Effects of Position and Orientation of the 72-Base-Pair-Repeat Transcriptional Enhancer on Replication from the Simian Virus 40 Core Origin

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A number of recent studies have reported that in papovaviruses such as simian virus 40 (SV40) and polyomavirus, the replication of the viral DNA *in vivo* is activated by the viral transcriptional enhancer or promoter sequences. Both viral and cellular transcriptional enhancers are well known for their ability to activate transcription in a position- and orientation-independent manner. In the present study, we investigated the effect of the position and orientation of the SV40 72-base-pair (bp) repeat enhancer on its replication activation function. We constructed plasmids containing one copy each of the SV40 core origin and enhancer placed in either order and orientation and at different distances from each other. We assayed the replication efficiencies of these plasmids in the presence of an internal control plasmid in COS-1 monkey kidney cells producing the SV40 T antigen required for replication. We found that the 72-bp repeat was capable of activating replication equally well in either orientation when placed 8 or 9 bp from the core origin. The activation of replication was totally abolished, and replication efficiencies in most instances were found to be lower than that obtained with the core origin alone, when the 72-bp repeat was separated from the core origin by distances of 99 bp or more. This was in direct contrast to the situation with polyomavirus, in which activation of replication by the homologous enhancer or by the SV40 72-bp repeat enhancer is known to be position independent. We also found that when the SV40 core origin and the 72-bp repeat enhancer were adjacent to each other, efficient activation of replication was obtained only if the end of the core origin containing the 17-bp A+T block was linked with the enhancer. In the other orientation of the core origin, activation of replication was either diminished or abolished. Hypotheses such as alteration of chromatin structure by the enhancer and interaction between *trans*-acting factors binding to the enhancer and the core origin mediating the activation effect are discussed.

In papovaviruses such as simian virus 40 (SV40) and polyomavirus, the origin of DNA replication and elements controlling the transcription of the early and late viral genes are located in a 400-base-pair (bp) region of the viral DNA known as the origin region. In SV40, the core replication origin has been mapped to a 64-bp portion of the origin region that includes sequences with dyad symmetry and an A+T-rich sequence (3, 8, 9, 10, 12, 19). The core origin also contains binding sites for the viral early protein large T antigen (48, 54, 55), which is required for the replication initiation process (53). The SV40 core origin mapped *in vivo* has been found to be both necessary and sufficient for replication *in vitro* (40, 49, 51). *In vivo*, however, the transcriptional control sequences located adjacent to the core origin have been found to have a profound effect on DNA replication in both of these viruses. In SV40, the transcriptional control sequences are organized as two sets of directly repeated sequences. The G+C-rich 21-bp repeat present in three copies is a major component of the viral early promoter (6, 15, 17, 22, 23). The 72-bp repeat present in two copies serves as a position- and orientation-independent enhancer of transcription from the homologous SV40 early promoter (2, 6, 17, 18) and heterologous promoters (1, 43, 59). The SV40 late promoter is known to be a multicomponent promoter that becomes active in the late phase of infection, and different studies have pointed to the participation of the 21-bp (5, 23, 28, 29) and the 72-bp (4, 14,

30, 36) repeats in the late promoter function. In addition, both the 21- and 72-bp repeats (especially the latter) are required for efficient infectivity of SV40 (17, 21, 27, 61).

The role of the 21- and 72-bp repeats in the replication of SV40 DNA *in vivo* has been examined by several investigators. In an earlier study, Bergsma et al. (3) reported that the 21-bp repeat enhances replication efficiency by 5- to 20-fold in a dose-dependent fashion and that, in the presence of the 21-bp repeat, the 72-bp repeat is totally dispensable for efficient replication. The enhancement of replication by the 21-bp repeat has been confirmed by more recent studies (33, 39, 40). These studies and another by DeLucia et al. (10) report additionally that the 72-bp repeat is also capable of enhancing the replication of SV40 DNA. Enhancement of replication by the 72-bp repeat is not observed if the intervening 21-bp repeat is left intact (33, 39, 40), in agreement with the earlier results of Bergsma et al. (3).

Unlike SV40, in which the core origin alone can initiate replication at a basal level, the polyomavirus core origin is incapable of initiating replication in the absence of the adjacent transcriptional enhancer region (11, 41, 44, 57, 58). The replication and transcription activation functions of the enhancer sequence correlate very well in polyomavirus. Only those subfragments of the 244-bp polyomavirus enhancer region that are capable of activating transcription are also capable of activating replication. The homologous polyomavirus enhancer can be replaced by heterologous enhancers such as those of SV40, Moloney murine leukemia virus, and the immunoglobulin heavy-chain gene for its

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FIG. 1. Nucleotide boundaries of the 106-bp fragment containing the core replication origin and the 94-bp fragment containing the 72-bp repeat enhancer. The core origin (O) and enhancer (E) cassettes are shown at the top, and their locations in the broader SV40 origin region are indicated at the bottom. The nucleotide numbers are according to Tooze (56). The arrows showing early (e) or late (l) indicate the orientations in which the cassettes face the early or late genes of SV40, which are located on opposite sides of the origin region. Symbols inside the O and E cassettes denote sequence landmarks that are also indicators of the orientations of these cassettes. The roman numerals I and II within O denote T antigen-binding sites I and II, respectively (48, 54, 55). The term AT denotes the 17-bp A+T block. The letters K and C within E denote the enhancer core elements with the sequences 5'-AAG(C/T)ATGCA-3' and 5'-TGTGGAA(A/T)GT-3', respectively, both of which occur in duplicate (16, 32, 61, 63). The 106-bp core origin fragment and the 94-bp enhancer fragment were taken out of a mutant of SV40 as described by Hartzell et al. (30).

replication activation function; and the cell type specificity associated with transcription enhancement by the immunoglobulin enhancer applies to its replication activation function also (7, 11). Also, both the polyomavirus and SV40 enhancers are capable of activating polyomavirus DNA replication in a position- and orientation-independent fashion (11).

Two of the most important properties of viral and cellular transcriptional enhancers are their bidirectionality and position independence (25). The SV40 72-bp repeat, for instance, is known to function as a transcriptional activator in *cis* in either orientation, and from distances as far as 4 to 5 kilobases (kb) from the promoter in question (1, 18, 43, 59). In the studies reported above on the activation of SV40 DNA replication by the 72-bp repeat enhancer, the enhancer and the core replication origin in their normal orientation were juxtaposed by deletion of the intervening 21-bp repeat (10, 33, 39, 40). Because of the known position and orientation independence of enhancers in their transcription activation function, and since in polyomavirus there is a direct correlation between the transcription and replication activation functions of the enhancer, we wanted to examine whether in SV40 the 72-bp repeat transcriptional enhancer displays position and orientation independence in its replication enhancement function. Toward this goal, we constructed plasmids in which the SV40 core replication origin and the 72-bp repeat enhancer were juxtaposed in the normal or reversed order and/or orientation with respect to each other, or separated from each other by various distances of intervening plasmid sequences. The replication efficiencies of these plasmids were determined accurately *in vivo* in the presence of an internal control plasmid.

MATERIALS AND METHODS

Plasmid construction. Plasmids were constructed by standard cloning techniques with recombinant DNA methods (42). Plasmids pE_lcat and pE_ecat, containing the bacterial chloramphenicol acetyltransferase (*cat*) gene (26), which we had made previously, served as good starting material for the plasmids to be constructed in the present study. Plasmids pE_lcat and pE_ecat were derived from plasmid pA₁₀cat₂ (37)

by replacing the SV40 early promoter sequence present in pA₁₀cat₂ with a 94-bp SV40 enhancer fragment in the late or early orientation, respectively. (The early and late orientations indicated by the subscripts e and l are opposite orientations of a given SV40 sequence and denote the polarity in which that sequence faces the early or late genes, respectively, in SV40 DNA [56].) This 94-bp enhancer fragment (E in Fig. 1), which includes one copy of the 72-bp repeat and a 22-bp adjacent sequence up to nucleotide 272, is known to be adequate for the transcription activation function of the SV40 enhancer region (16, 17, 27, 32, 43, 63).

A 106-bp fragment located between SV40 nucleotides 5172 and 34 (56) (O in Fig. 1) was used as a source of the core replication origin. This 106-bp fragment contains all the sequences obligatory for replication, including T antigen-binding site II and the 17-bp A+T block, and also contains T antigen-binding site I. This core origin fragment, with its ends modified with the appropriate oligonucleotide linker sequences, was cloned in either orientation at unique restriction sites located 9, 99, 499, or 3,800 bp upstream from the enhancer sequence in pE_lcat or 8, 99, or 3,800 bp upstream from the enhancer in plasmid pE_ecat (Fig. 2). Additionally, the core origin was also cloned in either orientation at the opposite side of the enhancer (between the enhancer and the *cat* gene) at a distance of 8 bp from the enhancer in plasmid pE_lcat (Fig. 2). All plasmid constructions were thoroughly tested and verified by restriction analysis and limited DNA sequencing (42). The plasmids constructed in this fashion are indicated at the top of each core origin insert in Fig. 2, with the plasmid nomenclature indicating the order and orientation of the core origin (O) and enhancer (E), with the number in parentheses indicating the distance (in base pairs) between them. For instance, plasmid pO_l(9)E_l has both the core origin and enhancer located in the late orientation upstream from the *cat* gene, with the origin and enhancer sequences separated by a distance of 9 bp.

Plasmid p2X21Ori, which was used as the internal control plasmid in the replication assays, was constructed by Kyung-Pyo Yoon in our laboratory as follows. A 160-bp fragment containing the SV40 core replication origin and the adjacent two copies of the 21-bp repeat were excised from plasmid pSVori2X21, described previously (3). This frag-

FIG. 2. Construction of plasmids containing the core replication origin (O) and the 72-bp enhancer (E) in either order and orientation and with different distances between them. The 94-bp enhancer (E) sequence shown in Fig. 1 was cloned in the late or early orientation upstream from the *cat* gene in a promoterless vector derived from plasmid pA₁₀cat₂ (37), resulting in plasmids pE_lcat and pE_ecat, respectively. The 106-bp core origin fragment shown in Fig. 1 was cloned in either orientation at different locations as indicated by the insert on the plasmid circles. These core origin inserts are at distances of 9, 99, 499, or 3,800 bp 5' or at a distance of 8 bp 3' from the enhancer sequence, as indicated by the numbers above the lines drawn inside the circles. The ends of the core origin fragment were modified to *Bgl*II ends for cloning at the *Bgl*II and *Bam*HI sites, to *Sal*I ends for cloning at the *Sal*I site, and with *Mlu*I ends for cloning at the *Nru*I site which had been converted to an *Mlu*I site. The end modifications were carried out with the respective oligonucleotide linkers with the following sequences: *Bgl*II, 5'-CAGATCTG-3'; *Sal*I, 5'-GGTCGACC-3'; *Mlu*I, 5'-GACGCGTC-3'. Linkers were purchased from New England Biolabs, Beverly, Mass. The notations inside the E or O cassettes are mainly for denoting their orientations and are described in the legend to Fig. 1. Each plasmid construct contained only one core origin insert and one enhancer insert. The names of the plasmids produced are indicated on top of each core origin insert (with the enhancer sequence to be combined with the core origin being already in place in the plasmid). Plasmid nomenclature indicates the order and orientation (early or late as indicated by subscripts e or l) of the O and E cassettes with respect to the *cat* gene, with the numbers within parentheses showing the distance (in base pairs) between the cassettes.

ment, with its ends modified to *Bam*HI ends, was cloned at the unique *Bam*HI site at nucleotide 375 of a derivative of plasmid pBR322 lacking the sequences between plasmid nucleotides 972 and 2066, resulting in plasmid p2X21Ori.

Plasmid replication efficiency assays. Replication assays were performed in T antigen-producing COS-1 monkey kidney cells (24) as described previously (3), with the modification of including an internal control plasmid as described by Li et al. (40) and Lee-Chen and Woodworth-Gutai (39). Briefly, COS-1 cells were grown in 100-mm plates overnight to about 25 to 30% confluence. Cells in each plate were transfected with 100 ng of the test plasmid and 25 ng of the internal control plasmid in the presence of DEAE-dextran (500 μ g/ml). (The control plasmid was used at a lower level in the transfections since it was found to replicate much more efficiently than the test plasmids.) At 24 or 48 h posttransfection, cells were harvested and low-molecular-weight DNA was extracted. The DNA preparations were digested with *Nco*I for linearization and with *Dpn*I to differentiate between the *Dpn*I-sensitive input DNA and the *Dpn*I-resistant newly replicated plasmid DNA (45). (The single *Nco*I site of the test plasmids is located within the *cat* gene [26], and that of the control plasmid is at nucleotide 37 of SV40 DNA [56].) The digests were fractionated by agarose gel electrophoresis, blotted onto nitrocellulose filters, and hybridized with a ³²P-labeled probe made by nick

translation of plasmid p2X21Ori (50). (The probe was made from the internal control plasmid rather than from one of the test plasmids to ensure an equivalent level of hybridization with all of the test plasmids.) The filters were washed, dried, and autoradiographed to reveal the radioactive DNA bands. Bands corresponding to the newly replicated test plasmids (~5.5 kb) and the internal control plasmid (~3.4 kb) were found to separate quite well because of their significant size difference. The positions of these bands were marked on the filters by superimposing the autoradiographs on them. The bands were cut out from the filters, and the amounts of radioactivity in them were measured with a Packard Tri-Carb model 3375 liquid scintillation spectrometer, giving a measure of the total replicated DNA in each case. The value obtained for each test plasmid was divided by that obtained for the control plasmid in that lane, giving the specific replication efficiency of the test plasmid, as described by Li et al. (40). The values determined in different experiments in this fashion were found to be highly consistent and reproducible, as reported by Li et al. (40).

RESULTS

Activation of replication by the enhancer at 24 and 48 h posttransfection. Figure 3 compares the replication efficiency of test plasmid pALori, containing the core origin alone,

TABLE 1. Replication efficiencies of plasmids containing the core origin alone (pALori) and combinations of the core origin and enhancer^a

Expt	Plasmid	Radioactivity (cpm) in DNA band ^b		Specific replication efficiency (test/control)	Relative replication efficiency (%)
		Test	Control		
1	pALori	11,196	43,406	0.26	100
	pO _l (9)E _l	52,360	22,137	2.37	910
	pO _e (99)E _l	3,448	29,505	0.12	45
	pO _l (499)E _l	1,043	29,133	0.04	14
	pO _e (499)E _l	1,647	18,141	0.09	35
	pO _l (3800)E _l	3,405	15,625	0.22	84
	pO _e (3800)E _l	1,118	12,344	0.09	35
	pO _l (8)O _l	5,094	25,462	0.20	77
	pO _l (8)O _e	22,737	9,904	2.30	884
	pO _l (8)E _e	49,457	16,874	2.93	1,128
	pO _e (8)E _e	15,350	23,113	0.66	256
	pO _l (99)E _e	8,735	29,498	0.30	114
	pO _e (99)E _e	1,518	22,590	0.07	26
	pO _l (3800)E _e	3,785	23,390	0.16	62
	pO _e (3800)E _e	2,837	22,402	0.13	49
2	pALori	316	2,770	0.114	100
	pO _l (99)E _l	450	4,462	0.10	87

^a Plasmid pALori contains the core origin alone. Other plasmids contain both the origin and the enhancer sequences. Plasmid nomenclature indicates the order of these sequences (with respect to the *cat* gene located 3'), their orientations, as shown by the subscripts (l for late and e for early), and the spacing (in base pairs) between them, as shown by the numbers in parentheses.

^b The relative replication efficiency values shown were calculated from the replication assays shown in Fig. 4 and 5. The autoradiographs were superimposed on the parent nitrocellulose filters, and the positions of the newly replicated DNA bands were marked. The bands were cut out and counted, giving a measure of the total amount of newly replicated DNA in each case. The value obtained for each test plasmid was divided by that obtained for the control plasmid in that lane, giving the specific replication efficiency value. The relative replication efficiency values shown are the specific replication efficiency values determined as above, with the value for plasmid pALori (which contained the core replication origin alone and lacked the enhancer) taken as 100%.

found to replicate as well or better, registering an 11-fold greater replication efficiency than pALori. This result indicates that the reversed orientation of the enhancer is as good or better than the normal orientation in its ability to activate replication. Thus, the SV40 72-bp repeat enhancer is truly bidirectional in its replication activation function, as has been established previously for its transcription activation function (1, 18, 43).

Orientation in which the core origin faces the enhancer is critical for activation of replication. It was shown above that plasmid pO_l(8)E_e, which had the core origin in the normal

orientation but had the enhancer in the reversed orientation, replicated very well, showing an 11-fold increase in replication efficiency over that of plasmid pALori, having the core origin alone. In contrast, plasmid pO_e(8)E_e, having both the core origin and the enhancer in the reversed orientation (Fig. 2), replicated less efficiently (Fig. 3 and 5), registering only a 2.5-fold increase in replication efficiency over that of pALori (Table 1). Another example with a structure similar to that of pO_e(8)E_e was plasmid pE_l(8)O_l. In this plasmid, the order of the core origin and the enhancer sequences was inverted (with respect to the *cat* gene located 3' to them) in comparison with that in the prototype plasmid pO_l(9)E_l. Although the enhancer and core origin were in the same (late) orientation with respect to the *cat* gene in both plasmids pO_l(9)E_l and pE_l(8)O_l, the orientation in which the core origin faced the enhancer was normal in the prototype plasmid pO_l(9)E_l and reversed in pE_l(8)O_l (Fig. 2). The results of the replication efficiency assays (Fig. 4 and Table 1) indicate that while the prototype plasmid pO_l(9)E_l replicated very well, plasmid pE_l(8)O_l replicated poorly, with its replication efficiency being somewhat less than that of plasmid pALori, which lacked the enhancer sequence altogether.

That the orientation in which the core origin faces the enhancer is critical for activation of replication was further illustrated by comparison of the replication efficiencies of plasmids pE_l(8)O_l and pE_l(8)O_e. In plasmid pE_l(8)O_l, the end of the core origin nearest to the T antigen binding site I faced the enhancer, and in pE_l(8)O_e, the other end (i.e., the one closest to the 17-bp A+T block) of the core origin faced the enhancer. These two plasmids differed drastically in their replication efficiencies, with the latter being about 11-fold more efficient than the former (Fig. 4 and Table 1).

The above results indicate that although the enhancer can operate in a truly bidirectional manner to activate replica-

FIG. 5. Autoradiograph showing the replication efficiencies of the E_e series of test plasmids, containing the enhancer (E) in the early orientation. Plasmid nomenclature indicates the orientation of the core origin (O) and its distance from the enhancer. The plasmids tested are indicated above each lane. Replication assays were performed as described in Materials and Methods. The DNA samples shown here were processed in the same experiment as those shown in Fig. 4a, with the same probe and hybridization conditions. Only the linearized, *DpnI*-resistant, newly replicated DNAs in the 48-h posttransfection isolates are shown. Lanes Mt and Mc, Test and control linear marker plasmid DNAs, respectively.

tion, the orientation in which the core origin faces the enhancer plays an important role. Efficient replication requires that the end of the core origin containing the 17-bp A+T block be linked with the enhancer.

Spacing of the core origin away from the enhancer abolishes activation of replication. We next proceeded to investigate the question of whether the 72-bp repeat enhancer is capable of activating replication when placed at various distances from the core origin. Plasmids constructed for this purpose contained the core origin present at distances of 99, 499, or 3,800 bp from the enhancer in the E_1 series and at distances of 99 or 3,800 bp from the enhancer in the E_e series of plasmids (Fig. 2). The results are shown in Fig. 4 for the E_1 series and in Fig. 5 for the E_e series. The quantitations of the relative replication efficiencies are presented in Table 1.

Our results show that in contrast with the efficient activation of replication brought about by the 72-bp repeat enhancer when it is close to the core origin, no activation was observed when the core origin and the enhancer were separated by distances of 99 bp or more. In addition, except in one case, the plasmids in which the core origin and the enhancer were spaced away exhibited replication efficiencies lower than that of the enhancerless plasmid pALori (Table 1). The relative replication efficiencies observed (determined with respect to that of pALori) were found to vary from 14% in one to 114% in another of these plasmids, with no clear patterns discernible based on the respective orientations of the core origin and the enhancer and the distance by which they were separated. What was consistent, however, was the lack of efficient activation of replication that we observed in these plasmids.

DISCUSSION

The results presented above indicate that in SV40, the enhancer exhibits orientation (but not position) independence in its activation of replication. Efficient activation of replication is obtained when the enhancer and the core origin are closely linked, but is abolished when they are separated by 99 bp or more. When they are juxtaposed, the enhancer functions in either orientation, but the core origin exhibits a clear preference for linkage through the end containing the 17-bp A+T block. The orientation independence of the enhancer is true to its form, since it is exhibited in transcriptional activation and in the activation of replication from the polyomavirus core origin, as referred to in the Introduction. However, our finding of position dependence of the SV40 enhancer vis-a-vis its interaction with the SV40 core origin is surprising. Both the SV40 and polyomavirus enhancers can activate replication in polyomavirus when placed at distances of up to 1,400 bp from the viral core origin (12). Regarding the position independence of enhancer sequences in transcription, activation of transcription is known to decrease as a function of distance from the promoter sequence; but even at a long distance of 4 kb at least a 10-fold enhancement of transcription was observed (60).

The difference in the position effect of the enhancer on replication in SV40 and polyomavirus indicates that the mechanism of activation of replication is likely to be different in these two papovaviruses. One of the reasons for this difference might be traced to the respective core replication origin. The SV40 core origin exhibits a significant basal level of activity, unlike the core origin of polyomavirus which is inactive in the absence of an enhancer sequence in *cis*. The reason for this difference is not clear. Both core origins contain sequences of dyad symmetry containing T

antigen-binding sites and an adjoining A+T block of similar length. One of the possible explanations includes the presence of a TATA box sequence of the viral early promoter in the SV40 (but not the polyomavirus) A+T block (11). In this connection, it is interesting that in Epstein-Barr virus (EBV), the origin of autonomous replication consists of a sequence with dyad symmetry (resembling a core origin) and an enhancerlike repeated sequence located 1 kb away (46). The distances between the two elements can be varied, but both elements are required in *cis* for replication, as in polyomavirus (46). It may be that if the core origin is inactive, as in polyomavirus (and perhaps EBV), replication is activated by an enhancer independent of its position. If the core origin can operate on its own to some extent (as in SV40), additional activation of replication by the transcriptional enhancer requires interaction from close proximity.

Whereas the mechanism of activation of replication by the transcriptional enhancer is likely to be different in SV40 than in polyomavirus, within SV40 itself there are many similarities between the 72-bp repeat enhancer and the 21-bp repeat promoter in this respect. Similar to the findings in the present study with the 72-bp repeat enhancer, Lee-Chen and Woodworth-Gutai have observed that the activation of replication by the 21-bp repeat is orientation independent and position dependent (39). Similarly, Innis and Scott have observed that separation of the core origin from the 21-bp repeats reduces replication efficiency (34). Also, both the 21-bp repeat (39) and the 72-bp repeat (this study) prefer linkage to the core origin in the same way (through the end of the core origin containing the 17-bp A+T block) for efficient activation of replication.

Both the 21- and 72-bp repeats play important roles in the generation of nuclease hypersensitivity and a nucleosome-free gap in the SV40 origin region (20, 34, 35). The generation of such an open chromatin structure by these elements could facilitate increased replication initiation activity in the neighboring core origin. In this connection, it is interesting that in the insertion mutants of Innis and Scott, the decrease in replication efficiency observed was associated with lower nuclease hypersensitivity in the core origin (34). In the naked DNA templates used in the *in vitro* replication assays, the core origin may be easily accessible to replication initiation factors without the need to form such a specialized open chromatin structure, explaining the lack of stimulation by the 21- and 72-bp repeats.

In vivo, although the viral origin region is free from histone (nucleosome) binding, there is evidence that it is available for binding by factors such as those required for initiation of replication and transcription. Two of these proteins or factors have been well characterized, one being the viral replication initiation protein T antigen, binding to defined sites in the core origin (48, 54, 55), and the other being the cellular transcription factor Sp1, binding to the GC box sequences in the 21-bp repeat (13, 33). Both direct and indirect evidence has been accumulated recently for the binding of other factors (mainly of host cellular origin) to the core origin and the 21- and 72-bp repeats (8, 9, 16, 32, 38, 47, 62, 63). In addition to binding, specific interaction between the bound factors may be required for efficient initiation of replication or transcription. There is *in vivo* evidence for a stereospecific interaction between transcription factors binding to the 72- and 21-bp repeat components of the SV40 early promoter and its TATA box component present in the 17-bp A+T block (52). Deletions in the 17-bp A+T block result in greatly reduced replication efficiency (8, 9, 19). The 21-bp repeat can compensate for this replication defect if it is

placed at an appropriate distance from the shortened A+T block (19). These results indicate that factors bound to the 21-bp repeat and the 17-bp A+T block interact with each other. A similar interaction could occur between factors bound to the 72-bp repeat and the 17-bp A+T block to activate replication from the core origin. Such an interaction could be either diminished or abolished when the 72-bp repeat enhancer or the 21-bp repeat promoter is separated from the core origin or placed at the opposite side of the core origin (39; this study).

One of the consequences of the close interaction between the 21-bp repeats and the core origin is that some of the replication initiation sites *in vivo* are found to be physically located within the 21-bp repeats (31). A similar contribution could be made by the 72-bp repeat enhancer when it is directly linked with the core origin. This and the other two hypotheses mentioned above are not mutually exclusive, and any of them or a combination could explain the position and orientation effects exhibited by the 21- or 72-bp repeats in their activation of replication.

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