Topography of Simian Virus 40 A Protein-DNA Complexes: Arrangement of Protein Bound to the Origin of Replication

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DNA binding regions I, II, and III at the origin of replication have different arrangements of A protein (T antigen) recognition pentanucleotides. The A protein also protects each region from DNase in distinctly different patterns. Footprint and fragment assays led to the following conclusions: (i) in some cases a single recognition pentanucleotide is sufficient to direct the binding and accurate alignment of A protein on DNA; (ii) the A protein binds within isolated region I or II in a sequential process leading to multiple overlapping areas of DNase protection within each region; and (iii) the 23-base pair span of recognition sequences in region II allows binding and protection of a longer length of DNA than the 23-base pair span in region I. We propose a model of protein binding that addresses the problem of variations in the arrangement of pentanucleotides in regions I and II and explains the observed DNase protection patterns. The central feature of the model requires each protomer of A protein to bind to a pentanucleotide in a unique direction. The resulting orientation of protein would protect more DNA at the 5' end of the 5'-GAGGC-3' recognition sequence than at the 3' end. The arrangement of multiple protomers at the origin of simian virus 40 replication is discussed.

The simian virus 40 (SV40) A protein (T antigen) regulates the initiation of DNA replication (14) by using a 65-base pair (bp) origin of replication centered at the unique SV40 *Bgl*I site (5, 13). The initiation event requires binding of A protein to the origin region because all *tsA* mutations that block initiation in vivo also block origin binding in vitro (17, 21). An enzyme of unknown origin then synthesizes a six- to nine-nucleotide RNA primer from the early strand within the 65-bp origin region (8). A detailed description of the interactions of the A protein with the functional origin will be required to understand how the A protein signals RNA priming and allows DNA elongation.

Tjian originally identified three A protein (T antigen) binding regions around the origin of SV40 DNA replication and recognized the importance of the pentanucleotide 5'-GAGGC-3' in the recognition process in regions I and II (19, 20). We extended these findings to identify multiple interactions of A protein within region II (16) and have found new recognition pentanucleotides in region III that all conform to the consensus sequence 5'-G(T)A(G)GCC-3' (4). Because the arrangements of recognition pentanucleotides in regions I and II are fundamentally different (4), the arrangement of A protein bound to the two regions is also likely to be different.

In the present study, we separate DNA binding regions and DNA binding sites within regions by cutting the DNA with appropriate restriction enzymes before protecting DNA from DNase by binding with A protein. In this way, we have mapped overlapping binding sites within both regions I and II. By relating the positions of DNase protection domains to the location of recognition pentanucleotides, we can deduce common empirical "rules" that explain the more complex patterns of DNase protection observed when A protein binds to intact DNA.

MATERIALS AND METHODS

Protein purification. SV40 A protein was purified from productively infected CV-1 cells as previously described (15).

DNase footprinting. An origin-containing fragment of SV40 DNA cut with *Hinf*I and *Bst*NI was used for DNase footprinting (6). The 3' ends of the early and late strands were labeled as previously described (4). For footprinting, different amounts of A protein were bound to 0.5 ng of the 270-bp origin fragment and 10 ng of unlabeled pBR322 DNA in 100 μ l of 0.02 M PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 7)-0.001 M dithiothreitol-0.0001 M EDTA plus varying concentrations of NaCl. After binding for 1 h at 0°C, 0.0005 U of DNase (Worthington Diagnostics) in 5 μ l of PIPES binding buffer with 0.1 M MgCl₂ and 0.05 M CaCl₂ was added for 5 min at 0°C in 0.005 M NaCl. Five- and 20-fold increases in DNase concentration

FIG. 1. DNase footprint of the origin region of SV40 DNA protected by A protein. The 270-bp *Hinfl/Bst*NI origin fragment was labeled on the late strand of the early end or the early strand of the late end as described in the text. SV40 A protein was added at monomer/or origin ratios of 0, 40, and 120 and at NaCl concentrations of 0.005, 0.075, and 0.15 M. Binding of protein to DNA and footprinting analysis were performed as described in the text. Binding regions I, II, and III are indicated by large brackets at the side of the gels. The nucleotide numbers at the ends of the brackets relate the limits of each region to the center of the inverted repetition in region II.

were required for similar degrees of cutting at 0.075 and 0.15 M NaCl, respectively. DNase activity was stopped by the addition of 105 μ l of 1 M NH_4Ac –0.2% sodium dodecyl sulfate–0.1 M EDTA, pH 6. The DNA was alcohol precipitated and analyzed with 8% polyacrylamide-urea gels (9) run at a constant temperature of 55°C.

DNase fragment assay. DNA was nick translated, using a kit from Amersham Corp. When appropriate, 0.2 μ g of DNA was cut with 1 U of various restriction enzymes (New England Biolabs) in the nick translation mixture. The fragment assay was performed as previously described (4). Protected fragments were eluted from gels and mapped by cutting with restriction enzymes as previously described (16).

RESULTS

DNase footprinting assay. We previously defined the limits of DNA binding regions I and II, using a large excess of protein in 0.005 M NaCl (16). In the present study, we have used a

variety of protein and NaCl concentrations to allow a more complete description of overall binding regions and to identify possible autonomous DNase protection sites within larger regions. Distinct DNase-sensitive sites outline three general regions of DNA binding (Fig. 1). Each of these regions also has a distinctive response to changes in NaCl and protein concentration.

Most of region I is well protected from DNase at all ionic concentrations. However, the early end of the region is only partially protected under low protein or high salt conditions. Dimethyl sulfate footprinting in the accompanying study (4) has shown that 0.15 M NaCl inhibits protein contact with the first recognition pentanucleotide in region I (PI-1). Hence, both approaches suggest that binding of A protein to PI-2 and -3 proceeds readily in the absence of binding to PI-1.

Region II is completely protected from DNase at 0.005 and 0.075 M NaCl but is more DNase sensitive at 0.15 M NaCl than is region I. A discrimination of internal binding sites is evident in region II but is more subtle than in region I. The brackets in the second-to-last well in each panel of Fig. 1 outline an area at the late end of region II that is less completely protected from DNase than the rest of the region at 0.15 M NaCl. Corresponding differences in the sensitivity of individual recognition pentanucleotides in region II to dimethyl sulfate are not shown in the accompanying study that used higher protein concentrations (4).

The A protein protects region III from DNase and induces a site hypersensitive to DNase in that region at 0.005 M NaCl but not at the higher NaCl concentrations tested. The late limit of region III is consistent with the location of an

exonuclease block under low salt conditions first reported by Shalloway et al. (12) and with the dimethyl sulfate protection described in the accompanying study (4). Binding to region III may explain the ancillary role of the region in DNA replication (1).

DNase fragment assay. The design of the footprint assay requires that significant amounts of protein bind to DNA to produce a clear-cut negative image of DNase protection on a background of labeled DNA. Smaller amounts of protein protect different DNA molecules to various extents to give an average result. Under such circumstances, it is difficult to distinguish binding of adjacent sites with similar affinities for protein. The problem is compounded when binding to adjacent sites results in overlapping DNase protection patterns. Individual binding sites within region II are difficult to identify

FIG. 2. Mapping of SV40 DNA protected from excess DNase in the fragment assay. DNA fragments were bound and protected from DNase by the A protein in 0.125 M NaCl as described in the text. Protected fragments were separated by gel electrophoresis, and size classes 1a, 2, 3, and 4 were individually eluted from a gel similar to that shown in the first well of Fig. 3. After alcohol precipitation, each class was cut with *StuI*, *AluI*, or *BglI*. The original eluted DNA and the products of digestion were analyzed by gel electrophoresis. The arrows indicate the predominant products and the numbers indicate their lengths in base pairs. The map locations are summarized in Fig. 7B.

without a fortuitous choice of binding conditions (Fig. 1). The DNase fragment assay, on the other hand, provides a positive image of protected DNA in the absence of background even at low protein/DNA ratios. In essence, each distinct size class of protected DNA represents a unique binding interaction or combination of binding interactions. Once completely mapped, the protected fragments provide a sensitive and specific assay for individual binding sites or a combination of sites.

Previous publications have identified multiple size classes of DNA fragments protected from digestion by an excess of DNase (16, 19, 20). Because the studies used different conditions and were not in complete agreement on the size or origin of the fragments, we have repeated and extended the mapping of the protected DNA. Each size class of protected DNA fragment described in the accompanying study (4) was eluted from a gel and cut with restriction endonucleases having only one site within the origin region. Figure 2 shows examples of the present results, and Fig. 7B summarizes the conclusions from this and other published sources (16, 18–20). DNA size class 1 (105 to 110 bp) was previously mapped in two independent studies that agreed on the location of the early limit of protected DNA but differed somewhat on the late boundary. The map location shown in Fig. 7 agrees with the footprint assay and with the results using *NcoI* discussed below. The restriction products of class 1a DNA, though indistinct (Fig. 2), localize 1a to the same position as the 75-bp fragment described by Tjian (20). Class 2 (60 to 65 bp) originates from the same position we previously reported (16). Class 3 (40 to 45 bp) is heterogeneous in origin. One subclass maps in region II as previously shown (16). A second subclass is cut by *StuI* and thus originates from region I. We do not know if *StuI* cuts this fragment into two subfragments of equal size or if one end of the fragment is distinct whereas the other end is more heterogeneous and escapes detection. The origin of class 4 (30 to 35 bp) is also heterogeneous. The major subclass originates from region I and shares one boundary with the class 3 fragment from region I. Its orientation is based on findings from the footprint assays. Cutting of the other subclass with *AluI* gives the same distinctive 10- and 12-bp doublet seen when fragments 2 and 3 are cut with *AluI*. This cutting pattern indicates that a part of class 4 DNA originates from region II and shares a common early boundary with class 2 and 3 DNA from region II. The *AluI* subfragment(s) establishing the late limit could not be identified. Thus, the late limit is based on the size of the total fragment relative to the early limit and is confirmed below. The results of the

fragment assay, summarized in Fig. 7B, demonstrate a complexity of binding patterns not evident in the footprint assay.

Independent binding of SV40 A protein to isolated subfragments of the origin. Restriction enzyme cutting of fragments of origin DNA after A protein protection from a vast excess of DNase was often incomplete (Fig. 2). To circumvent this problem, restriction enzymes were used to isolate and subdivide origin regions before binding and DNase protection by A protein at 0.125 M NaCl. Successful DNase protection allowed confirmation of the mapping of fragments discussed above and demonstrated the existence of multiple independent binding sites within regions I and II. Binding of the A protein to ends of restriction fragments did not lead to artifacts in the assay because no fragments were protected when pBR322 DNA or SV40 DNA with a deletion of both regions I and II was used in the assay at NaCl concentrations of >0.075 M (data not shown).

FIG. 3. Binding of SV40 A protein to isolated subregions of the SV40 origin. Nick-translated DNA was cut with single restriction enzymes before protection by A protein in 0.125 M NaCl as described in the text. All assays used a protein monomer/origin ratio of 100. Protected fragments were analyzed by gel electrophoresis. The arrows indicate the predominant products, and the numbers indicate their lengths in base pairs. The map locations of protected fragments are summarized in Fig. 7B and C.

Precutting of the origin with *NcoI* had no apparent quantitative or qualitative effect on the pattern of protected fragments (Fig. 3 and 7B). Thus, none of the major classes of fragments extend into region III. This result was expected because the footprint assay detects no protection of region III under similar ionic conditions.

Precutting of DNA with *StuI* destroys PI-2 and removes PI-1 from the origin. Nevertheless, the A protein protects the remaining truncated origin from DNase (Fig. 3 and 7C). The protection of size classes 1 and 1a is less efficient with truncated than intact DNA, and the span of protection is reduced by the truncation as expected. *StuI* cutting in region I reduces protection of class 2 DNA from region II only slightly at 0.125 M NaCl (Fig. 3) and not at all at 0.075 M NaCl. Protection of heterogeneous class 3 is reduced in amount because a portion of that size class originates from region I. Only a small part of class 4 DNA remains protected after truncation of region I (best seen in Fig. 4). Thus, most, but not all, of class 4 DNA originates from region I.

Cutting at single sites in region II with a

variety of enzymes prevents protection of classes 1, 1a, 2, and a part of 3 and leads to protection of distinct new fragments of DNA (Fig. 3). However, the presence of class 3 and 4 DNA from region I prevents complete analysis of the new fragments. Thus, enzymes were used in combinations to subdivide the origin further and to simplify fragment protection patterns for analysis.

Precutting the origin with *StuI* and *AluI* eliminates A protein protection of all the DNase fragments derived from intact DNA but results in protection of two new classes of fragments 29 to 32 and 46 to 50 bp in length (Fig. 4). These are the same lengths that were produced when classes 2 and 3 were cut with *AluI* after elution from a gel (Fig. 2). Precutting with *StuI* and *DdeI* reduced the size of both new classes by about 2 bp. Thus, the fragments originate from the distal part of region II (see Fig. 7D and E). Binding to the distal part of region II in the absence of the proximal part of region II and all of region I is 30 to 50% as efficient as in their presence under these conditions. This finding argues against a high level of cooperative binding between re-

FIG. 4. Binding of SV40 A protein to isolated subregions of the SV40 origin. Nick-translated DNA was cut with combinations of restriction enzymes before protection by A protein in 0.125 M NaCl. All assays used a protein monomer/origin ratio of 100. Protected fragments were analyzed by gel electrophoresis. The arrows indicate predominant products, and the numbers indicate their lengths in base pairs. The map locations of protected fragments are summarized in Fig. 7D, E, F, and G.

gions I and II under the condition of the experiment. We could neither identify nor exclude protection of the 35-bp, PI-3-containing fragment extending from the *StuI* site to the *AluI* site because of the background, 29- to 33-bp fragments from region II.

We precut DNA with *StuI* to suppress binding to region I and with *BglI* or *Fnu4HI* to subdivide the recognition pentanucleotides in region II. Precutting with these combinations eliminated protection of all the fragments usually seen when A protein is bound to intact DNA. Precutting with *StuI* and *BglI* resulted in protection of 28- and 45-bp fragments, whereas precutting with *StuI* and *Fnu4HI* led to protection of 32- and 49-bp fragments (Fig. 4). The map locations of these new fragments (see Fig. 7F and G) could be deduced because *BglI* has a single site in SV40 DNA and because *StuI* and *Fnu4HI* have only one site in the origin region. The 45-bp *StuI/BglI*- and the 49-bp *StuI/Fnu4HI*-protected fragments represent hybrids of regions I and II. These findings suggest that binding of A protein to PI-3 and PII-1 can lead to overlapping DNase protection under some circumstances. The 28-bp *StuI/BglI*- and the 32-bp *StuI/Fnu4HI*-protected fragments extend from the early limit of region II to the *BglI* cutting site within PII-2 and to the *Fnu4HI* site between PII-2 and PII-3, respectively. Previous studies have shown that PI-3 is not required for binding to PII-1 because the A protein protects the 28-bp fragment from DNase after binding to *BglI*-cut, purified region II DNA (16). We conclude that a single recognition pentanucleotide, PII-1, can direct the binding and proper alignment of A protein to the early half of region II. Under the same conditions, binding to the late half of region II is less obvious even though it contains two recognition pentanucleotides. An indistinct 36-bp fragment protected by A protein after precutting with *StuI* and *Fnu4HI* (Fig. 4) may represent weak protection of the late half of region II. However, we have not been able to obtain sufficient quantities of this fragment to map it directly.

Tandem binding within regions I and II. The addition of increasing amounts of A protein to intact origin DNA protects fragments of DNA of increasing size (16, 20). These findings reflect binding to a hierarchy of recognition sequences with different binding affinities (4). The hierarchy of sites is difficult to determine with intact DNA in the fragment assay because the smaller protected fragments are heterogeneous in origin. To simplify the binding pattern, we selectively suppressed binding either to region I by precutting with *StuI* or to region II by precutting with *AluI* and *BglI*.

Figure 5 shows that the A protein binds to isolated regions I and II in a sequential process

FIG. 5. Sequential binding of the SV40 A protein to isolated regions of the SV40 origin. Nick-translated origin DNA was divided into subregions with restriction endonucleases to allow independent analyses of binding to regions I and II by the fragment assay. After binding and DNase treatment at 0.075 M NaCl, protected fragments were analyzed by gel electrophoresis as described in the text. Binding to subregions isolated by cutting DNA with *StuI* to disrupt region I or a combination of *AluI* and *BglI* to disrupt region II were compared with binding to uncut, intact DNA. The numbers at the top of the wells indicate the ratio of A protein monomers to SV40 origins.

that protects tandem regions of DNA as more protein is added. With the exception of the 1 and 1a classes that represent hybrid fragments from both regions, the sum of the distinctive fragments resulting from binding to isolated regions I and II closely resembles the more complex pattern resulting from binding to whole DNA. With increasing protein concentration, binding proceeds from fragment 4 to fragment 3 in region I and from fragment 3 to fragment 2 in region II. Thus, at least two distinct binding steps occur within both regions.

Binding to region II after precutting region I with *StuI* was examined in greater detail by increasing the resolution of analysis by gel electrophoresis. The DNA fragments were separated over greater distances by increasing the bisacrylamide/acrylamide ratio from 1/30 to 1/20. A distinct 50- to 55-bp fragment became evident and was designated as class 2a (Fig. 6A). Classes

FIG. 6. Overlapping DNase protection sites in region II. (A) Protected fragments of DNA from intact DNA or *Stu*I-precut DNA were separated on a high-resolution gel at a bisacrylamide/acrylamide ratio of 1/20 in the Tris-borate buffer system described by Chandler and Gralla (3). Fragment classes 2, 2a, 3, and 4 from the *Stu*I-precut DNA were individually eluted from the gel. After alcohol precipitation, the fragments were cut with *Dde*I as indicated. (B) The original eluted DNA and the products of digestion were analyzed by gel electrophoresis. The arrows indicate the predominant products, and the numbers indicate their lengths in base pairs. The map locations are summarized in Fig. 7C.

2, 2a, 3, and 4 were isolated from a gel and cut with *Dde*I (Fig. 6B). The restriction patterns indicate that the four classes have a common early end and extend different lengths in the late direction (Fig. 6B and 7C). Fragment classes 2a, 3, and 4 do not originate from internal cutting of class 2 with DNase because fragments with a common late end are not evident in the gel analysis. These findings suggest that the A protein may bind to region II in a sequence of four steps from the early to the late direction.

DISCUSSION

The results summarized in Fig. 7 and previously published (4, 16, 18–20) lead to the inescapable conclusion that the SV40 A protein binds to multiple independent sites within origin regions I and II in an organized step-by-step process. In region I, dimethylsulfate and DNase footprinting show that A protein can bind to PI-2 and -3 without binding to PI-1. Precutting of region I with *Stu*I allows binding to PI-3 in the absence of PI-1 and -2. In region II, DNase footprinting demonstrates protection of the early side of the region before the late side. Precutting of region II with *Bgl*II or *Fnu*4HI allows binding

to isolated segments of DNA containing only pentanucleotides PII-1, PII-1 and -2, and possibly PII-3 and -4. The isolated pentanucleotides direct the proper alignment of bound protein because the limits of DNase protection of adjacent DNA are the same as those of protected fragments from intact DNA. These findings are consistent with our previous demonstration that the A protein binds to intact region II completely isolated from adjacent DNA to protect 30- to 35-, 40- to 45-, and 60- to 65-bp fragments (16). Furthermore, the A protein can not only bind DNA containing a single recognition pentanucleotide, but also contact and protect all of the pentanucleotides in regions I and II from dimethylsulfate simultaneously (4).

If we make several reasonable generalizations consistent with the accumulated results, a coherent model for A protein binding in the origin region is apparent. First, each protein monomer has a region that contacts a single pentanucleotide. Second, contact between the protein and nucleic acid sites requires and establishes a unique orientation of the protein relative to the direction of the pentanucleotide sequences. Finally, the association of monomers with penta-

FIG. 7. Map locations of regions and subregions of SV40 origin DNA protected from DNase. The locations of recognition pentanucleotides are shown at the top. Each pentanucleotide in region I or II is numbered to simplify description in the text. The location of restriction endonuclease cutting sites is shown, and the distance of landmarks in regions I and II from the *Fnu*4HI site in region II is indicated in base pairs. (A) Binding to intact DNA as determined by DNase footprinting in Fig. 1; (B) binding to intact or *Nco*I-cut DNA as determined by the fragment assays in Fig. 2, 3, and 6; (C) binding to DNA precut with *Stu*I as shown in Fig. 3 and 6; (D) binding to DNA precut by both *Stu*I and *Alu*I as shown in Fig. 4; (E) binding to DNA precut by *Stu*I and *Dde*I as shown in Fig. 4; (F) binding to DNA precut by *Stu*I and *Bgl*II as shown in Fig. 4; (G) binding to DNA precut by *Stu*I and *Fnu*4HI as shown in Fig. 4.

FIG. 8. Model for binding of the SV40 A protein to regions I and II of the SV40 origin of DNA replication. Regions I and II at the top were determined by DNase footprinting. Protein monomers bind to recognition pentanucleotides in a fixed directional relationship. The numbers below the diagrams of one of the protein monomers correspond to the number of DNA base pairs protected by that portion of the monomer. The lines at the bottom of the diagram correspond to DNase fragments from region II that are protected from DNase by the A protein.

nucleotides and adjacent DNA extends over a similar distance regardless of adjacent sequences, even though the adjacent sequences may influence the binding affinity and organization of the binding process.

Our present results indicate that the smallest protected sites in regions I and II are the 30- to 35-bp-long class 4 fragments shown in Fig. 7B and C. In both cases, a protein monomer would cover a recognition pentanucleotide 5'-GAGGC-3' (PI-2 or PII-1) and extend 10 to 15 bp beyond its 3' end and 15 to 20 bp beyond its 5' end. We will refer to the short extension as the head and the long extension as the tail of the protomer. Figure 8 shows the association of seven monomers in contact with the seven recognition pentanucleotides. The illustration of the protein-DNA interactions is intended to be a schematic rather than a literal representation of structure. The space occupied by the protein monomers corresponds to limits of DNase protection and not necessarily to the actual span of the protein. The direction of the pentanucleotides would result in a parallel overlapping arrangement of three protomers in region I. The tail of the third

monomer associated with PI-3 would extend into region II. This would account for our finding that the entire isolated region between the *Sma*I site in region I and the *Bgl*I site in region II can be but is not always protected from DNase. If all of the sites in region I were filled, no more than 50 to 55 bp of DNA would be protected. This conclusion is consistent with the results shown in Fig. 5.

In contrast, four monomers would bind to region II in an overall arrangement significantly different from that of region I. Binding of monomers to PII-1 and -2 or PII-3 and -4 would be similar to the overlapping and parallel binding to PI-1 and -2. However, each of these arrangements in region II would overlap each other in a head-to-head inverted manner. Together, they would protect 60 to 65 bp of DNA from DNase. The sequential binding of protomers to PII-1 through -4 would account for the protection of four fragments with a common early end (see Fig. 7). The spacial arrangement of the recognition pentanucleotides would require tight packing of the monomers with each monomer located 200 to 210° around the DNA helix from adjacent

monomers. The association of protein with contact areas on different faces of the helix can only be approximated in Fig. 8 because the shape of the monomers and the angle of their interaction with DNA is unknown.

Our model does not assume any state of protein assembly before binding but only describes the final outcome of binding. Interaction of monomers and dimers with pentanucleotides would more easily account for sequential binding within regions than would binding by preformed tetramers. Furthermore, a single species of tetramer would not easily accommodate interaction with the different arrangements of recognition pentanucleotides in regions I, II, and III and would not account for protection of variable spans of DNA in regions I and II. Although not in complete agreement, published studies suggest that all three forms of A protein can bind to DNA under some circumstances (2, 7, 10), but the precise location of the various forms bound to the origin has not been established.

The explanation for the differences in binding affinities of recognition sequences is unclear. The lower affinities of PI-1 and the pentanucleotides in region III probably reflect less favorable sequences within the actual contact pentanucleotides. The high binding affinities of PI-2 and -3 could result either from additional unmapped contact areas or from a protein-protein interaction between monomers located in a favorable orientation relative to one another. The latter possibility is consistent with the finding that cutting of PI-2 with *Sma*I diminishes binding to PI-3 (data not shown). In region II, there is a hierarchy of binding extending from the early to the late direction, even though recognition pentanucleotides PII-1 and -2 are equivalent to PII-3 and -4. Either a protein-protein interaction between regions I and II or a favorable sequence in the overlapping DNA of regions I and II could favor binding to the early half of region II. The difficulty in subdividing binding sites in region II by using DNase footprinting of intact DNA could indicate cooperative binding within region II. Finally, tertiary structure of DNA might also determine binding affinities.

Correlation of DNA binding patterns with initiation sites for RNA primers raises some interesting problems. Hay and DePamphilis (8) have shown that major RNA primers initiate within the contact pentanucleotide PII-3 and the adenine-thymine-rich area of region II. Complete binding to region II effectively blocks access to DNase over a span of 65 bp. It would seem difficult for an RNA primase to penetrate this obstacle to initiate replication. A number of explanations are possible. The A protein could have intrinsic RNA primase activity. Alternatively, it could interact with a cellular primase in

a highly specialized way to allow access to the origin. For example, a p53K cellular protein binds tightly to A protein and does not interfere with its binding to the origin (11). It is also possible that partial binding to PII-1 and PII-2 in region II of the origin is the active signal for replication whereas complete binding shuts down the process. Once binding and RNA priming have occurred, DNA elongation must proceed through the A protein binding region. In this case, the A protein might be released or perhaps become a part of a replication complex. In vitro systems will be required to define the details of the initiation process at the molecular level of understanding.

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