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Mammalian genomic sequences can substitute for the SV40 AT stretch in sustaining replication of the SV40 origin of replication

Ivo Galli[a], Sanae M.M. Iguchi-Ariga[b] and Hiroyoshi Ariga[a]

[a]*Faculty of Pharmaceutical Sciences* and [b]*College of Medical Technology, Hokkaido University, Kita-ku, N12 W6, Sapporo 060, Japan*

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The core of the SV40 origin of replication (*ori*) contains a stretch of adenine (A) and thymine (T) residues. This region is very conserved among the papova viruses, and is known to be extremely sensitive to mutations. So far, mutations have been found to drastically reduce, and in most cases abolish, replication. The AT stretch has been shown to be the target for several host cellular proteins that belong to the replication machinery. We reasoned that, in this light, there might exist cellular DNA sequences that can substitute for the SV40 AT stretch. To study this possibility, we digested mammalian genomic DNA and inserted the fragments instead of the SV40 AT stretch in a plasmid carrying the SV40 *ori* core. The resulting pool was analyzed by a 'replication trap' in CosI cells. We present evidence that there are indeed several mammalian sequences that can substitute for the SV40 AT stretch. All of them are rich in adenines and thymines but, surprisingly, these sequences differ from the wild-type SV40 AT stretch to such extent that at first sight they would seem unlikely to replicate. This is all the more impressive if we consider that another AT-rich sequence from the yeast TRP1 gene, which also carries a similar variation, cannot substitute for the SV40 AT stretch.

SV40 AT stretch; Mammalian genomic sequence; SV40 replication

1. INTRODUCTION

The functional core of the SV40 origin of replication (*ori* core) is a 63 base pairs long element [1–5] flanked by two auxiliary sequences that augment replication but are not indispensable [4,6–9]. The *ori* core consists of two distinct units. On its early-genes side, there is a GC-rich palindromic region that contains a binding site for the viral T antigen [10–12]. On the late-genes side, there is a 15 base pairs long AT-rich sequence (AT stretch). The actual functions of the AT stretch are not at all clear, although it is known to contain two of the major primer start sites for replication [10], to be a probable site of bending [13], to inhibit replication when multimerized [14], and to provide a TATA box for the early promoter [12,15]. At any rate, the AT stretch is strictly required for initiation of DNA synthesis: point mutations, deletions and insertions within the AT stretch drastically reduce, and in most cases abolish, replication [1,3,13]. On the other hand, the SV40 AT stretch has been reported to interact sequence-specifically with several host cellular proteins. In several cases, these proteins have been shown to recognize several (though not any) variants of the AT stretch [14,16,17]. Since all of the proteins that bind to the AT stretch are cellular proteins, it is only reasonable to assume that there might be cognate sequences (the AT stretch itself, or a variant thereof) in the cellular genome. AT-rich sequences, incidentally, have been recently shown to be a characteristic of several viral, prokaryotic and eukaryotic origins of replication [18–21]. An interesting question is then whether or not some of these cellular AT-rich sequences (or any other sequence, as a matter of fact) can substitute for the SV40 AT stretch in sustaining replication, despite the fact that most mutations decrease it, as mentioned above. We have studied this matter by substituting the SV40 AT stretch in the *ori* core with randomly cleaved mammalian genomic DNA.

Correspondence address: H. Ariga, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, N12 W6, Sapporo 060, Japan. Fax: (81) (11) 707-4490.

Abbreviations: SV40, simian virus 40; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

2. MATERIALS AND METHODS

2.1. *Plasmid construction*

This experiment is based on a pUC19 derivative that carries the SV40 origin core of replication and Auxiliary Sequence I (AUX1) (on the early-genes side). As described previously [14], the junction between the AT stretch and the rest of the *ori* core has been modified (two point mutations) to include an *Xba*I site, without altering the original distance between the two units. This modification does not affect replication. The SV40 AT stretch was released from this plasmid, pSVATØAUX1, by enzymatic restriction with *Xba*I and *Bam*HI (in the pUC19 polylinker). The resulting vector was then dephosphorylated with bacterial alkaline phosphatase. About 5 μg of genomic DNA extracted from mouse teratocarcinoma F9 cells were digested with restriction enzymes *Xba*I and *Mbo*I (which recognizes

the sequence GATC, leaving protruding ends compatible with *Bam*HI ends), and purified by organic extraction and ethanol precipitation. The expected length of the resulting fragments ranges from 100 to 1000 base pairs. By ligating the fragmented DNA with the vector prepared above, we created a pool of mutant SV40 *ori* cores containing random F9 genomic DNAs instead of the AT stretch. All manipulations were performed according to standard techniques [22]. The ligated pool was amplified by transformation in highly competent *E. coli* DH5α.

2.2. *Oligonucleotides*

Two complementary oligonucleotides corresponding to a possible variation of the SV40 AT stretch were inserted instead of the AT stretch in pSVATØAUX1 (see above) and tested for replication. The oligonucleotides were synthesized chemically with an Applied Biosystem DNA synthesizer, cleaved from their support by usual procedures and purified on a HPLC cartridge (Applied Biosystems). Their sequences are the following (from 5′ to 3′):

pm+ GATCCATTTTTTTCTCTTAT
pm− CTAGATAAGAGAAAAAAATG

2.3. *Cell culture, transfection, and screening procedure*

CosI cells were cultured at 37°C/5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Boehringer Mannheim). Ten μg of test plasmid were transfected to CosI cells (40% confluent) in 6 cm dishes by the calcium phosphate technique [23]. CosI cells sustain SV40 replication because they produce constitutively SV40 T antigen. Four to five hours after transfection, the cells were boosted with 20% glycerol for 3 min at room temperature, then incubated for 48 h. Low molecular weight DNAs were recovered by the Hirt method [24], and treated with the restriction enzyme *Dpn*I in order to dispose of non-replicated, methylated DNA. The remaining DNA, which had replicated in the mammalian cells, was then introduced in highly competent *E. coli* DH5α. Single colonies were amplified, the DNA was recovered and sequenced. We call this procedure a 'replication trap'.

The single clones of interest were then re-transfected to CosI cells to confirm the validity of the data. The DNA (10 ng, or 750 ng for the control clones lacking Auxiliary Sequence I) was co-transfected with 2 μg of a deleted form of pUC19 (CibiH) grown in the GM119 dam⁻ strain of *E. coli*. This plasmid cannot be cleaved by *Dpn*I, and provides a standard for the efficiency of transfection. After linearization with *Hin*dIII to make the identification of the bands easier, low molecular weight DNAs obtained from the Hirt extracts were treated with *Dpn*I (which leaves unmethylated DNA intact) and analyzed by Southern blotting [25] with a pUC-specific probe (random primer labelling). In addition, replication was assessed from *Mbo*I sensitivity: *Mbo*I cleaves unmethylated sites (hence, CibiH and replicated material are degraded), but not methylated (non-replicating material) or hemimethylated ones (input material).

Occasionally, mutations in regions neighbouring the SV40 origin of replication have been reported to render the DNA particularly prone to further modifications [1]. We felt therefore compelled to verify whether or not the replicated DNA recovered from CosI cells was identical to the input material. A fraction of the Hirt extracts was thus digested with *Dpn*I, but not linearized, and the resulting DNA was transformed in the C600 strain of *E coli* , in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). C600 bacteria carry the β-galactosidase gene, whose expression is inhibited by the *lac*I repressor. The introduction of a high-copy number pUC derivative, which carries the *lac* operon, titrates out the repressor available and therefore yields cells expressing the endogenous β-galactosidase gene (blue colonies). The internal control CibiH, on the other hand, lacks the *lac* operon sequences, and cannot bring about the same effect (white colonies) [26,27]. Blue colonies were then amplified, plasmid DNAs were recovered and sequenced. The DNA clone carrying oligonucleotides pm (one possible variation) was also tested according to the same procedure (10 ng transfected to CosI cells, with 200 ng CibiH).

3. RESULTS AND DISCUSSION

The experiments presented in this work are based on a pUC19 derivative that carries the SV40 origin core of replication (*ori* core) and Auxiliary Sequence I (AUX1) on the latter's early-genes side (Fig. 1). As described previously [14], the junction between the AT stretch and the rest of the *ori* core contains two point mutations to include an *Xba*I site, which, however, do not affect replication.

Our aim was to study whether or not cellular sequences can substitute for the SV40 AT stretch in sustaining replication of the SV40 *ori* core. The experimental procedure is described in detail in Materials and Methods, and is outlined schematically in Fig. 1. By digesting DNA from F9 cells with the restriction enzymes *Mbo*I and *Xba*I, we created a library of genomic sequences, whose length ranged from 100 to 1000 bp. These random sequences were inserted instead of the AT stretch in a plasmid carrying the SV40 *ori* core (Fig. 1) and amplified in bacteria; the resulting plasmid pool (10 μg) was transfected to CosI cells. The recovered, replicated (*Dpn*I resistant) DNA was back-transformed in highly competent *E. coli* DH5α (> 10^8 cfu/μg DNA). We call this selection of replicating material from a pool of mostly non-functional DNAs a 'replication trap'.

The results are displayed in Table I. Controls were taken by transfecting the plasmid pSVØ and a 'simulation pool' to CosI cells. As expected, plasmid pSVØ, which carries the SV40 *ori* core without the AT stretch, did not replicate (1 colony). (As a comparison, 1 μg of dam⁺ pUC19 digested directly, i.e. not transfected, with *Dpn*I under the same conditions of the Hirt extracts gave 3 colonies; data not shown.) On the other hand, it is clear that traces of a replication-competent plasmid (1 ng pSVATØAUX1) amidst large amounts of non-replicating material (10 μg pSVØ) still give rise to a positive signal (33 colonies). As for the pool of genomic sequences replacing the AT stretch in the SV40 *ori* core (F9(BX)ØAUX1), we obtained 376 colonies. We selected at random about 20 colonies and sequenced the plasmid DNA they carried. Of all the clones, about one-half corresponded to reconstituted wild-type SV40 AT stretches, flanked by genomic sequences (data not shown). One in four clones carried a stretch of 15–18 thymine residues (flanked by various sequences, see also Table II). The remaining one-fourth of the clones consisted of single specimens of various sequences, all rich in adenine and thymine residues, but different from the wild-type SV40 AT stretch.

As a matter of course, these single clones were transfected separately to CosI cells and re-assayed for replication. In order to standardize the efficiency of transfection, a pUC-derived plasmid (CibiH) was transfected together with the test samples. CibiH is shorter than the test plasmids, and can therefore be easily separated electrophoretically in a Southern assay; it was grown in the

dam⁻ *E. coli* strain GM119, so that (unlike the non-replicated input material of the test plasmids) it cannot be degraded by the *Dpn*I enzyme.

As described in Materials and Methods, Hirt extracts [24] were made 48 h after transfection, and replication was assayed by a Southern blotting [25] after *Dpn*I digestion and linearization (Fig. 2, upper panel). As shown in the central panel of Fig. 2, we included a further check by assessing MboI sensitivity of the replicated material (and of the internal control CibiH) and *Mbo*I resistance of the non-replicated (methylated) and input (hemi-methylated) material. (Since the transfected material was only 10 ng, signals due to MboI-resistant DNAs cannot be seen in this autoradiograph, but in a stronger exposure bands can be clearly seen at the position indicated; data not shown). In addition, we verified that the replicated DNA was identical to the input material (see Materials and Methods, and legend to Fig. 2).

The data indicate that all of the genomic sequences selected by back-transformation of the pool can indeed replicate autonomously: no artifact was therefore involved in the findings described in Table I. Interestingly,

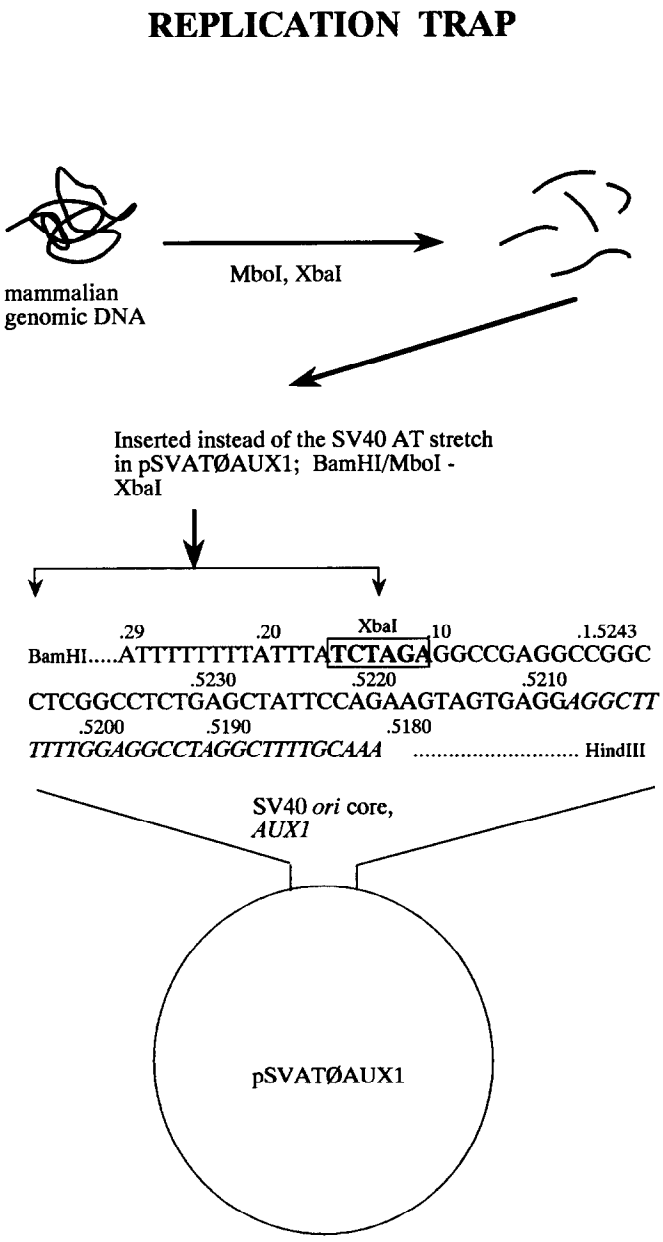

Fig. 1. Experimental strategy for the selection of genomic variants of the SV40 AT stretch. The experiments described here are based on pSVATØAUX1, a pUC19-derivative that carries the SV40 *ori* core and Auxiliary Sequence I (lower panel). Between the restriction sites for *Bam*HI and *Xba*I (in bold letters), there is the SV40 AT stretch. Between *Xba*I and *Hin*dIII, there are the palindromic SV40 Tag/IR unit (i.e. the second half of the *ori* core, Ø) [14] followed by Auxiliary Sequence I (AUX1, in italics). Numbers above the sequence indicate the respective positions in the wild-type SV40 genome. In the upper panel, a sketch of the experimental strategy is outlined.

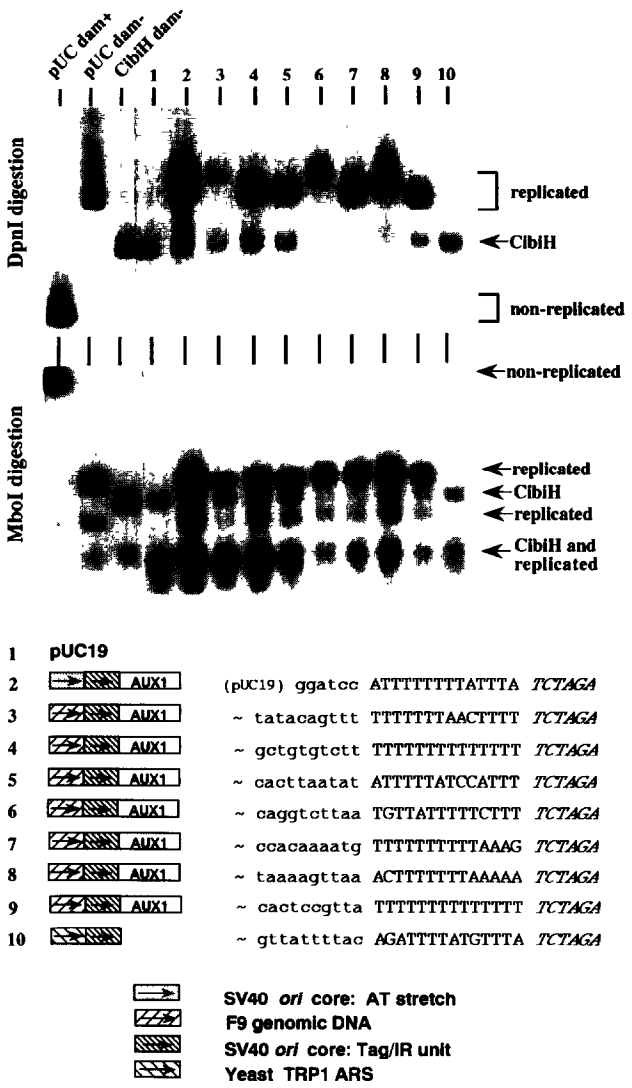

Fig. 2. Replication assays of clones carrying genomic sequences instead of the SV40 AT stretch. Single specimens of the clones of interest, identified in the 'replication trap' (see Table I), were transfected (10 ng) to CosI cells along with an internal control for transfection efficiency (CibiH, 2 μg). Replication was assessed in Southern blots from *Dpn*I resistance (upper panel) and *Mbo*I sensitivity (central panel) of Hirt extracts, first treated with *Hin*dIII to make the identification of the bands easier. 'Replicated' and 'CibiH' indicate the corresponding bands, to be compared with those of pUC19 (dam+ and dam−) and CibiH (dam−) similarly linearized with *Hin*dIII and digested respectively with *Dpn*I and *Mbo*I. 'Non replicated' indicates the position of non-replicating (methylated) and input material (hemimethylated) (linearized with *Hin*dIII). In this case, 10 ng transfected material give rise to very weak signals, which, however, can be seen in stronger exposures (not shown). In addition, the identity of the clones was verified by sequencing the plasmid DNA recovered from *E. coli* C600 back-transformed with *Dpn*I-treated Hirt extracts in the presence of X-Gal (see Materials and Methods). In the lower panel, the sequences of the functional genomic variants are shown aligned with the wild-type SV40 AT stretch. The mention (pUC19) indicates that from that point upstream, the sequence is that of the vector. A small wave sign, instead, indicates that the mammalian genomic sequence continues upstream. The *TCTAGA* sequence (*Xba*I site) in italics corresponds to the junction between the AT stretch and the SV40 Tag/IR unit (see Fig. 1). Downstream from this point, there is the rest of the *ori* core and, following that, Auxiliary Sequence I.

almost all of the clones found here (Fig. 2, lanes 3–9) seem to sustain replication as efficiently as the wild-type SV40 AT stretch (lane 2). The data presented infer that replication of the SV40 *ori* core does not require the integrity of its wild-type AT stretch quite as strictly as former studies have postulated [1,3,13]. Apparently, a sequence rich in adenine and thymine residues is needed (Fig. 2, lower panel), but not any sequence that fulfils this condition will do. In fact, an AT-rich sequence from the *S. cerevisiae* TRP1 gene [28], which contains a yeast Autonomously Replicating Sequence (ARS), cannot replace the SV40 AT stretch, although the two sequences are very much alike (Fig. 2, lane 10).

At this point, one could argue that replication of the test samples presented in Fig. 2 (lanes 3–9) may not be due to the genomic AT-rich sequences immediately adjacent to the rest of the *ori* core (SV40 Tag/IR unit), but to some sequence further upstream (since the genomic fragments are a few hundred base pairs long). Although this possibility cannot be excluded a priori, we believe that it is very unlikely because of the following observations.

First, although the sequences shown in Fig. 2 are part of longer AT-rich regions, we could not find any 'AT-box' further upstream that resembles a functional SV40 AT stretch [3,13].

Second, even if there were any such consensus, most probably it would not sustain replication. In fact, spacing between the SV40 AT stretch and the rest of the *ori* core results in total loss of replication [13]; this is true also when the spacing between the two units maintains the relative helical position (I.G., unpublished results).

Third, we have observed that, as described above, about one-fourth of the replicating clones carried a stretch of 15 to 18 thymine residues, flanked by various sequences. Of course, all of these clones, too, tested positive for replication when transfected to CosI cells

Table I

Replication trap

	Sample	Amount transfected	Colonies obtained
(1)	pSVØ	10 μg	1 colony
(2)	pSVØ pSVATØAUX1	10 μg 1 ng	33 colonies
(3)	F9(BX)ØAUX1	10 μg	376 colonies

Ten μg of a pool of plasmids carrying mammalian genomic DNA fragments instead of the SV40 AT stretch within the SV40 *ori* core (F9(BX)ØAUX1) were screened with a 'replication trap' in CosI cells. In this trap, non-replicated material is disposed of by *DpnI* restriction. Replicated, *DpnI*-resistant material is amplified by back-transformation in competent *E. coli*. Controls were taken with a non-replicating plasmid (1) and with a simulated pool of a large excess of non-replicating material and traces of replication-competent DNA (2).

(data not shown). A few examples are shown in Table II. The sequences upstream from these thymine stretches differ greatly from one another; some of them are even relatively poor in adenine and thymine residues.

Fourth, a hypothetical consensus can be drawn from the alignment of the sequences assumed to sustain replication shown in Fig. 2. We synthesized a pair of complementary oligonucleotides corresponding to one possible variation according to this hypothetical consensus (Fig. 3). The substitution of this new variant for the wild-type SV40 AT stretch did not affect replication at all, demonstrating that such variant sequences are sufficient for replication, as assessed from *Dpn*I resistance and *Mbo*I sensitivity (Fig. 3).

In the light of these observations, we assume that replication is due to the genomic sequences closest to the SV40 Tag/IR unit, although we do not exclude that their function may be promoted, at least in some cases, by the flanking regions [1].

In Fig. 2 (lower panel), the sequences assumed to sustain replication are showed aligned with the wild-type SV40 AT stretch. On the one hand, it is interesting to notice that all of these permissive sequences are AT-rich. Totally unrelated sequences were not isolated in the 'replication trap'. Most theoretically, one should not exclude that some unrelated sequences may be able to substitute for the AT stretch: variants that replicate poorly probably would not give a signal above background level in the screen.

On the other hand, it is also noteworthy that, in experiments involving point mutations of the SV40 AT stretch [1,3,13], the few mutants found to replicate carried only one mutated nucleotide at a time, which either regarded the central adenine residue (see Fig. 1, position 20), or the first three nucleotides (positions 27–29). Besides, in the latter case replication was greatly inhibited. This is the first time that sequences bearing rough deviations from the SV40 AT stretch, like those represented by the variants identified in this work, are found to implement replication of the SV40 *ori* core as efficiently as the wild-type.

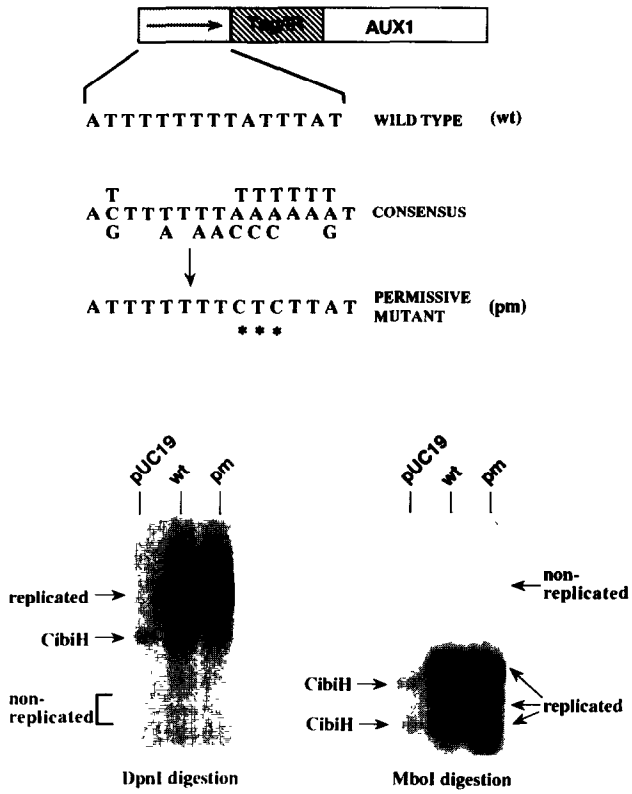

Fig. 3. Another permissive variation of the AT stretch. A comparison of the aligned sequences of the permissive variations found in the replication trap yielded a hypothetical consensus (upper panel). Among the many theoretically possible combinations, one sequence was chosen (upper panel), corresponding nucleotides were synthesized, and an SV40 *ori* core plasmid carrying that sequence in place of the wild-type SV40 AT stretch was constructed. Lower panel, replication activities of this construct and of the wild-type SV40 *ori* core (10 ng transfected with 200 ng CibiH to CosI cells) were assayed by *Dpn*I resistance and by *Mbo*I sensitivity. 'Replicated' and 'CibiH' indicate the corresponding bands. 'Non replicated' indicates the position of non-replicating (methylated) and input material (hemi-methylated). In this case, 10 ng transfected material give rise to very weak signals, which however can be seen in stronger exposures (not shown). Tag/IR, Tag binding site and inverted repeat region of SV40 *ori* core.

Table II

Functional genomic variants carrying a stretch of 15–18 thymines

~AACAACTGTTTGAACATATTAAGTTAATTTTTTTTTTTTTTTTTCTAGA
~AACACAGATACTGCCACACTCCCGTTATTTTTTTTTTTTTTTTCTAGA
~AACAACTGTTTGAACATATTAAGTTAATTTTTTTTTTTTTTTTTCTAGA
~TGGTTCGCCACCACGGCCTGGCCCTGTTTTTTTTTTTTTTTTTCTAGA

Among the functional genomic variants identified in the 'replication trap', many carried a stretch of 15–18 thymine residues instead of the wild-type SV40 AT stretch in the SV40 *ori* core. Four of them are shown here, with part of the flanking sequence upstream. A wave sign (~) shows that the mammalian genomic sequence continues upstream. The *TCTAGA* sequence (*Xba*I site) in italics corresponds to the junction between the AT stretch and the SV40 Tag/IR unit (see Fig. 1). Downstream from this point, there is the rest of the *ori* core and, following that, Auxiliary Sequence I.

These observations raise the following questions: what is the general rule that underlies the requirement of an AT stretch in replication of the SV40 *ori* core? Do the results presented here imply that a consensus sequence can be established, as proposed in Fig. 3? This is very tempting, especially since all of the proteins that bind sequence-specifically to the SV40 AT stretch actually present a certain degree of tolerance to mutations in their cognate sequence (at least in vitro) [14,16,17]. However, this idea conflicts with the observation that the AT-rich sequence from the yeast TRP1 gene [28] is totally inadequate as a substitute for the SV40 AT stretch, although it is no less homologous to the latter

than the functional genomic variants. In the case of the variants, it may also be that two or more of their deviations (from the SV40 AT stretch wild-type sequence) compensate for each other. All this told, we believe it would not be meaningful to propose a consensus.

In the SV40 *ori* core, the AT stretch serves two purposes: it influences the conformation of the surrounding DNA [29], and it is bound by host cellular proteins, some of which belong to the replication machinery [14,16,17,30,31]. Either event may not adhere to the 'classical' concept of sequence specificity as related to base-pairs composition, but to a broader concept that contemplates the overall structure of the AT stretch: stereospecificity. Stereospecificity may abide by some unknown rules, which at first sight may seem to yield loose requirements in terms of base-pairs composition. However, the instance of the AT-rich sequence from the yeast TRP1 gene discussed above implies that, in reality, requirements are by no means vague or ill-defined. It will be very interesting to determine the basic canons of this scheme. In fact, these criteria may apply to other systems than the SV40 origin of replication. First of all, as mentioned above, the sequences discussed in this paper are recognized by host cellular proteins that belong to the replication machinery. Secondly, these sequences are of cellular (mammalian) provenance, and have been conjectured to play a role in initiation of DNA synthesis also within the eukaryotic genomes [18–21]. The effects of the AT stretches described here on SV40 replication, and the notion of stereospecificity, may therefore have a counterpart in the mammalian replication system. This possibility is certainly worth exploring.

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