearly related biologically, the significance of their structural homology is less certain. For instance, the A and B2 regions of the T-ag-obd$_{131-260}$ are located at positions that are unrelated to the E2 regions responsible for site specific binding (Plate 3; green loops). Furthermore, in addition to the shared βαββαβ fold, the T-ag-obd$_{131-260}$ has a fifth β-strand (βE) and two additional helices (3_{10}A and αD) that are not present in either E2 or EBNA1. The additional α helices cover the "second side" of the central β sheet. This arrangement prevents the T-ag-obd$_{131-260}$ from forming an antiparallel β barrel similar to those formed by E2 and EBNA1.[21,161]

2. Homology with the RNA Binding Domain of U1A

The homology between the T-ag-obd$_{131-260}$ and the RNA binding domain of U1A protein is even more extensive than that with the E2 binding domain. As with the T-ag-obd$_{131-260}$, the RNA binding domain of U1A protein is flanked on both sides by α-helices.[8] The homology between these protein domains is interesting, given that T-ag binds RNA and is an RNA helicase.[335] Whether the T-ag-obd$_{131-260}$ interacts with RNA has yet to be determined.

An interaction between the purified T-ag-obd$_{131-260}$ and single stranded DNA was not detected using "band shift" assays.[180] Therefore, it is not possible to suggest that single-stranded DNA interacts with the T-ag-obd$_{131-260}$ in a manner analogous to RNAs interaction with the RNA binding domain of U1A.[290] It was proposed that the low level of T-ag-obd$_{131-260}$ interaction with single-stranded DNA is, in part, a consequence of masking of the B1 region by the C-terminus of the T-ag-obd$_{131-260}$.[239] The C-terminal helix of U1A has also been proposed to act as a lid governing interactions with RNA.[8]

3. Homology with Other Structurally Similar Proteins

Thirteen additional proteins were identified by the Dali server as being structurally related to the T-ag-obd$_{131-260}$ (Z score greater than two). It is of considerable inter-

* Plate 3 appears after page 504.

est that many of these proteins also contain βαββαβ folds. The shared βαββαβ fold constitutes a common structural motif that is present in many proteins involved in both mononucleotide, dinucleotide, and polynucleotide binding.[267,268,289] For example, it is present in human nucleoside diphosphate kinase[267] (sixth most structurally similar) (Z score of 3.7), the allosteric domain of *E. coli* aspartate carbamoyl-transferase[146] (tenth most structurally similar (Z score of 2.2), and *E. coli* DNA Pol I[287] (14th most structurally similar) (Z score of 2.0). These proteins bind dinucleotides, mononucleotides, and polynucleotides, respectively. Perhaps the structural homology between the T-ag-obd$_{131-260}$ and the DNA binding domain of the E2 protein, the RNA binding domain of U1A, and the 13 other members of the group reflect the presence of a common fold suitable for nucleotide binding. It will be interesting to establish whether the βαββαβ fold is a feature of additional protein domains that interact with other origins of replication.

III. NONORIGIN BINDING ACTIVITIES OF T-ANTIGEN

Although T-ag preferentially interacts with the SV40 origin, it also binds with lower affinity to non-origin containing duplex DNA and single-stranded DNA.[6,180,288,361,442,443] These interactions are critical for T-ag's ability to locate the SV40 origin and for its helicase activity. Indeed, in the context of T-ag, all mutations in the T-ag-obd that reduced nonspecific binding to duplex DNA also prevented origin specific binding.[354] In addition to the T-ag-obd, a region mapping between residues 269 and 522 is also involved in nonspecific binding.[229]

1. T-ag-obd Regions Involved in Nonspecific Binding

Within the T-ag-obd, elements B1(183–187), B4 (226–227), and certain residues in elements A and B2 (Plates 1, 2, and 5) were reported to be important for nonspecific DNA binding.[444] As noted previously, with the exception of B1, these elements form a continuous surface on the T-ag-obd$_{131-260}$. Furthermore, as with the GAGGC containing oligonucleotide, Luo et al.[239] reported that the A, B2, and B4 elements exhibited a significant chemical shift after binding a "control" double-stranded oligonucleotide (Figure 4). Therefore, both mutagenesis and NMR experiments indicate that most of the sites for nonspecific and sequence-specific double stranded DNA binding are located on the same surface of the molecule.

The position of the B1 element[351,354] is shown in Plate 2. Within this element, Ser 185 and His 187 were reported to be most important for nonspecific DNA binding. Surprisingly, interactions between the B1 element and duplex DNA were not detected in NMR studies employing the T-ag-obd$_{131-260}$[239] (Figure 4). Thus, it is possible that this region contacts DNA only in the context of the entire molecule.

2. Additional Regions Involved in Nonspecific DNA Binding

The purified T-ag-obd$_{131-260}$ has limited nonspecific binding activity.[180] Indeed, in addition to the T-ag-obd, it was demonstrated that additional T-ag residues (~269 to 522) are required for full nonspecific binding to double-stranded DNA.[229] However, this additional region (~269 to 522) is not an independent binding domain. Rather, it cooperates with the T-ag-obd to give rise

to wild-type levels of nonspecific double-stranded DNA binding.[229] It has been suggested that this region might also be involved in melting of the early palindrome.[229]

3. Regions of T-Ag Required for Binding to Single Stranded DNA

T-ag binds to single-stranded DNA in a sequence-independent manner.[180,361] Indeed, T-ag is unable to bind GAGGC repeats when present on single-stranded DNA.[6] However, little is known about the regions of T-ag involved in binding to single-stranded DNA. It has been proposed that the amino-terminal 246 amino acids of T-ag are largely responsible for single-strand DNA binding, although wild-type levels of binding require additional carboxy terminal sequences.[253] The importance of carboxy terminal sequences for binding to single-stranded DNA is suggested by several additional experiments. For example, T-ag molecules containing the C11A mutation (Pro 522 → Ser) bind to the SV40 origin, but they fail to interact with single-stranded DNA.[261] Furthermore, band shift experiments demonstrated that the purified T-ag-obd$_{131-260}$, which lacks carboxy terminal sequences, bound poorly to single-stranded oligonucleotides.[180] The Zn finger of T-ag, however, does not appear to be among the carboxy terminal sequences required for single-stranded DNA binding.[253]

It has been reported that single-stranded DNA is a poor competitor of origin-specific DNA binding.[6] Moreover, mutations (e.g., C6-2 (Asn 153 → Thr) in the T-ag-obd have been described that inactivate T-ag's site-specific binding activity but retain the ability to interact with single-stranded DNA.[261] These experiments suggest that in the context of T-ag, the interaction of single-stranded DNA with the T-ag-obd occurs via a mechanism distinct from that used to interact with duplex DNA.

IV. T-AG OLIGOMERIZATION EVENTS

It was observed that in the absence of ATP, T-ag forms oligomers when bound to the SV40 origin (Refs. 30, 126, 140, 280, 415; references therein). Using scanning transmission electron microscopy, Mastrangelo et al.[247] observed monomers through tetramers bound to the core origin (and monomers through trimers bound to site I). Tetramer binding was proposed to reflect the binding of four T-ag monomers to the four pentanucleotides in the core origin.[91,247,393] It was concluded that a critical step during initiation of replication is the assembly of T-ag monomers into oligomers at the SV40 origin. Related experiments demonstrated that in the absence of ATP, T-ag preferentially protects the PEN region, and not the AT or EP regions, from DNase I cleavage.[27,86,293]

Subsequent studies demonstrated that the addition of ATP stimulated the binding of T-ag to site II, 10- to 15-fold, and induced the formation of a larger multimeric nucleoprotein complex.[27,79,86,104] Furthermore, in the presence of ATP, the region protected from DNase I cleavage was extended symmetrically outward from site II (20 to 25 bp), a distance that includes both the AT and EP regions.[27,91,247,393] Nonhydrolyzable analogs of ATP also promoted binding to site II; therefore, stimulation of binding was not dependent on nucleotide hydrolysis.[27,86,315] These studies demonstrated that ATP binding regulates protein/protein interactions necessary for oligomerization and promotes additional interactions of T-ag with the SV40 origin.[76,246,293] The importance of

these interactions is underscored by the observation that at 37°C, the temperature at which DNA replication occurs, ATP is essential for efficient binding to the core origin.[79,246,293] However, even under these conditions, the T-ag-core origin complex is fairly unstable; the half-life of the complex is ~5 min.[3,274]

A. A Model for T-Antigen Oligomerization at the SV40 Origin

Electron microscopy studies revealed that in the presence of ATP, T-ag assembles as a bi-lobed structure on the SV40 origin.[79,81] Scanning transmission electron microscopy indicated that each lobe contains 6 monomers of T-ag.[246] Atomic force microscopy studies[245] and gel-based assays[76,293,412] have confirmed that, in an ATP-dependent manner, T-ag forms a double hexamer at the SV40 origin. Related studies indicated that all three core origin domains are important in the assembly of T-ag into a functional double hexamer.[24,293,412] Finally, experiments indicate that the SV40 core origin can be divided through the middle of the PEN into functional early and late halves.[293]

Based on observations from a number of laboratories, a model to account for double hexamer formation at the SV40 origin was proposed that is summarized in Figure 5.[25,76,246,292,293] Hexamer assembly is thought to initiate in the early half of the SV40 core origin (the side near the early palindrome[293] [Figure 5A]). Completion of a T-ag hexamer, owing to subsequent protein-protein interactions, is depicted in Figure 5B. Assembly of the second hexamer, on the late side of the core origin, is now initiated. This proposal is based, in part, on experiments indicating that formation of the early hexamer enhances the formation of the weaker binding late hexamer.[293,412] After the assembly of the second hexamer on the late side of the core origin, double hexamer formation is completed (Figure 5C). In this model, the double hexamers are depicted as two regular, hexagonal rings surrounding the core origin. Evidence that T-ag surrounds the DNA includes the observation that double hexamer formation protects the entire core origin from DNase I digestion.[27,86,293] The importance of this process is suggested by the finding that T-ag mutants, that do not assemble hexamers, fail to replicate viral DNA.[235,313]

A variation of this model has been proposed by Tegtmeyer.[254,389] According to this model, T-ag binds the four pentanucleotides (Figure 1) in two pairs. Members of a pair were suggested to be arranged in a head-to-head orientation;[389] pentanucleotides 1 and 3 constituted one pentanucleotide pair, while pentanucleotides 2 and 4 constituted a second pair.[180,389]. Owing to the structure of duplex DNA, the two pentanucleotides that form a pentanucleotide pair would be located on the same face of the duplex. Subsequent protein/protein interactions would then form the double hexamers.

1. Studies Suggesting a Modification of the Oligomerization Model

All four pentanucleotides are required for origin-dependent unwinding[77,179,292] and replication events.[77] Moreover, it was previously suggested that during double hexamer formation, all four pentanucleotides are simultaneously bound by T-ag.[65,91,247,442] Studies have, however, demonstrated that complete copies of all four pentanucleotides are not required for double hexamer formation.[24,412] Furthermore, the pentanucleotide

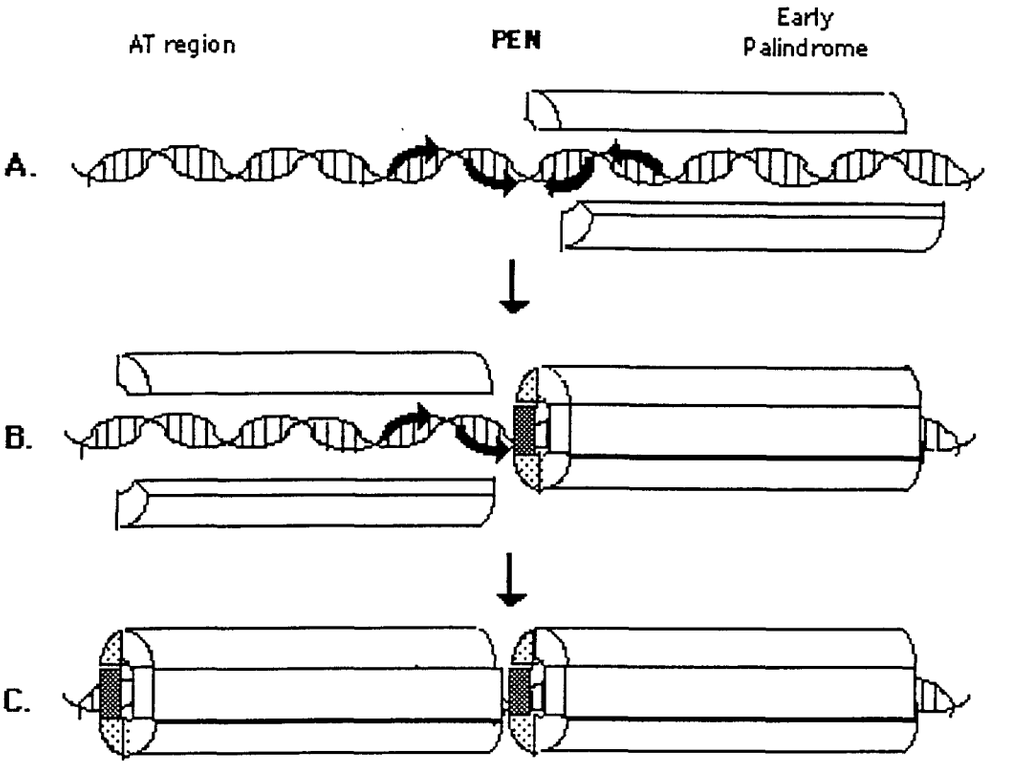

FIGURE 5. A model for hexamer and double hexamer formation at the SV40 core origin. The rectangles symbolize individual T-ag monomers. Regions in the core origin are indicated; the arrows depict the four pentanucleotides present in site II. The protein-DNA and protein-protein interactions required for hexamer and double hexamer formation are described in the text. It has been reported that T-ag covers ~20 bp at the 5′ end of a GAGGC and ~10 bp at the 3′ end.[389,393] Although, there is some uncertainty regarding the oligomerization state of the T-ag molecules used in these studies, it is clear that in the presence of ATP, T-ag bound to the central PEN extends outward to cover the entire core domain.[91,247,389,393]

requirements for T-ag oligomerization have been directly examined using a series of oligonucleotides having various subsets of the four pentanucleotides. It was determined that single pentanucleotides support near wild-type levels of hexamer formation.[179] Moreover, near wild-type levels of double hexamer formation were detected on oligonucleotides containing particular pairs of pentanucleotides (e.g., 1 and 3).[179,180] These experiments suggest that double hexamer formation on the core origin requires only two of the four pentanucleotides.

Studies of the T-ag-obd$_{131-260}$ interaction with the core origin provide additional evidence that only a single pair of pentanucleotides is required for initial assembly events.[180] Stable binding of the T-ag-obd$_{131-260}$ to the SV40 core origin required pentanucleotide pairs separated by ~ one turn of a DNA double helix and positioned in a head-to-head orientation.[180] Furthermore, 1,10 phenanthroline-copper footprinting techniques were used to demonstrate that the T-ag-obd$_{131-260}$ selectively bound to pentanucleotides 1 and 3. To account for the failure to bind to the second

pair of pentanucleotides, it was suggested that T-ag-obd$_{131-260}$ binding and dimer formation may result in a significant deformation of site II.[180] It was also proposed that T-ag-obd$_{131-260}$ binding to pentanucleotides 1 and 3 mimics nucleation of T-ag double hexamers.[180]

These studies raise the question "if single pentanucleotides support hexamer formation and certain pairs of pentanucleotides support double hexamer formation, what is the role of the second, unoccupied, pair of pentanucleotides during initiation"? Perhaps the second pair of pentanucleotides are utilized at a stage in between site-specific binding and DNA unwinding. According to this model, the second pair of pentanucleotides may be bound only after completion of certain structural distortions within the core origin. This hypothesis is analogous to one proposed to account for the binding of the Epstein-Barr virus EBNA 1 protein to oriP. It has been suggested that EBNA 1 binds in a two-step process; the first step involves site specific binding to the origin and the second step initiates structural changes in the DNA.[82] Alternatively, occupancy of the second pair of pentanucleotides may depend on binding of additional proteins required for unwinding. Clearly, much remains to be learned about T-ag oligomerization and the role of the pentanucleotides in this process.

B. Domains of T-ag Required for Oligomerization

An interesting question is "what is the minimal region of T-ag required for oligomerization"? Weisshart et al.[427] reported that a T-ag fragment missing much of the N terminus (T-ag$_{83-708}$) was capable of forming hexamers. Additional studies demonstrated that T-ag residues situated between 628 and 708 are not required for oligomerization.[229] These observations are consistent with a previous report indicating that a region between amino acids 114 and 152 and a C-terminal region up to residue 669 are necessary and sufficient for oligomerization.[265]

T-ag molecules containing mutations in the zinc finger element (Figure 2) fail to assemble into stable hexamers.[169,234,235] These studies suggest that the zinc finger region contributes to protein-protein interactions essential for the assembly of stable hexamers. In addition, the ATPase domain of T-ag is necessary for oligomerization; for example, mutations tsA58 and 5080 map to this region and are defective in this process (Refs. 313, 384 and references therein). The oligomerization defect in these T-ag molecules is believed to be due to their inability to bind ATP. It has been also suggested that phosphorylation of Threonine 124 is required for oligomerization;[264] however, others disagree.[252] Finally, mutations in the T-ag-obd can also disrupt oligomerization events; thus, the T-ag-obd also has a role in this process.[5,444]

1. Insights Into Oligomerization Based on the T-ag-obd$_{131-260}$ Structure

Because the T-ag-obd contains residues involved in oligomerization,[353] the positions of these residues within the T-ag-obd$_{131-260}$ are noteworthy. Both the class 2 (unable to bind either site specifically or nonspecifically to DNA) and class 3 mutants (repressed in their ability to unwind an origin-containing double-stranded DNA fragment) are defective in T-ag oligomerization on the SV40 origin[353] (Plate 4*).

Among the class 2 mutants, Ser 185 → Thr was severely abnormal in oligomeriza-

* Plate 4 appears after page 536.

tion, while Val 226 → Ala was abnormal and His 203 → Asn was only slightly abnormal in this process. Particularly with the class 2 mutants, it is not clear whether the mutant phenotype is the result of a direct effect on oligomerization or a more indirect effect such as structural alterations.[444] Class 3 mutants, Ala 168 → Val and Phe 183 → Leu, exhibit similar, defective oligomerization patterns.[353] Mutations such as Phe 183 → Leu are particularly interesting in view of reports that dimerization via β sheets is a common feature of α/β proteins (e.g., Ref. 269). Moreover, a single class 1 mutation (defined by their inability to bind site specifically to the SV40 origin), Phe197 → Leu, was also reported to have abnormal oligomerization activity.[353] It should be emphasized that while residues in the T-ag-obd may be necessary for oligomerization, they are not sufficient. Indeed, NMR and analytical ultracentrifugation experiments indicate that the T-ag-obd is a monomer even at very high protein concentrations (unpublished).

C. DNA Distortions Associated with T-ag Binding and Oligomerization

After binding of T-ag to the SV40 core origin, in the presence of ATP, the DNA structure is distorted.[28,291] Both topological and chemical assays were used to monitor the structural changes that take place. Topological assays demonstrated that binding initiates an untwisting (an increase in the number of bp per helical turn) of origin containing duplex DNA by 2 to 3 turns.[26,80,319] This observation was explained, in part, by the discovery that T-ag melts approximately 8 bp of DNA (nts 5210 to 5217 [Figure 1]) within one arm of the EP.[28,291] Furthermore, it was determined that the ~17 bp AT tract becomes structurally distorted, probably by untwisting the DNA, although the DNA remains essentially double stranded.[26,28,291] Based on these studies, it was concluded that the importance of the EP and AT regions during initiation of SV40 DNA replication lies in their ability to undergo structural changes essential for subsequent unwinding and initiation events.[18,24,28,291]

Melting of the 8-bp region in the EP region could be induced by nonhydrolyzable analogues of ATP, such as AMP-PNP.[28] Therefore, local melting of the EP region does not require ATP hydrolysis. Because the DNA helicase activity of T-ag (see below) is energy dependent,[78,144,366] it was proposed that melting of the EP by T-ag depends on an activity distinct from its DNA helicase activity.[291] However, complete distortion of the AT tract requires ATP hydrolysis, an indication that subsequent distortions of the SV40 origin are dependent on activation of T-ag's helicase activity.[28]

The structural distortions in the flanking sequences were further investigated using DNA molecules containing either the EP or AT regions. Parsons et al. showed that T-ag can melt the EP in the absence of the AT tract.[291] Furthermore, it was reported that although mutations in the AT region had little effect on structural changes in the EP, certain mutations in the EP were able to eliminate structural changes in both the EP and AT tract.[24,292] This pattern of structural changes was interpreted as indicating that the hexamers formed on the early-half of the core origin promote the assembly of late half hexamers.[292,293] This hypothesis is consistent with experiments indicating that each hexamer is responsible for promoting the structural changes in the nearest flanking region and that double hexamer formation is initiated on the early side.[24,293] However, Borowiec et al. reached an opposite conclusion regarding the sequence of events asso-

ciated with the structural distortions. Their studies indicated that after T-ag binding to site II, the distal portion of the AT tract was the initial site at which structural distortions occurred.[26]

Regardless of the order in which they occur, simultaneous structural distortions in the EP and AT tract are believed to depend on T-ag double hexamer formation at the core origin.[79,235,246] Consistent with this proposal, T-ag molecules containing mutations in the zinc finger bind to the central PEN domain but fail to to assemble into stable T-ag hexamers or to melt the adjacent EP region or to untwist the AT domain.[235] However, T-ag mutants have been described, the class 3 mutants, which are defective for oligomerization but are still able to melt the early palindrome.[353] Thus, at least for the early palindrome region, the structural distortions may be a natural consequence of the assembly of fewer than six T-ag molecules.[353] It has also been proposed that the SV40 origin may be at least partially untwisted before the double hexamer is completely formed.[80] In view of these studies, it is not certain whether complete oligomerization at the origin is necessary for the structural distortions in the EP and AT regions.[353,444]

1. Regions of T-ag Required for the Structural Distortions in the SV40 Origin

Recently, it was observed that the region of T-ag from 121 to 135 is important for AT tract untwisting and DNA unwinding but not for melting of the EP.[56] These studies demonstrated that separate regions of T-ag are responsible for the structural distortions within the AT and EP regions.[56] Moreover, in the context of full-length T-ag, it is known that mutations in the T-ag-obd can have significant effects on the structure of the flanking sequences.[353] Based on these studies, it was proposed that regions of T-ag involved in nonspecific DNA binding are also important for the structural distortions of the flanking sequences.[353]

Finally, for a given flanking sequence, it has been reported that T-ag interacts with only a single strand of DNA, via sugar-phosphate contacts, and that opposite strands are used in each flanking region.[340] These observations led to the suggestion that the structural changes in the EP and AT regions are mediated primarily through non-specific contacts with the sugar-phosphate backbone.[340]

D. SV40 Origin-Independent Oligomerization Events

T-ag binds to, and oligomerizes on, non-origin containing duplex DNA,[330] possibly via residues in the A and B2 regions required for nonspecific DNA binding.[239,444] T-ag also readily oligomerizes on single-stranded DNA in a sequence-independent manner.[180] Moreover, T-ag is known to form hexamers on synthetic replication forks.[341,429] Hexamers bound to synthetic replication forks were reported to interact primarily through the sugar phosphate backbone of DNA.[341]

Furthermore, in the absence of DNA, T-ag readily assembles into single hexamers in an ATP-dependent manner.[76,246,327,429] Additional studies indicate that while DNA is not essential for the protein/protein interactions that give rise to hexamer formation, it facilitates them.[76,293] The hexamers formed in the absence of DNA retain ATPase and DNA helicase activities;[76,429] however, preformed hexamers do not support replication and have low affinity for DNA.[76] This observation was the basis for the proposal that

T-ag binds DNA as a monomer and subsequently assembles into hexamers.[81] Support for this hypothesis was provided by studies demonstrating that inactive hexamers could be dissociated to active monomers by preincubation under conditions that favor separation into monomers (37°C, no ATP).[76]

SECTION 4. ORIGIN-DEPENDENT DNA UNWINDING AND INSIGHTS INTO T-ANTIGEN'S HELICASE ACTIVITY

I. T-ANTIGEN-DEPENDENT UNWINDING FROM THE SV40 ORIGIN

SV40 T-ag was reported to be an ATP-dependent DNA helicase (for reviews of helicases see Refs. 23, 236, 248) that translocates along the leading-strand template in a 3′ to 5′ direction.[78,144,366,432] In view of T-ag's helicase activity, it was postulated that in the presence of a topoisomerase that can remove positive superhelical turns, a single-stranded DNA binding protein and ATP, T-ag might promote extensive SV40 origin-dependent DNA unwinding. Indeed, these components were demonstrated to catalyze T-ag-dependent bidirectional unwinding.[78,104,439] The product of the unwinding reactions was a highly unwound, covalently closed circular molecule that was termed Form U. Electron microscopic studies of Form U demonstrated that it consisted of a population of molecules having unwound regions of various sizes.[104] An obvious function of origin-specific unwinding was the establishment of two replication forks containing regions of single-stranded DNA that can serve as substrates for DNA synthesis events.[78] Indeed, studies demonstrated that initial DNA synthesis events take place on a topological isomer related to Form U.[43] Additional experiments revealed that T-ag unwinds DNA at a rate of approximately 200 bp/min.[275]

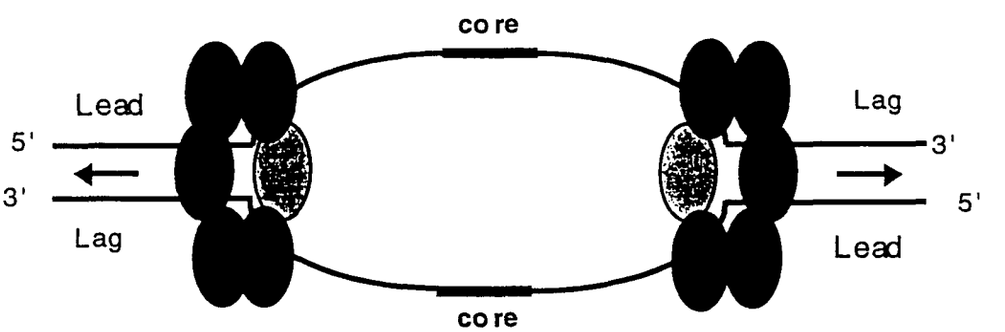

FIGURE 6. A model depicting T-ag-dependent, bidirectional DNA unwinding. T-ag hexamers at the replication forks are represented by the six-membered rings. The SV40 core origin is symbolized by the rectangle labeled "core". Templates for leading (Lead) and lagging (Lag) strand DNA synthesis are indicated. The SSB molecules required for unwinding[78] are not depicted. The T-ag hexamers are shown encircling duplex DNA at the replication forks. However, given the paucity of data, it would be equally valid to draw the T-ag hexamers surrounding single-stranded DNA and moving in a 3′ to 5′ direction.

Although *E. coli* SSB was originally used in the unwinding studies, SSBs from many organisms, including the heterotrimer present in humans (HSSB;[437] also termed replication factor A (RFA) or replication protein A (RPA)[123,436,438]) support the unwinding reaction. The three subunits present in HSSB include a 70-kDa subunit that binds to single-stranded DNA and subunits of 32 and 14 kDa.[34,118,192,440] It has been suggested that the 34-kDa subunit might be involved in DNA unwinding.[215] Presumably, the requirement for an SSB during unwinding is to prevent reannealing of the unwound single-stranded regions.[78]

Regarding the core origin requirements for DNA unwinding, it was observed that plasmids containing base substitutions that inactivated the core origin in both *in vivo* and *in vitro* replication assays also inactived these molecules in the *in vitro* unwinding assays.[77] Related studies confirmed that an intact origin, including the four pentanucleotides in site II, are required for origin-specific unwinding.[78,104,144,292] However, under certain conditions, the helicase activity of T-ag can initiate at internal sites on non-origin-containing linear fragments. Thus, T-ag-dependent unwinding is not strictly dependent on the SV40 origin.[330] Nevertheless, at low T-ag to DNA ratios, such as in the presence of competitor DNA, T-ag-mediated unwinding is origin dependent.[144,420]

It has been determined that origin unwinding requires the DNA binding, zinc finger, and ATPase domains of T-ag.[31,235,238,443] These regions correspond, roughly, to those required for helicase activity and oligomerization (~amino acids 131 and 616).[443] Finally, experiments indicate that those T-ag molecules that initially bind the origin also catalyze the unwinding reaction.[274] Thus, after melting of the EP, it is unlikely that additional T-ag molecules are recruited to the origin.

II. EFFORTS TO ESTABLISH THE MECHANISM OF SV40 UNWINDING

Little is known about how T-ag is converted from a double hexamer that distorts the SV40 core origin (Figure 5) into functional helicases that catalyze bidirectional unwinding at replication forks (Figure 6). Helicases are proteins that use the energy of NTP or dNTP hydrolysis to catalyze the unwinding of duplex DNA. The T-ag helicase activity is most active in the presence of ATP or dATP, although dTTP, UTP, and dCTP function to lesser extents;[144,432] furthermore, single-stranded DNA stimulates nucleotide hydrolysis.[139] Regarding stimulation of this activity on the SV40 origin, it was suggested that the AT domain may activate the helicase activity of T-ag. How might this domain activate the helicase activity of T-ag? Giacherio and Hager[139] reported that single stranded dT homopolymers stimulate the ATPase activity of T-ag. Therefore, it was proposed that at some stage in the unwinding process, the AT region stimulates the ATPase and helicase activities of T-ag, thereby promoting catalysis of an extended unwound region.[291]

Given that T-ag forms relatively stable double hexamers at the SV40 origin, what additional event(s) is necessary to initiate unwinding? One possibility is binding of the HSSB to the ~8 nt single-stranded region formed in the E.P.[25,172] It has been demonstrated that the minimum binding site for HSSB on single-stranded DNA is 6 to 10 nucleotides.[19,22,172] Once bound to the ~8 nucleotide single-stranded DNA region, the HSSB may reorient and elongate to form a more stable 30 nucleotide complex.[20,199] Binding of additional HSSB molecules may extend the single-stranded region, releasing T-ag bound to GAGGC pentanucleotides, and perhaps positioning T-ag molecules over

the AT-rich region in a manner that activates T-ag's helicase activity. However, additional studies are needed to reveal how unwinding is actually initiated at the SV40 origin. Indeed, it is noted that an alternative model for unwinding has been proposed. This model posits that once a double hexamer forms on the SV40 origin, it remains in place and DNA is threaded through the intact complex.[429]

III. T-ag UNWINDING AT REPLICATION FORKS

When functioning at replication forks as a helicase, T-ag is likely to assemble as a hexamer.[79,104,246,291,313,316,341,429] This conclusion is based, in part, on electron microscopy studies that revealed structures the size of T-ag hexamers at forks in DNA molecules undergoing unwinding.[104,330,429] Indeed, most DNA helicases assemble to form oligomeric structures, generally dimers or hexamers.[236] One reason for oligomerization is that this process provides helicases with multiple DNA and nucleotide binding sites.[236,237] Like other helicases, a cycle of nucleotide binding, hydrolysis, and product release is likely to drive the T-ag helicase through energetic states that are necessary for unwinding.[237] Related studies indicate that DNA binding generally occurs within the central hole of a given helicase (e.g., Ref. 111). Therefore, it is of considerable interest that T-ag forms a sixfold ring-like particle containing a central channel.[327] Furthermore, the central ring was reported to be large enough to accommodate either single- or double-stranded DNA.[327]

Regarding the interaction of T-ag hexamers with the single-stranded/double-stranded DNA at replication forks, ethylnitrosourea studies indicated that these interactions are primarily through the sugar-phosphate backbone of one strand of DNA, an interaction that may be related to the $3' \rightarrow 5'$ directionality of the DNA helicase activity of T-ag.[341] However, others have reported that individual hexamers interact with both of the single-stranded DNAs at the replication fork.[429] These studies illustrate that although progress has been made, we are far from understanding how T-ag catalyzes unwinding at replication forks. Indeed, the mechanism of DNA unwinding by any helicase is not presently understood, although models have been proposed.[236,237,341,441]

IV. INSIGHTS INTO T-ag'S UNWINDING AND HELICASE ACTIVITIES BASED ON THE T-ag-obd$_{131-260}$ STRUCTURE

A. Unwinding

T-ag molecules containing the C8A mutation (Lys 224 $\rightarrow$ Glu) have near wild type levels of helicase activity and selectively bind the SV40 origin.[261,375] However, molecules containing this mutation are not capable of origin unwinding or initiation of DNA replication.[242,261] The position of Lys 224 in the T-ag-obd$_{131-260}$ is indicated in Plate 5*. It is interesting that Lys 224 is situated close to the regions of the molecule (A and B2) that bind to DNA. As previously discussed, the B3 region is also required for DNA unwinding.[444] Inspection of Plate 2 reveals that Lys 224 is in between the A/B2 and B3 regions; therefore, this surface of

* Plate 5 appears after page 536.

the molecule may play a critical role in DNA unwinding.

Phosphorylation of Thr 124 is also essential for SV40 replication[337] and for complete unwinding of the SV40 replication origin.[252,260] A threonine to alanine mutation at position 124 gives rise to T-ag molecules with a phenotype similar to those containing the C8A mutation (Lys 224 →Glu), that is, wild-type helicase and oligomerization activities but defective in unwinding.[252,260] These experiments demonstrate both the importance of Thr 124 in origin-specific unwinding and that intact origin binding, oligomerization, and helicase activities are not sufficient to catalyze unwinding.

The class 2 (unable to bind DNA or oligomerize) and class 3 (defective in oligomerization and unwinding) mutants (Plate 4)[444] are also defective in unwinding a linear duplex molecule. However, it is not surprising that molecules that cannot bind to DNA or properly oligomerize cannot unwind DNA. It is interesting that the class 4 mutations (mostly overlapping the B3 region [Plate 2])[444] are selectively defective in unwinding covalently closed duplex DNA molecules. Finally, as in previous examples, it is important to consider that in certain instances the mutant phenotype may be due to indirect structural alterations.[351]

B. Helicase

Little is known about how either prokaryotic or eukaryotic helicases work.[237,248,430] Therefore, it is of considerable interest that the structures of two prokaryotic helicases were published recently.[382] A similar structural information for a eukaryotic DNA helicase is not yet available, although the structure of the hepatitis C virus RNA helicase domain is known.[451]

The T-ag-obd$_{131-260}$ is a significant portion (~25%) of the region of T-ag required for helicase activity (131 to ~616).[443] Regarding the amino acids within the T-ag-obd required for helicase activity, those residues required for sequence-specific binding are not needed for helicase activity.[354] Alternatively, all T-ag-obd residues necessary for nonspecific contacts with DNA are required for helicase activity.[354] As previously discussed, these include certain residues in elements A (Ala–149 and Phe–159) and B2 (His–203) as well as residues in elements B1 (Ser–185 and His–187) and B4 (226–227).[354] It was proposed that these residues make contacts, either direct or indirect, with the sugar-phosphate backbone of DNA.[354] The positions of these residues in the T-ag-obd$_{131-260}$ are indicated in Plate 5. Because the T-ag-obd$_{131-260}$ binds poorly to single-stranded DNA,[180] it is possible that while functioning as a helicase this region of T-ag makes contact with duplex DNA at the replication fork. This hypothesis implys that a second region is largely responsible for interactions with single-stranded DNA.

The PcrA helicase[382] is also a 3′ to 5′ helicase and like the T-ag-obd$_{131-260}$,[239] it may interact with DNA via loops. It is noteworthy that the PcrA helicase contains a subdomain that is structurally very similar to the ATPase domain of the *E. coli* recA protein.[382] Indeed, it was suggested that all helicases contain recA-like domains.[382] In light of this proposal, it is interesting that antibodies against large T antigen interact with recA protein.[339] Moreover, a region of homology has been noted between amino acids 372 to 648 of T-ag and 36 to 352 of recA.[339] These observations raise the possibility that prokaryotic and eukaryotic helicases may share basic mechanisms, such as DNA binding and the processes use to couple ATP hydrolysis to the conformational changes necessary for helicase activity. Perhaps the region of T-ag with homology to Rec A, a region that includes much

of the nonspecific DNA binding domain (Figure 2), is responsible for binding to single-stranded DNA.

SECTION 5. DNA SYNTHESIS IN THE VICINITY OF THE SV40 ORIGIN

I. INTRODUCTION

After addition of T-ag to an otherwise complete SV40 replication reaction, nascent DNA formation is detected only after an incubation period of approximately 10 min.[122,378,435,439] During this "presynthesis stage", multiple protein-protein and protein-DNA interactions take place, culminating in the formation of a complex capable of initiating DNA synthesis.[25,28,78,104,319,399,439] The proteins required for initiation of DNA synthesis and their interactions are discussed in this section. Moreover, insights into initiation of DNA synthesis, derived from studies of nascent DNA formation in the vicinity of the SV40 origin, are described.

II. INITIATION OF DNA SYNTHESIS

A. Proteins Required for Initiation of SV40 DNA Synthesis

The isolation of cellular proteins necessary for initiation of SV40 DNA synthesis was accomplished via fractionation of extracts that support SV40 replication *in vitro*[174,211,256,402,425,436] It was demonstrated that in addition to the three proteins required for origin-specific DNA unwinding (T-ag, Topo I, HSSB), initiation of DNA synthesis required the pol α-primase complex.[174,273,276,400,424,436] Indeed, a model system for studying bidirectional initiation of DNA synthesis, the monopolymerase system, was established by including the pol α-primase complex in with T-ag, Topo I, and HSSB.[174,436] While not an essential protein for initiation, it was discovered that the activity of T-ag isolated from particular vectors, such as the Ad5SVRll2, was increased by removal of particular phosphates by human protein phosphatase 2A.[410,411]

1. The Function(s) of Proteins Required To Initiate DNA Synthesis

Some of the functions of T-ag in the initiation process, such as binding to the origin and initiation of structural changes, were described in previous sections. It is thought that Topo I acts as a swivel at the replication forks, releasing positive supercoils generated as a result of replication fork progression.[7,240,450] Indeed, preferential binding of human Topo I to superhelical DNA has been demonstrated.[240]

The pol α-primase complex[216,422,423] contains four subunits of molecular masses 180, 68, 58, and 48 kDa. The largest subunit is necessary for DNA catalysis while the two smallest subunits are responsible for primase activity (reviewed in Ref. 423). The primase activity is present in the 48-kDa subunit, whereas the 58-kDa subunit is thought to stabilize the primase interaction with the 180-kDa subunit.[9,70,328] The role of the 68-kDa subunit is not known. In the presence of nucleotide cofactors, the pol α–primase complex synthesizes the first nascent strands at the SV40 origin.[41,44,92,174,249,272,400,403,424,436] Furthermore, the pol α-primase complex is required for

subsequent priming of every Okazaki fragment (see below). Dilution experiments indicated that the pol α-primase complex dissociates from the template following every priming event.[275] However, in contrast to this "distributive mechanism", it has been suggested that the pol α-primase complex is tethered to the replication complex.[38] This arrangement would enable a single pol α–primase complex to catalyze multiple initiation events in a processive manner.

The HSSB promotes DNA unwinding and is known to stimulate the activity of the pol α-primase complex.[118,190,255,402] While many SSBs promote unwinding, only the HSSB is able to support high levels of DNA synthesis.[34,190,249] Moreover, antibodies directed against any of the HSSB subunits inhibited SV40 DNA replication *in vitro*.[119,192,408] Thus, in addition to the 70-kDa subunit that binds to single-stranded DNA, the 32- and 14-kDa subunits provide activities necessary for DNA synthesis.[106,249]

2. Evidence for Protein-Protein Interactions Among Initiation Factors

It is likely that the pol α-primase complex is recruited to the partially unwound SV40 origin owing to specific protein-protein interactions with T-ag[36,68,69,105–107,135,136,255,274,275,336,355] and possibly HSSB.[106,255] Additional studies have revealed that T-ag interacts with the 180-, 68-, and 48-kDa subunits of the pol α-primase complex.[36,69,105–107] It is noted that experiments by Weisshart et al.[427] demonstrated that the interaction of the pol α-primase complex with the first 82 amino acids of T-ag is not an absolute requirement for DNA replication. The HSSB[69,106,255] and Topo I[352] also associate with T-ag. Studies have also shown a direct interaction between HSSB and the pol α-primase complex[106,118,190] and with the primase subunits.[106] Moreover, it has been demonstrated that very precise interactions between T-ag, HSSB, and the pol α-primase complex are required for the synthesis of RNA primers.[249,275] These observations have led to the suggestion that a precise "priming" or "preinitiation" complex assembles at the SV40 origin.[42,68,106,255,399,435]

B. Interactions Governing Species-Specific Initiation Events

Extracts prepared from primate cells, but not other mammalian cells, support SV40 DNA replication *in vitro*.[221] However, SV40 DNA replication will occur in non-primate extracts (e.g., extracts from mouse cells) provided the extracts are supplemented with the human pol α-primase complex. These experiments demonstrated that the pol α-primase complex is the major host factor responsible for determining species-specific replication *in vitro*.[113,183,273,276,336,436] Subsequent studies established that the 180-kDa subunit of the pol α-primase complex is largely responsible for species-specific replication of SV40.[365] In contrast, the species specificity of polyoma DNA replication *in vitro* is primarily mediated by mouse primase — particularly the catalytic 48-kDa subunit.[36,113,336] Experiments also indicate that the HSSB (RPA) may participate in the determination of species-specific replication.[34,183,255] It has also been determined that the protein-protein interactions between the pol α-primase complex, HSSB, and T-ag are species specific only in the context of the SV40 origin.[36,336]

III. ELONGATION FACTORS AND THEIR ROLE IN CATALYZING A POLYMERASE SWITCH

Once initiated by the nonprocessive pol α-primase complex, DNA synthesis proceeds bidirectionally from the SV40 origin both *in vivo*[75,128] and *in vitro*.[221,377,434] Owing to its association with T-ag, the pol α-primase complex is believed to move away from the SV40 origin with the replication fork.[78,92,104,431] Evidence that T-ag remains part of the replication complex includes the finding that certain T-ag-specific monoclonal anitbodies block elongation from SV40 minichromosome templates *in vitro*.[367,431] However, subsequent replication events depend on additional proteins that serve only as elongation factors (reviewed in Refs. 171, 257).

An important early observation was that SV40 replication *in vitro* depended on a 36-kDa protein termed proliferating cell nuclear antigen (PCNA),[305] a protein that plays several essential roles in eukaryotic nucleic acid metabolism (reviewed in Ref. 189). As PCNA had been previously shown to increase the processivity of polymerase δ (pol δ),[33,307,387] these studies indicated that pol δ is required for SV40 replication. Indeed, replication systems reconstructed with purified proteins have demonstrated that pol δ is required for the synthesis of the bulk of nascent DNA.[211,256,425] Pol δ contains a catalytic subunit of 125 kDa and a smaller 50-kDa subunit.[210] Moreover, the multisubunit complex termed replication factor C ((RFC),[403] also termed activator 1 (A I)[213]) was demonstrated to be essential for SV40 replication *in vitro*. RFC (140, 40, 38, 37 and 36-kDa subunits[48,72,407]) mediates the recognition of nascent DNA molecules, presumably synthesized by the pol α-primase complex and has an associated DNA-dependent ATPase activity.[213,401,404] In addition, RFC is required for efficient loading of PCNA onto the RFC primer complex.[131,212,305,401,405] Once the RFC-PCNA primer recognition complex forms, it is capable of recruiting pol δ (or pol ε [see below]) to DNA.[212,400,404] Experiments indicate that the association between pol δ and PCNA is mediated by the amino terminal 182 amino acids of the 125 kDa catalytic subunit of pol δ,[455] although evidence for an interaction with the 50-kDa subunit has also been presented.[456] The net effect of these interactions is a polymerase switch (Figure 7) between the pol α-primase complex and pol δ.[212,400,401] Once the polymerase switch has occurred, pol δ is able to catalyze extensive DNA synthesis owing to its association with

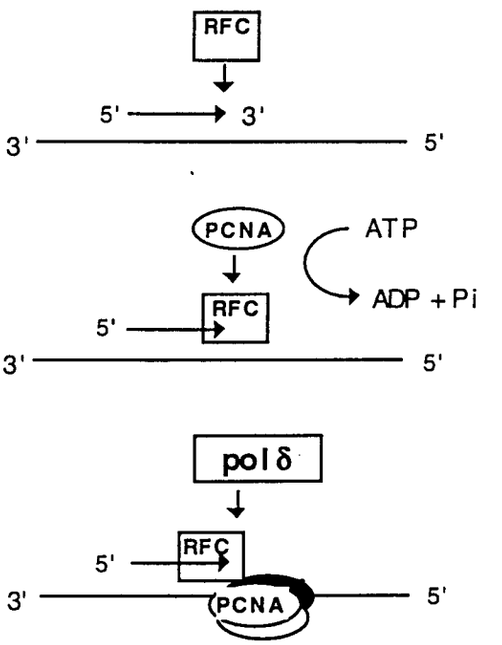

FIGURE 7. Components of a polymerase switch. After synthesis of a nascent DNA molecule (arrow) by the pol α-primase complex, the 3′ terminus is recognized by the RFC complex. Once bound, RFC recruits PCNA, shown as a homotrimer, and subsequently pol δ. RFC-dependent ATP hydrolysis is required for recruitment of pol δ.[212,401]

PCNA. The ability of PCNA to act as a sliding clamp is due to its forming a homotrimer, "donut like", structure that tethers the polymerase to the DNA.[151,205] Indeed, by analogy with the *E. coli* γcomplex, it was proposed that RFC functions as a clamp loader, while PCNA is believed to act as a sliding clamp,[281] a hypothesis that was recently confirmed.[452] It has also been suggested that a preformed RFC-PCNA complex can slide along a template until it encounters a 3′-OH containing primer-template junction.[301] Finally, one reason for employing a polymerase switch is that Pol δ is not associated with a DNA primase;[358] therefore, the pol α-primase complex is needed to initiate DNA synthesis.

An interesting question is, What prevents the pol α-primase complex from competing with pol δ for a given primer? It was determined that in cooperation with HSSB, the RFC-PCNA primer recognition complex regulates which polymerase recognizes the primer. The pol α-primase complex is inhibited by the RFC-PCNA complex, whereas pol δ is stimulated by it.[114,211,256,404,405,418] Furthermore, by binding to single-stranded DNA and preventing nonspecific binding events by RFC and PCNA, HSSB increases RFC–PCNA recognition of primer templates and thereby functions as a specificity factor for primer recognition.[249,404]

These and related studies demonstrated that unlike prokaryotic replication, two DNA polymerases are required for eukaryotic replication.[88,190,211,305,358,400,402,425] There is also evidence that a third DNA polymerase, polymerase ε (pol ε), may be required for eukaryotic DNA replication.[4,46,270,302] Like pol δ, pol ε requires RFC and PCNA for the elongation of primed DNA templates.[214] However, a unique role for pol ε during SV40 replication has not been detected.[214,416,458]

Replication systems reconstituted with purified RFC, PCNA, pol δ, and the components of the "monopolymerase system" (T-ag, HSSB, Topo I, and the pol α-primase complex) mimic many of the features of replication reactions performed in crude extracts.[114,405,424] It is stressed that pol δ, RFC, and PCNA are elongation factors that are not required for origin unwinding, formation of the preinitiation complex, or formation of the first nascent strands at the replication origin.[44,403] Indeed, it has been suggested that because prokaryotic replication factors can replace pol δ and its accessory proteins in *in vitro* replication reactions,[249,401] there may be few, if any, specific interactions between the elongation factors and the proteins involved in the initiation of replication.[117] Nevertheless, experiments indicating that many of the proteins involved in SV40 DNA replication are found assembled into a functional complex[219,241] can be cited as an argument that interactions do take place.

IV. FACTORS REQUIRED FOR MATURATION OF OKAZAKI FRAGMENTS

Maturation of Okazaki fragments into complete lagging strands requires the removal of the RNA primers, DNA synthesis across the resulting gaps, and subsequent ligation events (reviewed in Ref. 11). "Maturation activities" include RNase H1,[147,174] a 44-kDa double-strand specific 5′ to 3′ exonuclease (FEN–1, MF–1) that aids in the removal of RNA primers[147,157,174,191,406,416] and DNA ligase I.[174,416]

Experiments suggest that the bulk of a given RNA primer is removed by an endonucleolytic cleavage near the RNA/DNA junction by RNase H1.[170] After cleavage a single monoribonucleotide is left at the RNA/DNA junction that is likely removed by the 5′ to 3′ exonuclease.[174,406] Exactly

how DNA synthesis is coupled with RNA primer removal is not understood. However, once the gap resulting from RNA primer removal is repaired, the mature Okazaki fragments are ligated by DNA ligase I.[147,416] An additional "maturation event" is decatenation of DNA molecules. It has been reported that while the swivel activity necessary for replication fork progression can be provided by either topo I or topo II, only topo II can decatenate newly synthesized daughter molecules.[174,450]

V. CHARACTERIZATION OF NASCENT DNA FORMED IN HeLa CELL CRUDE EXTRACTS

As the proteins involved in SV40 replication were isolated and used to reconstitute purified replication systems, considerable progress was made in terms of establishing the mechanism of nascent DNA synthesis (reviewed in Refs. 38, 374). However, for certain studies of nascent DNA formation, replication reactions employing HeLa cell crude extracts afforded distinct advantages. One advantage was the greater efficiency of nascent DNA formation in crude extracts. A second advantage was that when using replication reactions containing HeLa cell crude extracts, nascent DNA formation could be limited to the vicinity of the SV40 origin.[43] Therefore, these reactions were used to further characterize the mechanism of nascent DNA formation.

To study nascent DNA synthesis in the vicinity of the SV40 origin, the SV40 *in vitro* replication system[220,377,434] was modified so that pulse and pulse-chase experiments could be conducted.[43] Pulse assays are essentially SV40 replication assays in which the dNTPs and rNTPs (with the exception of ATP) are initially withheld from the reaction. In most experiments, these components were added to the reaction after a preincubation period (~15 min) designed to allow formation of the "preinitiation complex". DNA synthesis was initiated by the addition of the pulse components and either chased with an excess of cold dNTPs or stopped after a brief period of time (e.g., 5 s).[43,44]

A. Evidence that Initiation Occurs on a Particular Unwound Species

Initial pulse-chase experiments were used to demonstrate that the first species to incorporate labeled deoxyribonucleotides comigrated with a topological species related to Form U (Form $U_{R\ [Replication]}$).[43] It was proposed that Form U_R is the initial substrate for SV40 DNA synthesis and that initiation occurs on an unwound topological form. It is thought that Form U_R is less extensively unwound than Form U, a topological species generated in the absence of DNA replication. Limited unwinding in Form U_R may reflect the formation of the preinitiation complex at the SV40 origin. Consistent with this proposal, generation of Form U_R required ATP but not dNTPs or the other rNTPs.[43] Thus, replication per se is not required for formation of Form U_R.

Related experiments indicated that once the poorly defined "preinitiation complex" forms, it does not leave the vicinity of the SV40 origin unless nucleotides are available for DNA synthesis.[43,44] These, and related experiments, led to the proposal that origin unwinding and nascent DNA synthesis are coupled events.[44,275,399]

B. Discovery and Characterization of Primer-RNA/DNA

When nascent DNA formed in the pulse reactions was analyzed on acrylamide gels,

it was discovered that in addition to Okazaki-sized fragments (~200 nt[96]) a small (~30 nt) RNA/DNA hybrid species was also present.[44,45] This small RNA/DNA hybrid was termed primer-RNA/DNA.[44,45] Related studies of SV40 DNA synthesis conducted in isolated nuclei in the presence of a potent inhibitor of the pol α-primase complex, butylphenyl-dGTP, also suggested the involvement of a small RNA/DNA hybrid molecule in Okazaki fragment formation.[283,284] Moreover, evidence for a small RNA/DNA hybrid species was obtained from early studies of polyoma virus DNA replication.[116] The presence of an ~10 nt long RNA fragment in a covalent linkage to DNA was one of several indications that primer-RNA/DNA is formed by the pol α-primase complex.[44,92,283,284]

Primer-RNA/DNA was further characterized using hybridization techniques.[92] These experiments demonstrated that at all time points, primer-RNA/DNA was synthesized from templates for lagging strand synthesis. To explain the selective formation of primer-RNA/DNA on templates for lagging strand synthesis, it was proposed that T-ag, moving on the leading strand template in a 3′ to 5′ direction, selectively positions the pol α-primase complex to lagging strand templates,[44,92] a conclusion supported by related studies of the pol α-primase complex.[68,256,272,400,424] Moreover, hybridization studies demonstrated that primer-RNA/DNA is initially formed in the vicinity of the SV40 origin and at subsequent time points at progressively distal locations.[92]

When primer-RNA/DNA was discovered, it was generally believed that Okazaki fragments were synthesized entirely by the pol α-primase complex (reviewed in Ref. 134). However, in view of primer-RNA/DNA, it was no longer clear how Okazaki fragments were synthesized. Therefore, to further define Okazaki fragment formation, advantage was taken of the fact that sera from certain patients with lupus erythematosus contain antibodies that neutralize PCNA.[259,386] Anti-PCNA serum (AK[386]) was added to a pulse assay and its affects on nascent DNA formation were determined.[44] Surprisingly, it was observed that primer-RNA/DNA was the only nascent DNA species formed in the presence of the anti-PCNA serum.[44] In light of these studies, it was concluded that a PCNA-dependent polymerase was required for the formation of every Okazaki fragment.[44,92] Moreover, it was proposed that every Okazaki fragment was formed as a result of a polymerase switch between the pol α-primase complex and a PCNA-dependent polymerase.[92] Nethanel and Kaufman also concluded that two polymerases are required for lagging strand synthesis and that Okazaki fragment formation may entail a polymerase switch.[283–285] Primer-RNA/DNA was also detected in replication systems reconstituted with purified proteins.[114,272] Confirmation that Okazaki fragments are not the exclusive products of the pol α-primase complex require RFC (A I), PCNA and pol δ for formation, was provided by additional studies employing purified proteins.[114,275,418]

It is interesting to speculate why nature designed such a complicated RNA-DNA primer. It was observed that pol δ will extend a DNA oligonucleotide hybridized to a single-stranded template but not an RNA primer of the exact sequence and size as the DNA primer.[211] Thus, RNA molecules do not serve as primers for pol δ. These and related observations led to the suggestion that eukaryotic cells may have evolved a system to prevent priming of DNA synthesis by random fragments of RNA.[232] Only DNA primers, such as those at the ends of primer-RNA/DNA, are able to serve as primers for the polymerases that catalyze the bulk of DNA synthesis.

1. The Use of Primer-RNA/DNA to Locate the Template Position(s) At Which the Pol α-Primase Complex Initiates DNA Synthesis In Vitro

When primer-RNA/DNA was discovered, little was known about how the pol α-primase complex selects a site at which to initiate synthesis. Therefore, primer-RNA/DNA was used in experiments designed to establish the template sequence requirements for initiation of DNA synthesis.[45] Primer-extension experiments demonstrated the existence of an initiation signal that was located within those template sequences that encode primer RNA (Figure 8). More precisely, it was situated immediately proximal to the nucleotide encoding the 5′ end of the RNA primer. Comparison of many primer-RNA/DNA start sites suggested a consensus initiation signal of 3′-$N_1T_2T_3$-5′; the nucleotide at position 1(N_1) was proposed to encode the 5′ end of the RNA primer. With the exception of the pyrimidine-rich 3′-NTT-5′ consensus signal, computer analyses of initiation sites failed to suggest additional sequence features that are likely to be components of the initiation signal[45] (and unpublished results). Consistent with these observations, several previous studies indicated that pyrimidine residues are critical for initiation by the pol α-primase complex (reviewed in Ref. 45). Moreover, it was observed that under the reaction conditions employed, the average distance between neighboring molecules was ~19 nucleotides, although the distribution ranged between 2 and 59 nucleotides.[45] An average distance of ~19 nts between initiation sites is consistent with a dinucleotide consensus sequence for the pol α-primase complex.[45]

Having detected an initiation signal for DNA synthesis, using a model system for eukaryotic replication, it was compared with prokaryotic initiation signals. There was

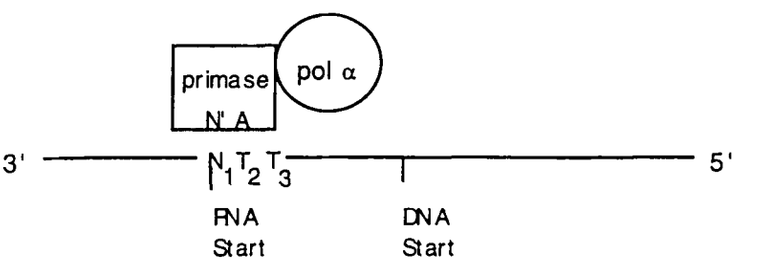

FIGURE 8. Summary of the initiation model presented in Bullock et al.[45] Template sequences encoding the RNA start site, position 1, had no detectable sequence specificity; therefore, this position was labeled N. 98% of the start sites had a T at position 2, while ~72% of the start sites had a T at position 3. The "A" associated with the primase subunit represents an ATP molecule that was suggested to be the first nucleotide bound to the primase. Binding of ATP was proposed to stabilize the primase and to promote the binding of the second nucleotide complementary to the nucleotide at position N_1. After primase synthesizes an ~10 nt. RNA primer, pol α begins DNA synthesis at the DNA start site. It is noted that footprinting studies indicate that the pol α-primase complex protects ~9 nt of the primer and ~12 nt of the template in the duplex primer/template region, as well as ~13 nt of single-stranded template region[70,392,404]

enough similarity between the prokaryotic and eukaryotic template sequences to suggest an evolutionary link between the initiation signals.[45] Support for this hypothesis was provided by a reanalyses of previously published *in vivo* start sites.[326] Given the extensive enzymatic and functional homology between prokaryotic and eukaryotic replication systems,[249,372,401] perhaps it is not surprising that an evolutionary link exists between initiation sites.

a. A Model for Initiation by the Pol α-Primase Complex

The 3′-NTT-5′ consensus signal was considered in terms of possible insights into the mechanism of initiation. Given that 98% of the start sites had a T residue at position 2, it was suggested that this nucleotide plays a critical role in the initiation process.[45] Results presented by Sheaff and Kuchta[344] provided additional insights into the initiation mechanism. For example, they reported that the first NTP to bind the pol α-primase–DNA complex becomes the second nucleotide of the primer.[344] One important consequence of formation of the initial pol α-primase-DNA-NTP complex is that it helps to determine the identity of the 5′-terminal NTP.[344]

These studies suggested a model for primase-mediated initiation of SV40 replication[45] that is summarized in Figure 8. It was proposed that the pol α-primase complex scans lagging strand templates looking for an initiation signal. After recognition of a 3′-$N_1T_2T_3$–5′ signal, an ATP molecule binds to the primase subunit opposite the thymine at position 2. Binding of ATP was suggested to stabilize the bound primase subunits and to promote the binding of a second nucleotide (N′) opposite position 1 (Figure 8). Studies by Sheaff and Kuchta and others (reviewed in Ref. 45) indicated that binding of the first two nucleotides induces a dinucleotide to form within primase. Indeed, it was reported that the rate-limiting step during RNA primer formation is after assembly of the E-DNA-NTP-NTP complex, but before — or during — dinucleotide synthesis.[344]

If conditions are correct, primase extends the dinucleotide 8 to 9 nts: at this point, the RNA primer is switched to the active site of pol α and the DNA primer is synthesized. It has been suggested that the relatively uniform length of primer RNA reflects the inherent processivity of DNA primase (e.g., Refs. 45, 206). Others have observed shorter primer RNA molecules (1 to 9 nt) and suggested that template sequences must indirectly determine the nucleotide site where DNA synthesis begins.[326,449]

There are still many unanswered questions regarding the mechanism by which the pol α-primase complex initiates DNA synthesis in HeLa cell crude extracts. For example, it is surprising that no sequence bias was detected at position 1. Given that most previous studies suggested that purines are present at the 5′ termini of RNA primers (e.g., Refs. 398, 448), one would have predicted a bias toward pyrimidines at template position 1. It has been suggested[158,326] that this discrepancy is due to a one base error in the primer RNA/DNA mapping studies[45] (i.e., the actual initiation site is opposite the thymine labeled as position 2 in Figure 8). If this hypothesis is correct, then most (~98%) primer-RNA/DNA molecules are initiated with an ATP molecule. If true, however, the data are not as obviously consistent with studies indicating that the first NTP to bind the pol α-primase–DNA complex influences where primase initiates.[344] If the primer-RNA/DNA mapping studies were off by one base, then

the thymine residue at position 2 (present at 98% of the start sites), and not the thymine at position 3 (present at 72% of the start sites), is the primary determinant of where RNA primers initiate.

Alternatively, in certain previous studies, the degradation of Okazaki fragments may have led to reports of a bias toward purines at the 5′ termini of primer RNA molecules. Indeed, because RNA primers are believed to be removed by maturation factors during completion of Okazaki fragment formation (reviewed in Ref. 11), it follows that full-length Okazaki fragments are relatively poor reagents for mapping initiation sites. As previously noted,[45] mutagenesis studies are needed to confirm the sequence features of initiation sites. Additional studies are required to address the affect of nucleotide concentrations on initiation site selection.[200] Furthermore, the model reproduced in Figure 8 does not address the fact that approximately 72% of the start sites had a T residue at position 3 and that many start sites had additional T residues beyond this site.[45] What role(s), if any, are played by these thymines is currently not clear. Because the HSSB preferentially binds to pyrimidine residues,[199] it was proposed that these residues may help to recruit HSSB and thereby stimulate primase[249] and the pol α-primase complex.[190,255,272]

At certain locations the trinucleotide 3′-NTT-5′ is present but is not used as an initiation signal.[45] In general, these "inactive sites" are immediately downstream of active initiation sites. Therefore, at certain locations upstream initiation sites may be dominant over downstream initiation sites. Alternatively, the inactive initiation sites could reflect that some unidentified component of the initiation signal is lacking at these sites, or that certain flanking sequences can inhibit initiation events. Additional mutagenesis studies are required to resolve these issues.

2. A Model for Okazaki Fragment Formation

The experiments summarized in the previous sections suggested a model for Okazaki fragment synthesis that is presented in Figure 9. According to this model, each primer-RNA/DNA molecule primes the formation of an individual Okazaki fragment. The observation that functional initiation sites occurred every ~20 nt,[45] rather than once every 200 nt (the average size of a eukaryotic Okazaki fragment),[96] was suggested to reflect the utilization of different initiation sites on different template molecules.[45] The utilization of different start sites on different templates is depicted in Figure 9A. It was proposed that once a given primer-RNA/DNA molecule is formed, it serves as a substrate for a polymerase switch with a PCNA-dependent polymerase (presumably pol δ) (Figure 9B). When loaded on the template, the PCNA-dependent polymerase reinitiates DNA synthesis and continues until contact is made with the previously synthesized Okazaki fragment. At this point, the maturation factors are employed to remove the RNA primer and to aid in repair of the resulting gap (Figure 9C). Regarding the recruitment of maturation factors to sites of primer removal, it has been demonstrated that PCNA physically interacts with the 5′ to 3′ exonuclease and helps to stimulate its activity.[226]

In support for the model summarized in Figure 9, it was noted[45,92] that studies of the fidelity of DNA replication are consistent with a limited role for the pol α-primase complex during lagging strands synthesis.[92] For example, it was demonstrated that in HeLa cell crude extracts replication fidelity is similar for SV40 leading and lagging strands.[320] However, the pol α-primase complex purified from HeLa cells does not contain an associated 3′-to-5′ exonuclease ac-

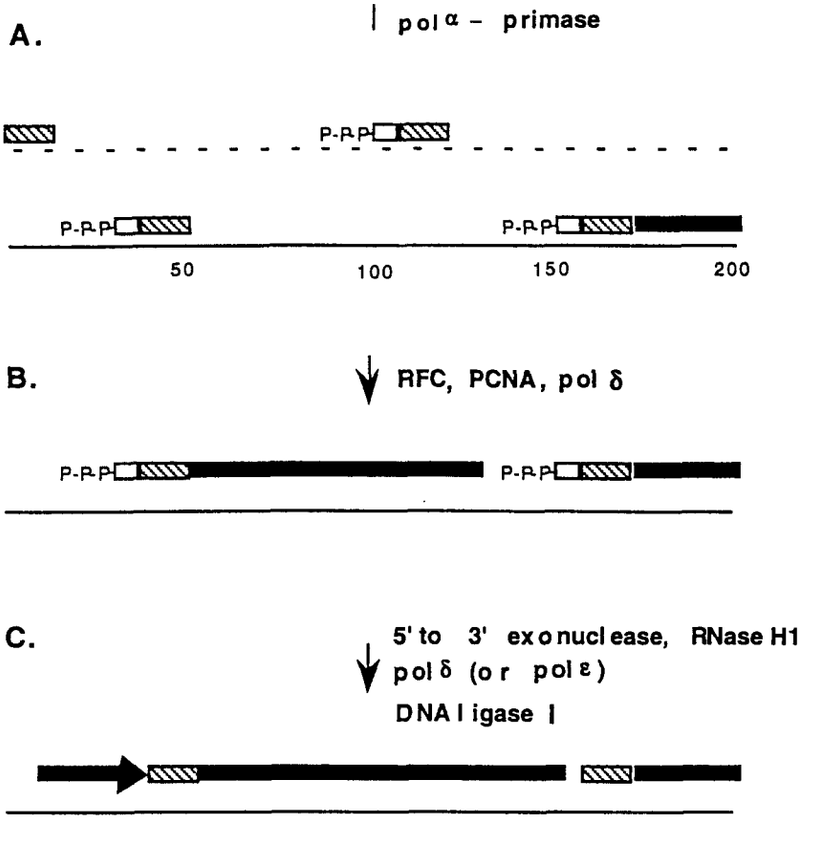

FIGURE 9. A model depicting the function of primer RNA/DNA during Okazaki fragment synthesis. (**A**) Synthesis by the pol α-primase complex of primer RNA/DNA molecules at different sites on different templates is symbolized by the association of primer RNA/DNA molecules with both the solid and dotted lines. Primer RNA is depicted by the white rectangles, while primer DNA is depicted by the striped rectangles. (**B**) After synthesis, primer-RNA/DNA molecules were proposed to serve as substrates for a polymerase switch with a PCNA-dependent polymerase.[44,92] (**C**) To complete Okazaki fragment formation, primer RNA molecules are removed via a 5′ to 3′ exonuclease and RNase H1,[174] and the resulting gaps filled in with either pol δ (or pol ε) and subsequently sealed with DNA ligase I (reviewed in Ref. 11).

tivity.[53] Therefore, if the pol α-primase complex catalyzed a significant percentage of the lagging strand, the fidelity of lagging strand synthesis would be expected to be lower than that for leading strand synthesis.[45] Pol δ (and Pol ε) are both high-fidelity polymerases that contain 3′ to 5′ exonuclease activities.[387,391] Therefore, it was suggested that either polymerase could extend primer-RNA/DNA and synthesize lagging strand DNA at an error rate similar to that of the leading strand.[92]

However, this model is not consistent with data from the Kaufmann laboratory indicating that DNA primers face short gaps (~10 to 20 nts) at their 3′ ends.[284,285] In view of this data, it is difficult to imagine how pol δ can synthesize the bulk of a given Okazaki fragment. Therefore, an alternative, "nested discontinuity" model for lagging-strand DNA synthesis, was proposed by Nethanel and Kaufmann.[283–285] This model suggested that several primer-RNA/DNA molecules are assembled into a single

Okazaki fragment after intervening primer-RNA molecules are removed and the resulting gaps filled by DNA polymerase δ (or perhaps ε). While the "nested discontinuity" model for Okazaki fragment formation is consistent with certain experiments, such as the occurrence of initiation sites every ~20 nt,[45] the relatively large contribution of the pol α-primase complex to lagging strand synthesis makes it difficult to account for the equal fidelity of leading and lagging strand synthesis events. However, it has been proposed that during primer-RNA removal, some of the lagging strand DNA synthesized by the pol α-primase complex is removed.[11,232] If repaired by a PCNA-dependent polymerase, removal of this DNA may help to explain the equal fidelity of leading and lagging strands synthesis events. Future studies will establish the exact mechanism of Okazaki fragment formation.

3. Evidence that SV40 DNA Synthesis Does Not Initiate in the Core Origin In Vitro

Perhaps the most interesting region in which to study initiation of DNA synthesis is in the vicinity of the SV40 origin. Hybridization experiments, using the entire nascent DNA population formed during a 5 s pulse as a probe, indicated that DNA synthesis does not initiate within the SV40 core origin *in vitro*.[44,92] Rather, these studies revealed that initiation occurs on templates for lagging strand DNA synthesis. In contrast, previous *in vivo* studies of SV40 replication suggested that initial synthesis events take place within the core origin.[159] Therefore, to improve the resolution of the *in vitro* experiments, the initiation sites for primer-RNA/DNA, formed in the vicinity of the SV40 origin, were mapped to the nucleotide level.[41] These studies demonstrated that start sites for DNA synthesis were greatly suppressed over the SV40 core origin. Relatively weak start sites were detected over the 21-bp repeats and T-ag binding site I (Figure 10); distal to these regions, stronger start sites were detected. The same conclusion was drawn whether the primer-RNA/DNA molecules were isolated from pulse assays or "continuous-labeling reactions", in which the nucleotides in the pulse mix are added with T-ag, and the reactions stopped 15 min later.[41] By conducting experiments with primer-RNA/DNA molecules isolated from the continuous labeling reactions, the possibility was eliminated that the locations of the initiation sites reflect extensive template unwinding prior to initiation of DNA synthesis.[41]

It was concluded that DNA synthesis initiates *in vitro* outside of the SV40 core origin on templates for lagging strand DNA synthesis.[41,42,44] Evidence that DNA synthesis initiates outside genetically defined origins in other replication systems has been reviewed.[41,44,373] However, a fundamental difference between SV40 DNA replication *in vivo* and *in vitro* is that DNA assembles into minichromosomes *in vivo*,[125] but there is no evidence of chromatin assembly *in vitro* under standard assay conditions.[57,357,371] This fundamental difference in the two systems may be related to the disparity in the results obtained using the *in vivo*[159] and *in vitro*[41,44] replication systems.

It is interesting to speculate how leading strand DNA synthesis is initiated in the *in vitro* replication system. It was previously proposed that after formation of the first nascent strands at the replication origin, a polymerase switch occurs to initiate leading strand synthesis.[89,249,256,306,400] Therefore, it is possible that origin proximal primer-RNA/DNA molecules, formed on lagging strand templates on either side of the core origin, are the nascent DNA molecules used to prime leading strand synthe-

sis.[41,44,92,284] After undergoing a polymerase switch with a primer-RNA/DNA molecule, pol δ could catalyze processive leading strand synthesis. In contrast, pol δ molecules involved in lagging strand DNA synthesis would encounter previously synthesized nascent DNA molecules and would terminate synthesis.

4. The Influence of Sequences Flanking the Core Origin on the Initiation of DNA Synthesis

The SV40 core origin is flanked by sequences that facilitate DNA replication *in vivo* (see Ref. 94 for a review). These sequences include the 21-bp repeats and enhancer sequences on the late side of the origin and a 17-bp region, termed site I, on the early side of the origin. Binding site I extends between nucleotides 5208 and 5192[325] and contains two pentanucleotides oriented as direct repeats. In the absence of ATP, T-ag preferentially interacts with site I. However, T-ag interactions with site I are not essential for DNA replication.[99,279,394] Site I is thought to be mainly involved in autoregulation of early transcription.[100,318]

How the sequences flanking the core origin stimulate replication *in vivo* is not understood.[94] One hypothesis is that owing to the interaction of the 21-bp and 72-bp repeats with certain transcription factors, the distribution of nucleosomes is changed, resulting in an increase in the accessibility of the origin region to replication initiation factors.[57,58] Alternatively, the bound transcription factors may help recruit proteins required for initiation, such as HSSB.[160,224] The observation that flanking sequences from different papovaviruses can substitute for one another suggests that the interactions necessary to stimulate DNA replication are not very precise.[16]

It is uncertain whether the flanking, or as they are sometimes termed, auxiliary sequences, play a similar stimulatory role during replication *in vitro*. For example, previous studies suggested that the 21- and 72-bp repeats do not have a significant effect on SV40 DNA replication *in vitro*.[222,375] In contrast, it has been reported that the auxiliary sequences stimulate replication.[152,154,447] Others have noted limited stimulation of replication *in vitro* only by sequences within binding site I.[222,375] Recent experiments indicated that the auxiliary sequences, and transcription factors bound to these sequences, play a very limited role during initiation of DNA replication *in vitro*.[42] It has been suggested that these discrepancies may be explained by variations in the reaction conditions.[153]

In related experiments, it was observed that after removal of the flanking regions, previously active initiation sites are no longer functional when juxtaposed next to the core origin.[42] One interpretation of these results is that the unwound region must attain a particular size before DNA synthesis can initiate. Alternatively, components of the preinitiation complex, such as the pol

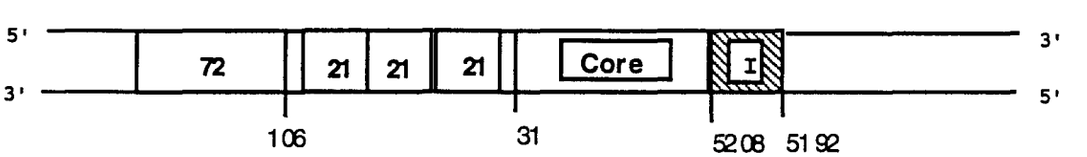

FIGURE 10. Sequence features flanking the SV40 core origin. The core origin, T-ag binding site I, the 21-bp repeats and one of two copies of the 72-bp "enhancer element" are indicated.

α-primase complex, may be positioned beyond the limits of the core origin where they cover, and thereby inactivate, origin proximal initiation sites.

SECTION 6. REGULATION OF INITIATION

I. INTRODUCTION

Having established the basic enzymology and mechanisms required for initiation of SV40 replication, there is increasing interest in the regulation of these events. As initiation involves many steps, and each step is a potential site of regulation, defining the steps involved in regulation is a complicated problem.

Regarding biochemical mechanisms for regulation, it is known that the activities of many of the proteins involved in SV40 DNA replication are affected by posttranslational modifications. For example, the activity of SV40 large T-ag is regulated both positively and negatively by phosphorylation.[308,428] The importance of phosphorylation of Thr 124 to unwinding[252,260] was previously discussed. Indeed, it has been suggested that DNA unwinding may be a key control point for initiation of DNA replication.[104,274,322] Phosphorylation of Thr 124 *in vitro* or *in vivo* depends on a cyclin-dependent kinase and either cyclin B[251,252] or cyclin A,[1] respectively. These observations provide important clues into how SV40 DNA replication is coordinated with the cell cycle.

In addition to Thr 124, T-ag undergoes additional phosphorylation events at two separate clusters; one situated at the amino terminus (that contains Thr 124) and a second cluster at the carboxy terminus (for reviews see Refs. 125, 308). These phosphorylation events regulate additional activities of T-ag, such as differential recognition of site I and site II[262,337] and interactions between T-ag domains.[54,332,454] However, posttranslational modifications are not limited to T-ag. For example, the pol α-primase complex[282] and HSSB[37,101] are phosphorylated in a cell cycle dependent manner. Furthermore, phosphorylation affects the activity of the pol α-primase complex on SV40 origin-containing DNA[413] and the activity of Topo I (reviewed in Ref. 421).

Initiation of SV40 replication is also regulated by numerous protein-protein interactions. For example, T-ag associates with p107,[109,120,121] an interaction that blocks the binding of T-ag to the core origin.[3] Regarding T-ag's interaction with p53, stable T-ag-p53 complexes are not required for replication of SV40 in culture,[230] and *in vivo* studies indicate that wild type p53 is not a negative regulator of SV40 DNA replication in monkey cells.[414] However, p53 inhibits SV40 replication *in vitro* by binding to T-ag and blocking its assembly onto the core origin (e.g., Refs. 132, 195, 419). Other biochemical activities of T-ag are enhanced owing to interactions with p53.[385] Protein-protein interactions regulate other critical stages of replication. For instance, p21, the product of a p53-inducible gene, is both an inhibitor of cyclin-dependent kinases[115,150,156,445] and blocks the ability of PCNA to activate DNA pol δ.[130,225,417]

The assembly of the SV40 genome into chromatin provides an additional level of regulation. For instance, when assembled into minichromosomes, SV40 DNA replication *in vitro* is initiated efficiently only if the origin region is free of nucleosomes.[57,149,173,312] Clearly, the regulation of initiation of SV40 DNA replication is a complicated process, and large pieces of the puzzle remain to be solved.

II. CONCLUDING REMARKS

Limited structural information is now available about the proteins involved in the initiation of SV40 DNA replication. In addition to the solution structure of the T-ag-obd$_{131-260}$,[239] the structure of the single-stranded-DNA binding domain from the 70-kDa subunit of HSSB has been determined.[22] Structural information is also available for Topo I isolated from different organisms (e.g., Ref. 228; reviewed in Ref. 421). Continued progress in determining the structures of proteins involved in SV40 replication will permit insights into initiation, and its regulation, at the molecular level. It will be tremendously interesting to determine the extent to which nature conserved the initiation process in other eukaryotic replication systems, such as those catalyzed by the ORC complex (e.g., Ref. 14).

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