A 69-Base-Pair Monkey DNA Sequence Enhances Simian Virus 40 Replication and Transcription through Multiple Motifs

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We have determined that a 69-base-pair (bp) monkey DNA sequence, previously found to enhance simian virus 40 replication, has transcriptional enhancer activity as well. Consensus recognition sites for the transcription factor AP-1, present at each end of this sequence, are partially responsible for its replication- and transcription-enhancing activities. Other motifs within the 69-bp monkey sequence also act to increase the levels of replication and transcription. The activity of the monkey sequence is augmented by the presence of a simian virus 40 21-bp repeat. The 69-bp sequence enhances transcription but not replication from a distance.

We conclude that the stimulation of replication and transcription can be uncoupled, suggesting that different mechanisms may be involved.

The cis-acting elements that control simian virus 40 (SV40) DNA replication and transcription are located in the same region of the viral genome, from approximately nucleotide (nt) 5171 to 272. The elements of the early promoter include the TATA box (4), the 21-base-pair (bp) repeats (14, 15), and the 72-bp repeats (3, 19, 34). The minimal sequence that can support detectable levels of replication is an approximately 64-bp region called the core origin (21, 27, 43). We and several other groups have shown that the 21-bp repeats as well as the 72-bp repeats can enhance DNA replication (7, 10, 12, 23, 30, 31). Similar findings indicating that transcriptional regulatory elements may facilitate core origin function have been obtained for polyomavirus, BK virus, adenovirus, bovine papillomavirus, and Epstein-Barr virus (11, 12).

Furthermore, protein factors that function in both replication and transcription have been isolated. CCAAT-binding transcription factor, a cellular protein that activates transcription from promoters that contain a CCAAT box, is identical to nuclear factor I, a cellular DNA-binding protein required for the initiation of adenovirus replication in vitro (25). Nuclear factor III, a protein from HeLa cells that stimulates the initiation of adenovirus DNA replication, has been shown to be functionally identical to octamer transcription factor 1 (37).

Our laboratory has used evolutionary variants of SV40 as tools to determine the sequence elements and arrangements that confer high-level replication. Evolutionary variants are generated by serially passaging SV40 in permissive monkey cells at high multiplicities of infection. Under these conditions, the viral DNA recombines at high frequency with itself and with cellular DNA, generating a heterogeneous collection of naturally arising variants (6). We have previously shown that alterations that occur in the regulatory regions of abundant species from later passages enhance replication efficiency (29). Of particular interest is a 69-bp non-SV40 DNA sequence present in SV40 evolutionary variants cloned from passages 40 (ev 1108 [45]) and 45 (ev 1104 [21]). We cloned monkey genomic DNA containing this sequence and found that the 69-bp non-SV40 sequence is in fact monkey DNA that recombined with viral DNA (Fig. 1A). The genomic clone contains a perfect match (nt 190 through 249) to nt 10 through 69 of the non-SV40 DNA present in the variants (Fig. 1B). The genomic DNA includes a consensus recognition site for the transcription factor AP-1 (2, 28) from nt 243 to 249, which was duplicated and inverted during the recombination events that generated the variants. The duplication resulted in the 69-bp sequence having an AP-1 site at each end (nt 1 to 7 and 63 to 69). A match of 9 of 10 nt to the consensus recognition site for the transcription factor

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FIG. 1. Sequences of monkey genomic clone (A) and non-SV40 (B) DNA present in SV40 variants ev 1108 and ev 1104. Potential AP-1- and AP-4-binding sites are overlined. The segment of the genomic clone that appears in the SV40 variants is underlined. (C) Consensus AP-1 and AP-4 recognition sequences (2, 28, 33).
In an earlier study, we showed that the 69-bp sequence stimulates replication from the SV40 origin (29). To determine whether the 69-bp sequence might also enhance transcription, we tested for transcriptional enhancer activity by inserting it into plasmids that carry the gene for chloramphenicol acetyltransferase (CAT) as a reporter (Fig. 2). BSC-1 cells were transfected with 3.5 μg of plasmid DNA by the DEAE-dextran method (32). Cells were harvested at 48 h posttransfection, extracts were prepared, and CAT activity was assayed (18). To control for variations in transfection efficiencies, each experiment was repeated several times (each time in duplicate) and CAT activities were normalized to the amount of plasmid DNA in cells at the time of harvest (1). Plasmid pC139cat (Fig. 3, lane 1) consists of the promoter-origin C139 (the SV40 origin of replication plus one 21-bp repeat) driving the CAT gene. The 69-bp sequence increased the CAT activity of pC139cat about 10-fold when placed upstream and proximal and about 4-fold when placed downstream and distal to the promoter in either orientation (Fig. 3, lanes 1 to 5). The monkey DNA also enhanced the activity of the triosephosphate isomerase promoter (5) about 12-fold in either orientation (Fig. 3, lanes 6 to 8). Primer extension analysis demonstrated that transcription was initiated at the normal SV40 early-early start sites in these constructs (data not shown). The presence of the 69-bp monkey sequence upstream of C139 did not alter the position of transcription initiation, nor did this sequence contain any start sites of its own. Therefore, the 69-bp monkey sequence functions as a transcriptional enhancer, not as a promoter.

AP-4 (33) is present from nt 221 to 230 of the monkey genomic sequence and nt 41 to 50 of the 69-bp sequence. Recombination between the viral and genomic DNAs occurred at a 7-bp sequence common to both parental DNAs (nt 57 to 63 of the SV40 21-bp repeat region and nt 250 to 256 of the monkey genomic sequence). An A+T-rich region (71% A+T from nt 105 to 195) is adjacent to the other recombination junction. Computer analysis through GenBank detected no other significant similarity between the 589-bp monkey genomic sequence and any published mammalian sequence.

FIG. 2. (A) Derivation of insert fragments. Plasmid pCl39H carries a portion of the regulatory region of the passage 45 evolutionary variant v 1104 (21). Open horizontal arrows denote the SV40 origin of replication (SV40 nt 3171 to 37) and 21-bp repeat segments and their orientations (pointing in the direction of early transcription). Dots represent GC consensus boxes, which are recognition sites for the transcription factor Sp1 (14). Wavy lines represent monkey sequences. HindIII and HindIII restriction sites are indicated. (B) Vector pSV0cat(Cla). CAT coding sequences are represented by a solid box. The arrow indicates the direction of transcription. The 3' noncoding region derived from SV40 is represented by an open box. The small t antigen splice sites (dotted lines) and polyadenylation site are indicated.

FIG. 3. Transcriptional enhancer activity of the 69-bp monkey sequence. A representative CAT assay is shown. Relative CAT activities were determined by averaging results from several experiments and comparing them with the CAT activity of the basal plasmid pC139cat. Orientation of the monkey (host) sequence (direct, 1-69H; inverse, 69-1H) is indicated below the C139cat or TPlcat transcription units. The monkey sequence was inserted upstream of C139cat at the unique ClaI site or downstream of C139cat at the unique BamHI site. Lanes: 1, pCl39cat; 2, p1-69H · C139cat; 3, p69-1H · C139cat; 4, pCl39cat · 1-69H; 5, pC139cat · 69-1H; 6, pTPlcat; 7, p1-69H · TPlcat; 8, p69-1H · TPlcat.
To determine the significance, if any, of the consensus AP-1 sites, we constructed a set of mutations of the monkey sequence (Fig. 4C) and assayed their relative replication efficiencies (Fig. 4A) and CAT activities (Fig. 4B). Replication efficiency (Fig. 4A) was determined by cotransfecting COS-1 cells with equimolar amounts of a test plasmid (CAT vector, upper band) plus a smaller competitor plasmid (lower band). Plasmids carrying the SV40 origin replicate in COS cells because of endogenous T antigen (17). Plasmid DNA was extracted 48 h posttransfection (24), digested with HindIII, separated by agarose gel electrophoresis, transferred to nitrocellulose (42), hybridized to labeled pUC13, and quantitated by densitometry. The competitor plasmid serves as an internal standard for replication efficiency. The presence of the entire 69-bp monkey sequence enhanced replication in either orientation about 35- to 40-fold (Fig. 4A, lanes 2 and 3) and increased CAT activity about 10-fold (Fig. 4B, lane 3; Fig. 3, lane 3). We deleted the terminal AP-1 sites by digesting the 69-bp monkey sequence with HindIII, which cuts within the AP-1 sites and leaves a 3'-nt 5' overhang, and then trimmed the overhang with mung bean nuclease and added ClaI linkers before insertion into pC139cat. A more extensive deletion was produced by treating the sequence with nuclease BAL 31. We observed a 9- to 15-fold enhancement of replication with either the mung bean-generated constructs (p6-64H·C139cat and p64-8H·C139cat; Fig. 4A, lanes 4 and 5) or the BAL 31-generated construct (p57-9H·C139cat; Fig. 4A, lane 6). This level was lower than with intact AP-1 sites but still much greater than the basal level, indicating that at least one other replication-enhancing motif is contained in the monkey sequence. Also, CAT activity was still enhanced about 2.5-fold over the basal level (Fig. 4B, lane 6), suggesting that at least one other transcription-enhancing motif exists in the monkey sequence.

Deletion of either of the terminal AP-1 sites (p57-1H·C139cat and p69-14H·C139cat; Fig. 4A, lanes 7 and 8; Fig. 4B, lanes 4 and 5) resulted in a level of replication efficiency and CAT activity between that of the intact 69-bp sequence and that of the constructs which have lost both AP-1 sites. Thus, both AP-1 sites contribute to the replication- and transcription-enhancing activities of the monkey sequence. Addition of a single AP-1 site to the promoter-origin increased replication efficiency nearly threefold and increased CAT activity slightly (p7-1H·C139cat; Fig. 4A, lane 9; Fig. 4B, lane 2).

To identify other functional elements within the monkey sequence, we constructed a series of unidirectional deletions and assayed their relative replication efficiencies (Fig. 5). In the direct orientation, sequences that stimulate replication were located between nt 22 and 35 (compare p22-35H·C139cat and p36-57H·C139cat) and nt 42 and 57. In the inverse orientation, functional motifs were located between nt 9 and 21 (compare p57-9H·C139cat and p57-22H·C139cat) and nt 34 and 57. Whereas deletion of nt 44 to 57 decreased replication only about 1.7-fold (compare p57-9H·C139cat and p43-9H·C139cat), deletion of nt 34 to 45 reduced replication activity about 5-fold (compare p57-
34H · C139cat and p57-46H · C139cat), suggesting that although both deletions disrupt the putative AP-4 site, nt 34 to 45 likely contain at least part of another functional motif.

To determine if an interaction with upstream promoter elements is required for the monkey sequence to enhance replication and transcription, we tested some of the constructs in the absence of the 21-bp repeat (Fig. 6). The 69-bp sequence still enhanced replication, but to a lesser extent than in the presence of the 21-bp repeat (p1-69H · ORIcat and p69-1H · ORIcat; Fig. 6A, lanes 2 and 4 [compare Fig. 4A, lanes 2 and 3]). Deletion of both AP-1 sites decreased replication efficiency about four- to sixfold, but the level was still two- to threefold greater than the basal activity (p6-64H · ORIcat and p64-6H · ORIcat; Fig. 6A, lanes 3 and 5), again suggesting the presence of at least one additional replication-enhancing element within the monkey sequence.

The 69-bp sequence also enhanced CAT expression in the absence of upstream promoter elements, about sixfold in either orientation (Fig. 6B, lanes 2 and 4). However, no increase in CAT activity was detected when both AP-1 sites were deleted (lanes 3 and 5), in contrast to the 2.5-fold enhancement observed in the presence of the 21-bp repeat (compare Fig. 4B, lane 6).

In this study, we demonstrated that a 69-bp monkey DNA sequence previously shown to stimulate replication (29) also has transcriptional enhancer activity. More importantly, we found that both activities are conferred in part by consensus recognition sites for the transcription factor AP-1 and that a single AP-1 site stimulates replication in the presence of a 21-bp repeat. Deletion analysis of the 69-bp sequence demonstrates that in addition to the AP-1 sites, other functional elements in this monkey DNA sequence act to stimulate replication and transcription.

The differences observed between the constructs in the direct and inverse orientations of the monkey sequence derivatives (Fig. 5) suggest that a precise spatial arrangement of functional motifs may be required for the stimulation of replication. Perhaps some of the motifs stimulate replication only when positioned close to the 21-bp repeat-origin region or in an orientation-dependent manner. A requirement for a stereospecific alignment of functional elements, as has been found for the SV40 early promoter (44), is also a possibility. There is not an absolute requirement for a 21-bp repeat, since the 69-bp sequence enhances replication in its absence (Fig. 6), but the presence of a 21-bp repeat does augment the replication activity of the monkey sequence.

The potential AP-4-binding site within the 69-bp sequence is an interesting feature. The presence of an AP-1 site in close proximity is consistent with the results of Mermod et al. (33), who found that in the five cases analyzed, AP-4 recognized sequences that overlap with AP-1-binding sites. A requirement for multiple motifs to enhance replication suggests a commonality with the enhancer model of transcription enhancement (13, 16, 36). However, certain qualitative and quantitative differences between the patterns of stimulation of replication and transcription were observed. In all constructs in which a derivative of the 69-bp sequence is placed proximal to the promoter-origin, the degree of enhancement of replication is greater than that of transcription, and this difference is especially striking when both AP-1 sites are deleted from the monkey sequence. Namely, in p57-9H · C139cat, replication is enhanced 15-fold while transcription is enhanced only 2.5-fold (Fig. 4). When both AP-1 sites are deleted, the monkey sequence does not detectably enhance transcription in the absence of a 21-bp repeat but still enhances replication two- to threefold (Fig. 6). If transcription factor Sp1 (14) is acting through the 21-bp repeat in this system, it may be said that without its AP-1
The possibility that trans-acting transcription factors regulate cellular DNA replication furthers some speculative thoughts. Since several cellular factors recognize the AP-1 binding site, preparations of AP-1 consist of multiple proteins (9). Might some members of the AP-1 family regulate only replication while others regulate only transcription, or could some proteins regulate both processes? With which other factors might the DNA-binding proteins interact to regulate replication? One of the DNA-binding proteins that recognizes the AP-1 site is the Fos-associated protein p39, the product of the jun oncogene (8, 39, 40). Jun and Fos form a heterodimer which binds AP-1 sites (22, 26, 35, 41) and stimulates transcription (8, 40, 41). It would be interesting to know if Fos participates in the regulation of cellular DNA replication as well. A direct link between Fos and Jun and the regulation of DNA replication is an attractive hypothesis and could help to explain the role of these proteins in the control of cell division.

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