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SHORT COMMUNICATION

Replication of the Minute Virus of Mice

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The minute virus of mice (MVM) genome is a linear single-stranded length of approximately 5000 nucleotides of DNA with unique terminal palindromic sequences at both ends. The left (3′) hairpin is used to prime the initiation of DNA synthesis on parental single-strand DNA while the right (5′) hairpin or stem-plus-arms structure can also prime the initiation of DNA synthesis during synthesis of dimer and higher oligomers as well as synthesis of progeny single strands. Previous studies have shown that if viral duplex DNA was input into an *in vitro* DNA replication system using extracts from uninfected HeLa cells, the 5′ end of the molecule was able to form a hairpin and initiate DNA synthesis by DNA polymerase **δ** (Cossons *et al.* (1996), *Virology* 216, 258–264). In this study, the effect of the deletion of known *cis*-acting genetic elements upon the initiation of DNA replication was studied using a series of MVM mutants with deletions within the 5′ terminal region. Mutants containing deletions of elements A (nucleotides 4489–4636), B (nucleotides 4636–4695), and either one or both of the 65-bp repeats (nucleotides 4720–4785 and 4785–4849) were used as template in the *in vitro* DNA replication system. When element A was deleted, the efficiency of initiation decreased significantly. Subsequent removal of element B, leaving just the two 65-bp repeats, restored levels of initiation back to those seen in the wild-type genome. In the absence of either A or B both 65-bp repeats were necessary for efficient initiation, and removal of one of these repeats caused a decrease in efficiency. Thus, element B appeared to have a negative regulatory effect (in the absence of element A), and element A appeared to have a positive regulatory effect, at least in the presence of element B. These data demonstrate, for the first time, a complex interaction between these *cis*-acting regulatory elements which can function as both positive or negative regulators in the initiation of MVM DNA replication.

One of the genetic characteristics of the parvoviruses, including the minute virus of mice (MVM), is that the genome consists of a linear, single-stranded length of DNA approximately 5000 nucleotides in size with unique terminal palindromic sequences at both ends. This feature confers upon the virus the ability to form a hairpin or stem-plus-arms structure at the right (5′) end and a hairpin or "rabbit-earred" structure at the left (3′) end (*1*). The formation of these secondary structures is a property believed to explain their role as replication origins for viral DNA replication (*2–4*).

In vivo studies on MVM have previously shown several sequence elements which are important in maintaining or enhancing the efficiency of replication. Salvino *et al.* (*4*) showed that if one of the 65-bp repeats that are present 94 nucleotides (nt) inboard of the 5′ terminal palindrome was deleted, replication was inhibited 10- to 100-fold in comparison to the wild type. Tam and Astell (*5*) further showed that the presence of two genetic elements (identified as A and B) further upstream were necessary for the efficient replication of the genome and that two regions within these fragments (*Rsa*I A and *Rsa*I B) were able to activate a DNA replication-deficient genome (*6*). These sites were also shown to be bound in a sequence-specific manner by host cell proteins. Apart from indicating that there are multiple sequence elements required for MVM replication, these studies have also shown that host cell proteins (and not just the virally encoded NS-1 protein) play a crucial role in the activation of DNA replication.

Previous work showed that when viral duplex DNA with termini in the extended configuration (confirmed by Southern blot analysis; data not shown) was input into an *in vitro* DNA replication system using extracts from uninfected HeLa cells, the right end (5′) of the molecule was able to form a hairpin which was used to initiate DNA synthesis. This initiation of DNA synthesis from a

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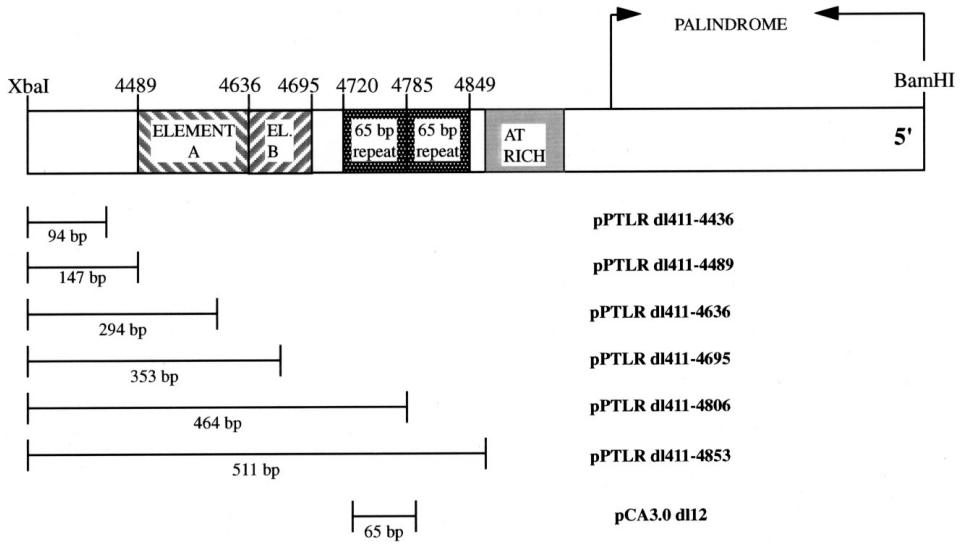

FIG. 1. Schematic diagram of the 5′ terminal region of the MVM DNA sequence from the *Xba*I site (4342) to the *Bam*HI site (5149). Significant regulatory sequence elements are indicated as shaded blocks. Element A, nucleotides (nt) 4489–4636; element B, nt 4636–4695; two 65-bp repeat regions, 4720–4785 and 4785–4849, respectively; AT-rich region, nt 4877–4902; palindrome nt 4944–5149. Each horizontal line approximately represents the deleted area of the 5′ terminus and does not correspond exactly to the size or position of the deletion. The size of the deletion is indicated below the line. The pPTLR deletion mutants are described in more detail in Tam and Astell (*5*); pCA3.0 dl12 is described in more detail in Salvino *et al.* (*4*); pMM984 is described in Merchlinsky *et al.* (*10*).

hairpin primer was carried out by DNA polymerase δ specifically and was inhibited if the terminal 82 nt (5067–5149) of the 5′ region were deleted (*7*). These studies led us to examine in more detail the role of known *cis*-acting genetic elements on the formation of the hairpin structure and upon the initiation of DNA synthesis from the 5′ terminal palindrome.

In order to do this, a series of MVM mutants was utilized with deletions within the 5′ terminal region, together with a mutant containing an internal deletion of the 65-bp repeat region (Fig. 1). All plasmids were linearized beforehand with *Bam*HI to release the 5′ palindromic end, as this was previously found to be important in the ability of the template to initiate DNA synthesis (*7*). The series of six linearized mutants with deletions increasing in size from 94 to 511 bp was used as template in an *in vitro* DNA replication assay (*7*–*9*) and then digested with *Eco*RV prior to separation by electrophoresis on a 1.5% agarose gel. The results (Fig. 2A) gave the expected digestion products. The fragment containing the 5′ terminal palindrome region (Fig. 2A, labeled 5′) decreased in size consistent with the size of the deletion (Fig. 2A, lanes 1–6; Fig. 2B, lane *8*) as did the corresponding novel band (labeled with an asterisk) previously shown to be an alternate secondary structure (*7*). The putative 5′ hairpin structure was still able to form and initiate DNA synthesis in two plasmids (pPTLR dl411-4436 and pPTLR dl411-4489 in Fig. 2A, lanes 1 and 2, respectively), as shown by the appearance of the novel band, coupled with initiation of DNA synthesis. The novel band, although not apparent with the other plasmids (Fig. 2A, lanes 3–6), was present in much lower amounts and either was obscured by the 3′ terminal fragment (see below) or was at too low a level to readily detect in this one-dimensional analysis. The same type of analysis performed upon the pCA3.0 dl12 mutant (Figs. 1 and 2B) showed no effect upon the production of the putative hairpin structure (asterisk) and associated initiation of DNA synthesis. The wild-type plasmid pMM984 was also analyzed (Fig. 2B, lane 7). pMM984 is a fully infectious clone, containing the entire MVM coding sequence in a pBR322 background (*10*) and was included as a comparison to indicate wild-type levels of initiation of DNA synthesis and hairpin formation. The pattern of hairpin formation and initiation of DNA synthesis seen with this plasmid is similar to that seen with the deletion mutants (pPTLR dl411-4436 and pPTLR dl411-4489) mentioned above.

To confirm that the novel band was in fact a secondary structure, the products of an *in vitro* DNA replication assay, using the deletion mutants, were subjected to two-dimensional electrophoresis on a neutral/alkaline gel (*11*). In the first dimension, a secondary structure should migrate faster than its duplex counterpart due to its smaller conformation. However, in the second alkaline dimension, a retardation in migration relative to the duplex forms is expected due to the denaturation of this structure. The smallest fragment seen in the one-dimensional gels (Fig. 2A, bottom band, containing only vector sequences) was run off the bottom of the gel to increase resolution of the 5′ and 3′ termini-containing fragments. The results (Figs. 3A–3H) show the duplex forms migrating on an arc with the associated secondary structure migrating as a retarded fragment (asterisk). This indi-

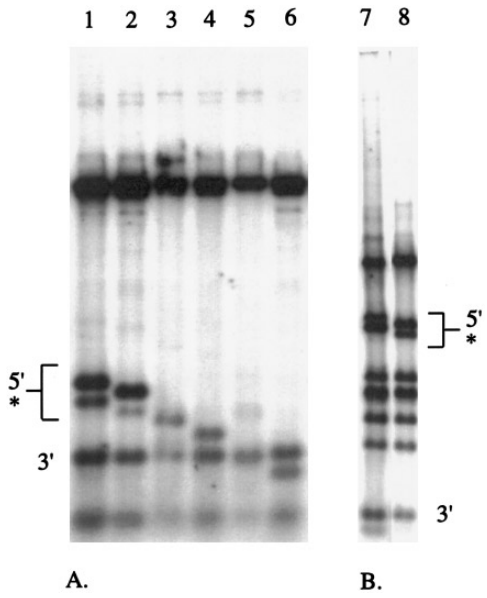

FIG. 2. *In vitro* DNA replication of plasmids containing deletions within the 5′ terminal region of MVM (Fig. 1). (A) Lane 1, pPTLR dl411-4436; lane 2, pPTLR dl411-4489; lane 3, pPTLR dl411-4636; lane 4, pPTLR dl411-4695; lane 5, pPTLR dl411-4806; lane 6, pPTLR dl411-4853. (B) Lane 7, pMM984 (wild type), lane 8, pCA3.0 dl12. All plasmids were linearized prior to the *in vitro* DNA replication reaction by digestion with *Bam*HI. After the *in vitro* reaction was complete, the pPTLR plasmids were digested with *Eco*RV, and plasmids pCA3.0 dl12 and pMM984 were digested with *Pst*I. Cell extracts for the *in vitro* replication reaction were prepared from log phase HeLa S3 cells, grown in suspension, as described previously (*8*). The *in vitro* reaction was performed as detailed in Cossons *et al.* (*7*). Digestion products from the reaction were run on a 1.5% agarose gel. The fragments containing the 5′ terminal palindrome sequence in the extended (5′, upper fragment) and the hairpin (*, lower fragment) configuration are indicated. The 3′ terminus-containing fragment (3′) is also indicated.

cated that the formation of the hairpin and initiation of DNA synthesis were still able to take place, despite deletions within *cis*-acting elements known to inhibit the replication activity of MVM *in vivo* (*4, 5*). However, an effect upon the efficiency of initiation was clearly seen. A second retarded band was seen in some samples (Figs. 3A, 3D, 3G, and 3H), indicative of an additional secondary structure being formed. Although its migration position would be consistent with the formation of a secondary structure at the 3′ terminus, there is no evidence to support this and further clarification would be required.

Previous work showed that, although the presence of the free end of the 5′ terminal palindrome alone was sufficient for the initiation of DNA synthesis from a hairpin, the presence of the free end of the 3′ terminal palindrome on the same plasmid increased the proportion of hairpin DNA being produced and subsequent initiation from the 5′ end (*7*). In addition, deletion of sequences within the 5′ terminal region was shown to affect the replication efficiency of MVM minigenomes (*5*). Therefore, it was anticipated that deletions of *cis*-acting sequence elements might affect the efficiency of initiation of DNA synthesis. To determine the extent of incorporation of nucleotides into each fragment, the autoradiographs shown in Figs. 2A, 2B, and 3A–3H were scanned using transmittance densitometry. The incorporation of nucleotides per basepair into the 5′ extended (E) and hairpin (H) forms was calculated, correcting for background levels of repair synthesis, which remain constant regardless of the extent of replication (data not shown). If initiation of DNA synthesis from the hairpin is inhibited, then the ratio of E:H forms of the 5′ terminus would increase, indicating a decreased efficiency of initiation of DNA synthesis. Previous work showed that the E:H ratio remained the same regardless of whether the se-

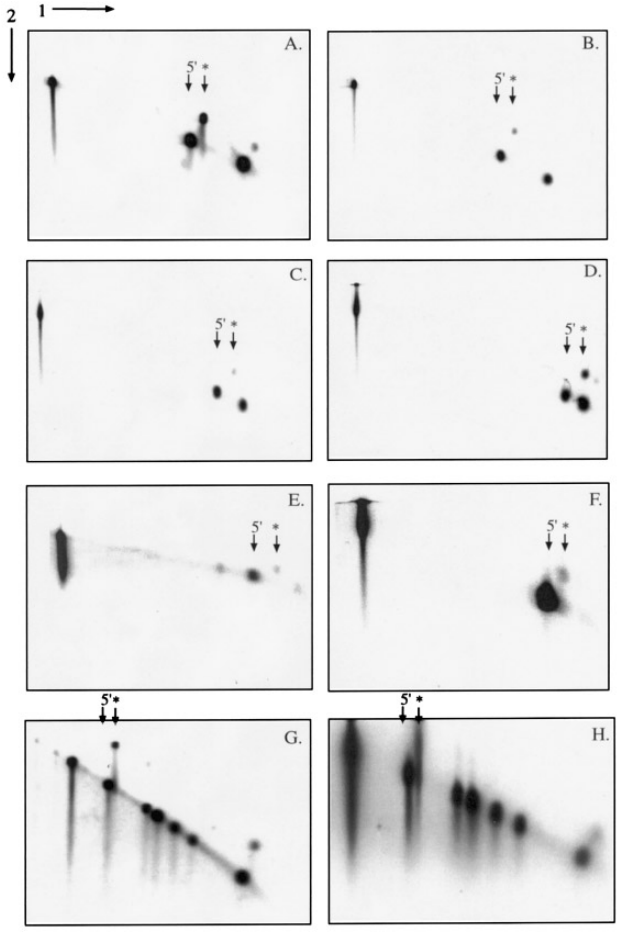

FIG. 3. Anomalous migration patterns in two-dimensional neutral/alkaline gels. (A) pPTLR dl411-4436. (B) pPTLR dl411-4489. (C) pPTLR dl411-4636. (D) pPTLR dl411-4695. (E) pPTLR dl411-4806. (F) pPTLR dl411-4853. (G) pMM984. (H) pCA3.0 dl12. All plasmids were treated as described in the legend to Fig. 2. After the products were subjected to electrophoresis in the first dimension in a neutral 1.5% agarose gel, the gel was stained in ethidium bromide, and the appropriate lane was cut from the gel using a clean scalpel blade. The gel slice was then equilibrated in alkaline running buffer (11) for 30 min, rotated 90°, and placed at the top of a second gel support at right angles to the direction of migration. A 1.5% alkaline agarose gel was then poured around the gel slice and electrophoresis carried out at 4° (11). The direction of electrophoresis in each dimension is indicated (arrows). The extended (5′) and hairpin (*) configurations of the 5′ terminal region are indicated.

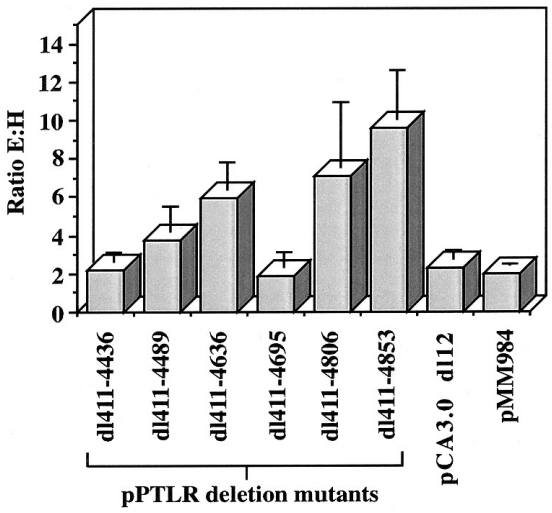

FIG. 4. The ratio of incorporation of radiolabeled nucleotides into the extended (E) configuration of the 5′ terminal region in comparison to the hairpin (H) form. The autoradiographs shown in Figs. 2A, 2B, and 3A–3H were scanned using transmittance densitometry. The incorporation of nucleotides per basepair into the 5′ extended and hairpin forms was calculated, correcting for background levels of repair synthesis. These data represent the average values of at least three separate experiments. Error bars indicate the standard error of the data.

quence was cleaved within 1788 bp (pMM984 *Bam*HI–*Pst*I, E:H ratio 2.58, Fig. 4), 807 bp (pMM984 *Bam*HI–*Xba*I, E:H ratio 2.58), or 750 bp (pPTLR dl411-4436 *Bam*HI–*Eco*RV, E:H ratio 2.14, Fig. 4) from the 5′ terminus (data not shown). This suggested that the main effect was upon initiation rather than upon elongation, although some effect upon the elongation activity could not be ruled out. When the E:H ratio of incorporation was plotted, it could be seen that the deletion of certain sequences significantly affected the efficiency of initiation from the hairpin (Fig. 4). Previous studies with these deletion mutants (*5*) indicated that the inhibitory effect of deleting specific MVM DNA elements upon full-length synthesis was not due to the decrease in size of the genome.

Deletion of one or both of the 65-bp repeat regions in addition to elements A and B (pPTLR dl411-4806 and pPTLR dl411-4853, respectively) reduced the efficiency of initiation to significantly lower levels in comparison to the wild-type pMM984 (Figs. 3E–3G and 4). Removal of just one 65-bp repeat had no significant effect upon the efficiency of initiation of DNA synthesis (pCA3.0 dl12; Figs. 3H and 4). Deleting element A (pPTLR dl411-4636) decreased the efficiency of initiation of DNA synthesis (Figs. 3C and 4), by comparison to wild-type levels (pMM984; Figs. 3G and 4), suggesting that its presence has a positive regulatory effect upon this reaction. When element B was also removed (pPTLR dl411-4695), leaving just the two 65-bp repeat regions, there was a marked increase in the efficiency of initiation from the hairpin form (Figs. 3D and 4), similar to levels seen in the wild type (pMM984; Figs. 3G and 4). Deleting 147 bp (pPTLR dl411-4489) resulted in a slight increase in the E:H ratio in comparison to the wild type (Figs. 3B and 4). Although no *cis*-acting sequences have been mapped to this region previously, this effect may be due to differences in the sensitivities of the cell systems used, as previous work has shown differences between COS7 and A9 cells in their ability to support efficient replication of these deletion mutants (*5*). A deletion of 94 bp (pPTLR dl411-4436) did not show any significant effect upon the proportion of DNA synthesis initiating from the hairpin form of the 5′ terminus (Figs. 3A and 4).

Thus, if element B was present, with both 65-bp repeats (pPTLR dl411-4636), efficiency of initiation decreased significantly (Figs. 3C and 4). When mutants containing elements A, B, and either one (pCA3.0 dl12; Figs. 3H and 4) or both (pPTLR dl411-4436, pPTLR dl411-4489; Figs. 3A, 3B, and 4) of the the 65-bp repeat regions were used as template in the *in vitro* DNA replication reaction, the efficiency of initiation was not significantly affected. In addition, when both of the 65-bp repeat regions were present, but with neither element A nor element B (pPTLR dl411-4695; Figs. 3D and 4), initiation of DNA synthesis also occurred at wild-type levels. This indicated that the presence of element B in the absence of element A had an inhibitory effect upon the initiation of DNA synthesis from a hairpin, while removal of element B restored wild-type levels of initiation, as did the presence of both elements A and B.

It is also apparent that, in the absence of either A or B elements, both 65-bp repeats are necessary for efficient initiation. When a partial copy (44 bp) of one of the 65-bp repeats was present (pPTLR dl411-4806), efficiency was decreased (Figs. 3E and 4). However, if both A and B elements were present with just one copy of the 65-bp repeat (pCA3.0 dl12; Figs. 3H and 4), levels were restored to that of the wild type *in vivo.* Thus, it is possible that just a complete copy of the 65-bp repeat would be sufficient to restore efficient replication. Previously Salvino *et al.* (*4*) had reported that deleting one of the 65-bp repeats decreased the levels of replication to 10% of that seen in the wild type *in vivo.* However, this was for full-length replication of MVM in infected cells. In contrast, Tam and Astell (*5*) showed that the one 65-bp repeat deletion within a minigenome did not appear to affect the efficiency of replication. It is possible that the initiation event described here is independent of events occurring during full-length replication or that viral proteins may somehow interact with the 65-bp repeats and/or with other proteins.

Thus, elements A and B act in different ways to regulate initiation of DNA replication from the hairpin. Element B appears to have a negative regulatory effect on the efficiency of initiation (in the absence of element A), while element A has a positive regulatory effect, at least in the presence of element B. In the absence of both elements A and B, 44 bp of one of the 65-bp repeats was not

sufficient for efficient initiation. In previous studies using the same plasmid constructs as those used here, deletion mutants pPTLR dl411-4436 and pPTLR dl411-4489 were able to support replication in COS-7 cells, but replication was significantly reduced when element A was deleted and completely abolished when both elements A and B were deleted (*5*). Further work indicated that two *Rsa*I restriction fragments, *Rsa*I A (nucleotides 4431–4579) and *Rsa*I B (nucleotides 4579–4662), contained within these elements, were able to activate DNA replication of a minigenome lacking these elements (*6*). However replication could be restored to 100% of the wild-type levels only if both *Rsa*I A and *Rsa*I B were present, indicating that the activation effect of these elements was additive.

The data presented here are consistent with the hypothesis that both elements A and B are required for efficient MVM replication. Furthermore, they also demonstrate an apparent negative regulatory effect of element B, in the absence of element A, when both 65-bp repeats were present. Previous work (*6*) showed that *Rsa*I A and *Rsa*I B could stimulate MVM replication by 60% and 20–30%, respectively. However, these levels of activation refer to full-length synthesis of MVM when these elements were supplemented with an NS-1-complementing expression vector, in which case the role of these elements during initiation could be different. Host cell proteins (MRF B3 and B4) have been identified, which bind to regions within elements A in a sequence-specific manner and which have been postulated to bind in a cooperative manner (either before or after binding to the DNA), thus activating the processing of the right terminus (*6*). The data presented here agree with such a model of protein cooperativity but indicate that, if these same proteins are involved in the initiation of DNA synthesis, then there may be a different role for them in the initiation activity compared to full-length DNA synthesis.

Studies in eukaryotes have shown that origins comprise two components: a core component which represents the minimal essential *cis*-acting sequence required to initiate DNA replication and one or more auxiliary components which affect the efficiency but not the mechanism of replication (*12*). This study has demonstrated that the presence of elements A and B, together with both 65-bp repeat regions, significantly affects the efficiency with which the initiation of DNA synthesis from the hairpin takes place. As this activity is known to occur in the absence of viral proteins (*7*), and host cell proteins are known to bind specifically to these regulatory sequence elements (*6*), it is possible that the initiation of synthesis is controlled in some way by this interaction. Tam (*13*) has postulated that the A/B region facilitates the formation of hairpin structures at the 5′ end of the genome by denaturing the genome and initiating strand separation. This model is distinct from that proposed earlier by Rhode and Klaassen (*14*) in which initiation of DNA synthesis occurred inboard of the 5′ end and included incorporation of nucleotides in this region.

In contrast to the work done on full-length DNA synthesis (*5*), the present study showed that element B on its own appeared to have an inhibitory effect in the initiation assay. This difference could be due to the presence of the NS-1 protein in the activation studies, which may, in turn, affect the interaction of host cell proteins with these regulatory sequences. It is also conceivable that the events involved in initiation and full-length synthesis are biochemically distinct. Both positive and negative regulatory *cis*-acting elements, bound by host cell proteins, have been identified in bovine papillomavirus type 1 (*15*) and control the efficiency of DNA replication. If full-length synthesis and initiation are indeed distinct activities, then it is conceivable that MVM DNA replication is controlled by events occurring at the level of initiation of DNA replication, which is in turn regulated by a complex system of both positive and negative control elements.

One possible mechanism could be the binding of a host cell protein to element A, allowing initiation at normal levels. This could prevent the binding of other proteins either to element B or to the 65-bp repeats. In the absence of element A, a different protein (or the same protein but with reduced affinity) would be able to bind freely to element B and exert a negative effect upon initiation. Removal of element B would consequently remove the inhibition and levels of initiation could then be restored to wild-type levels. The effect of the 65-bp repeats is overcome by the effect of either element A or B. It is also possible that these elements simply represent binding sites of differing affinities for the same protein, in a manner similar to the binding of SV40 T-antigen to the viral origin region (*16*). Previous work performed on the *in vitro* replication of adeno-associated virus DNA indicated that the inability of uninfected HeLa cell extracts to support full-length synthesis was due to some defect in elongation during strand displacement synthesis (*17, 18*). This could be due to any number of reasons, including the requirement for some component which alters the interaction of host cell proteins at regulatory sequences, allowing elongation and full-length synthesis to occur.

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