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Uncoupling of SV40 tsA replicon activation from DNA chain elongation by temperature shifts and aphidicolin arrest

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ABSTRACT

To synchronize SV40 replicons, simian cells infected with a tsA mutant were restricted at 40°, to complete ongoing replication and returned to 32°, to activate new replicons in the presence of the DNA chain elongation inhibitor aphidicolin. Upon further incubation at 40° without the drug, ^{3}H-dT was incorporated into SV40 FI DNA, almost to the extent seen with cells recovered in the absence of the drug. To determine whether DNA synthesis would begin from the origin, following the temperature-shifts-aphidicolin regimen, chains subsequently pulse-labeled with (α-^{32}P)dGTP in isolated nuclei were analyzed for size distribution and genomic location. These chains reached up to 300-400 nucleotides in size, unlike the control which featured comparable amounts of label in long chains and Okazaki pieces. The nascent DNA of the drug-treated system could be chased into longer chains, indicating that it was a replicative intermediate; and it hybridized preferentially to an origin proximal fragment of Atu_I- restricted SV40 DNA, demonstrating partial replicon synchronization.

The data prove that T-antigen activates the SV40 replicon independent of DNA chain elongation and suggest means to study the mechanism of DNA chain priming at the origin.

INTRODUCTION

Considerable knowledge exists about the location and structure of the SV40 replicon origin (1-4) and about the interaction of the origin DNA sequences with the cognate replicator protein, the viral gene A product (5-7). Yet, virtually nothing is known about the DNA chain priming mechanism at the origin and the actual initiation site(s) of the leading (continuous) DNA strands. Lack of efficient *in vitro* systems which initiate new replication rounds impeded progress on these latter subjects. To circumvent this difficulty, we have developed a procedure to synchronize SV40 replicons at the origin. In it, SV40 tsA (5, 8) -infected cells are restricted at 40°C to complete ongoing replication and returned to 32°C to initiate new replicons in the presence of the DNA elongation inhibitor, aphidicolin (9, 10). Here we describe some properties of SV40 nascent DNA synthesized following this regimen, either *in vivo* at 40°C, or in isolated nuclei.

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MATERIALS AND METHODS

Materials - Aphidicolin was a gift from Dr. Todd, of the Imperial Chemical Industries Ltd. and from Prof. Ikegami of Hiroshima University. Methyl-^{3}H thymidine was purchased from NRC-Negev and (α-^{32}P)dGTP from the Radiochemical Centre, Amersham. Atu$_{I}$ restriction endonuclease (11) was purchased from Bethesda Research Laboratories.

Cells and Virus - SV40 tsA 209 strain (8) was obtained from Prof. Winocour and was grown in BSC-1 African green monkey kidney cells. To prepare stocks, cell plates were infected close to end-point dilution and incubated for 14 days at 32°C. The cell debris were suspended in the medium and sonicated.

Synchronization of SV40 tsA replicons - Confluent BSC-1 cells were infected as follows: two ml of 1:5 diluted SV40 tsA 209 stock were added per 150 mm plate. Following two hours of incubation at 32°C, medium was added and incubation continued. The infected cells were subjected to the following regimens: (A) continuous incubation for 72 hours at 32°C, (B) additional incubation at 40°C for 40 min, (C) further incubation at 32°C for 60 min without or (D) with 2 µg/ml aphidicolin. The cells were then labeled at 40°C with ^{3}H-dT, or served as a source of nuclei. In the latter case the protocol was somewhat modified: continuous incubation at 32°C was for 60 hours, restriction at 40°C lasted 60 min and aphidicolin was employed at 10 µg/ml.

In vivo SV40 DNA synthesis - The medium was removed and the plates (60 mm) washed thrice with 3 ml of isotonic buffer (0.15 M NaCl, 0.01 M Tris HCl, pH 7.5, 0.01% each of $MgCl_2$ and $CaCl_2$) and covered then with 1 ml of the same buffer containing 50 µCi of ^{3}H-dT (43 Ci/mmole). After 20 min incubation at 40°C the plates were chilled, the radioactive medium removed and the cells washed with 5 ml of isotonic buffer at 0°C. The cells were lysed and the viral DNA extracted according to Hirt (12). Incorporation into viral DNA was determined by measurement of acid insoluble radioactivity after sedimentation in alkaline sucrose gradients (13).

SV40 DNA synthesis in isolated nuclei - At the end of the particular regimen the medium was removed, nuclei prepared and pulse labeled with (α-^{32}P) dGTP (200 Ci/mmol), similar to ref. 14, except that the cytosol was from wt SV40 infected cells (15) and the concentration of dGTP was 0.1 µM. Following shaking for 2 minutes at 30°C the reaction was stopped, the viral DNA extracted and (RI)DNA isolated by sedimentation through a neutral sucrose gradient (13, 15). Subsequent fractionation of the nascent DNA chains according to size was carried out by Sepharose 6B gel filtration (15).

DNA-DNA hybridization - Nascent DNA chain aliquots of up to 2000 cpm of ^{32}P were hybridized, essentially as previously described (16), to Atu_I-restricted SV40 DNA (11) blotted on nitrocellulose strips (17) containing the equivalent of 0.5 µg SV40 DNA. The filters were autoradiographed at -70°C using pre-flashed (18) Agfa Curix No. 2 X-ray film and CaWo rapid intensifiers. Radioactive bands were excised from the filters and counted in toluene scintillation fluid.

RESULTS

Aphidicolin does not block the recovery of SV40 tsA replicons from 40°C restriction

Temperature sensitive gene A mutants of SV40 complete ongoing replication rounds at the restrictive temperature but do not initiate new ones. Upon downshift to permissive conditions, initiation of replication resumes (5, 8). Yet this property of SV40 tsA mutants alone cannot serve to synchronize replicons, since recovery from restriction is slow relative to the replicon generation time (8, 1). It was expected, however, that origin-synchronized replicons would accumulate after a specific and reversible inhibition of DNA chain elongation during the recovery period. The effect of aphidicolin on the recovery of SV40 tsA replicons was therefore investigated. This drug competes with dCTP over an allosteric site in DNA polymerase α (10) and was not expected to directly interfere with the activation of the replication origin by the viral gene A product. Indeed, in vivo and in vitro experiments described below confirmed this assumption.

In the first experiment, SV40 tsA 209 (8) - infected BSC-1 cells were subjected to regimens A through D (METHODS) and labeled with ^{3}H-dT for 20 minutes at 40°C. The viral DNA was then extracted (12) and sedimented through alkaline sucrose gradients. As shown, the presence of aphidicolin during the preincubation at 32°C did not block the recovery from 40°C restriction (Table 1 and Fig. 1, A-D). Thus, both regimens C and D (recoveries from restriction without or with aphidicolin, respectively) exhibited similar extents of recovery, about 50% of the nonrestricted control (A) and well above the background of the "restricted-only" cells (B). Furthermore, the DNA of regimen D was matured to F1 DNA to an extent which was close to that of the controls, A and C. The measured recoveries were solely due to replicons activated by T-antigen prior to the labeling at 40°C. This preactivation occurred in regimen D under conditions which caused greater than 90% inhibition of SV40 DNA synthesis (Ref. 19 and our unpublished results).

Table 1 - Extent of recovery and maturation of SV40-tsA replicons following temperature-shifts and aphidicolin-arrest

Regimen	^{3}HdT incorporation cpm in total DNA	cpm in F1 DNA	% recovery	% maturation
A	15,729	11,728	100	75
B	1,148	-	7	-
C	8,585	6,076	55	71
D	7,020	3,848	45	55

SV40 tsA 209 infected cells were subjected to the following regimens: A-Normal, B-restricted, C-restricted and recovered without or D-with aphidicolin. The cells were shifted afterwards to 40°C and labeled for 20 min with ^{3}H-dT in the absence of the drug. Recovery is expressed as incorporation into total viral DNA compared to A. Maturation is expressed as F1 DNA out of the total in that regimen.

To determine whether DNA precursors would become incorporated preferentially into origin-proximal regions, following the temperature-shifts and aphidicolin-arrest, the size distribution and genomic location of corresponding pulse-labeled nascent DNA chains were examined. However, in view of the time-lag needed to equilibrate the intracellular ^{3}H-TTP pool and the low number of counts expected in vivo, these experiments were conducted in vitro, with isolated nuclei (20-22). The nuclei system was considered more suitable for assessing the degree of replicon synchrony as well as for eventual analyses of the young leading chains of the synchronized replicons. The nuclei were isolated sixty hours post-infection from tsA 209 SV40-infected cells which were subjected to the four regimens described above, except that aphidicolin was employed at 10 µg/ml. The nuclei were

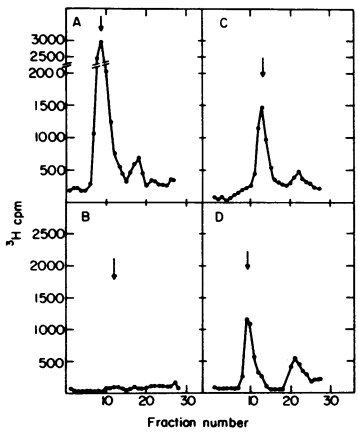

Figure 1 - Incorporation of ^{3}H-dT into SV40 tsA 209 DNA following temperature-shifts and aphidicolin arrest. The ^{3}H-dT labeled viral DNA of the various regimens described in Table 1 was sedimented in alkaline sucrose gradients with (^{14}C) F1 DNA marker (designated with an arrow).

pulse-labeled with (α-^{32}P)dGTP in the presence of cytosol from wt SV40-infected cells (15) as described in METHODS and similar to previous protocols (22, 14). Following the in vitro labeling, the viral DNA was extracted from the nuclei and further purified by sedimentation through a neutral sucrose gradient. As shown (Fig. 2 Table 2), comparable extents of recovery from 40°C restriction were observed in vitro whether or not aphidicolin was employed. Less than 3% of the normal value was incorporated into the "restricted-only" (RI) DNA region. Note that the nuclei were supplemented with cytosol from wt SV40-infected cells. Regimen B may have served therefore to monitor the in vitro activation of restricted tsA replicons by any wild type T-antigen in the cytosol. It was not clear, however, whether the low level of incorporation seen with regimen B was actually in (RI) DNA and if so, did it reflect in vitro activation of new replicons by functional T-antigen (23) or residual in vivo replicon activation under the restrictive conditions (24).

Absence of long nascent chains from (RI) DNA pulse-labeled in temperature shifted and aphidicolin-arrested nuclei

The in vitro labeled (RI) DNAs (regimens A, C and D) were heat-denatured and their dissociated nascent chains fractionated by Sepharose 6B gel filtra-

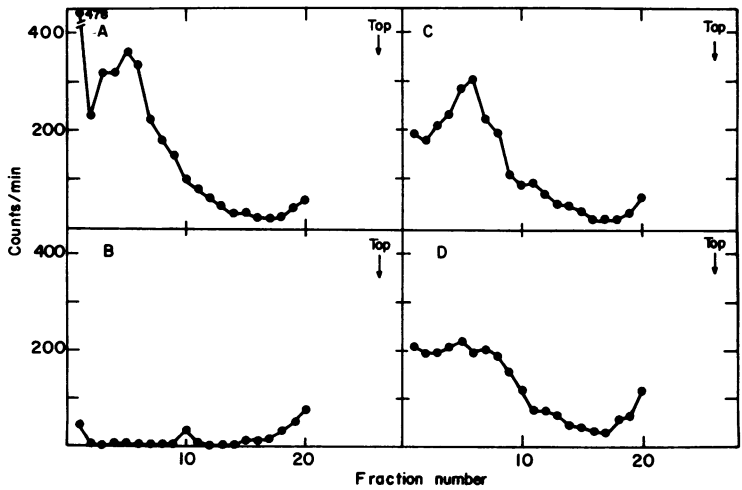

Figure 2 - Incorporation of (α-^{32}P)dGTP into SV40 tsA 209 (RI) DNA synthesized in isolated nuclei following temperature-shifts and aphidicolin-arrest - SV40 (RI) DNA was pulse-labeled for 2 min at 30°C with (α-^{32}P)dGTP in nuclei isolated from SV40 tsA 209-infected cells following regimens A-D (METHODS), extracted and sedimented through a neutral sucrose gradient.

Table 2 - Effect of aphidicolin on the recovery of SV40 tsA replicons - determination in isolated nuclei

Regimen	^{32}P-dGMP incorporated in (RI) DNA cpm	% recovery
A	2832	100
B	81	3
C	2219	78
D	2081	73

SV40 (RI) DNA was pulse-labled with (α-^{32}P)dGTP for 2 min at 30°C in nuclei isolated from tsA 209-infected cells which had been subjected to the regimens described in Table 1. The DNA was extracted from the nuclei, sedimented through a neutral sucrose gradient and radioactivity determined by Cerenkov counting.

tion (15). As shown (Fig. 3), regimen D-nascent chains had an unusual elution profile, with mostly short "Okazaki-like" chains of less than 300-400 nucleotides. The other preparations exhibited the commonly observed distribution between comparable peaks of long nascent chains and Okazaki pieces (25-27). The short nascent chains of regimen D could be chased into longer ones, similar to those of regimen C (Fig. 4), suggesting that the incorporation seen with regimen D was into replication intermediates rather than arte-

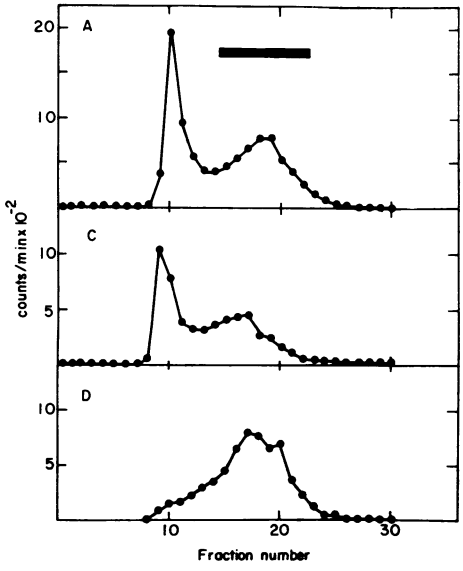

Figure 3 - Size-distribution of SV40 tsA 209 nascent DNA chains synthesized in isolated nuclei following in vivo temperature-shifts and aphidicolin-arrest - SV40 tsA 209 (RI) DNA preparations described in legend to Fig. 3, were heat denatured and their dissociated nascent chains fractionated on 12 ml Sepharose 6B columns (15). Regimen assignments (A, C and D) as in METHODS.

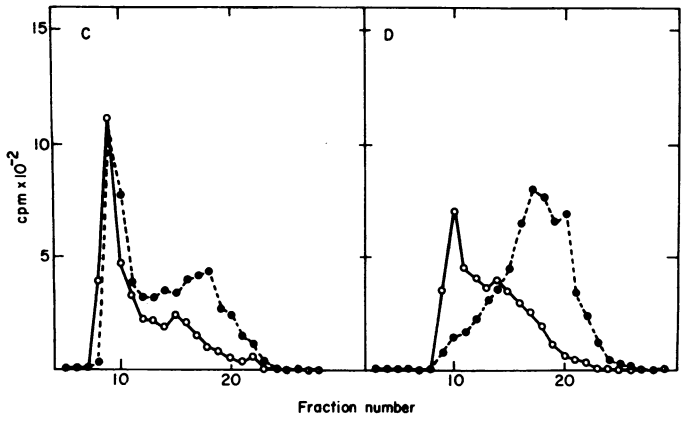

Figure 4 - Size distribution of pulsed and pulsed-chased SV40 tsA 209 nascent DNA chains from temperature-shifted and aphidicolin arrested replicons. Nascent DNA chains from regimen C or D replicons which were pulse-labeled with (α-^{32}P)dGTP for 2 min or further chased with nonlabeled dGTP for 90 min were fractionated by Sepharose 6B gel filtration as in Fig. 5. •---• pulse, o——o pulse chase.

factual degradation products.

Preferential incorporation of DNA precursors into origin-proximal regions following temperature-shifts and aphidicolin arrest

To determine the genomic location of the post-Sepharose 6B short-chain fractions (Fig. 3, area under bar) they were hybridized to an Atu_I-restricted SV40 DNA probe (11) immobilized on a nitrocellulose filter (17). The distribution of hybridized chains among the various fragments is presented in an autoradiogram and plotted as relative hybridized cpm/bp versus distance from the replicon origin (Fig.5). In both the (A) and (C) control preparations, the origin-proximal fragment G usually hybridized to the short chains at about twice the average efficiency of the other fragments. Such a phenomenon was also observed with SV40 wt short nascent DNA chains. In contradistinction, the hybridization pattern of regimen D chains revealed a ca. 10:1 preference of the origin over terminus fragment. Yet, exclusive hybridization to origin-proximal fragments was not observed with these chains.

Attempts to determine the fork-polarity of the short nascent chains of regimen D (RI) DNA led to an ambiguous outcome. It was expected that these short chains would consist of both Okazaki pieces and short leading chains in equal proportions and hybridize to both template strands of origin-

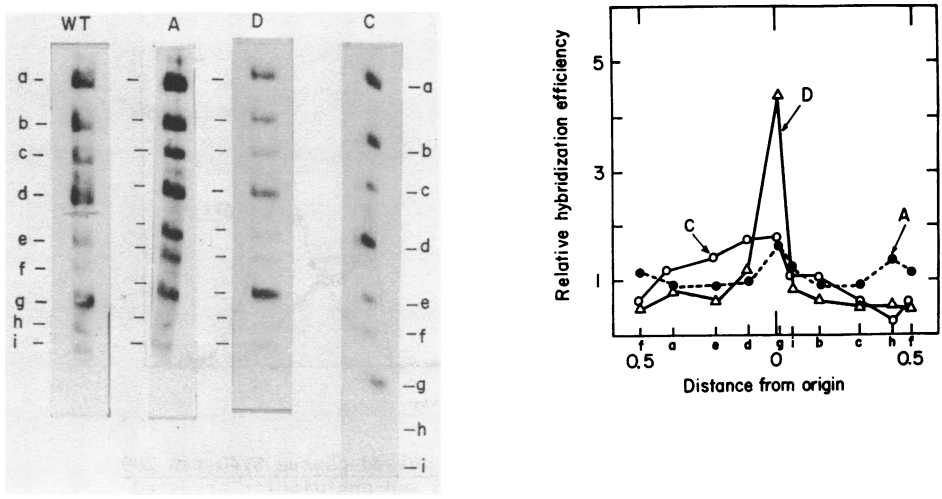

Figure 5 - Genomic distribution of SV40 tsA 209 short nascent chains which were pulse-labeled in vitro following in vivo temperature-shifts and aphidicolin-arrest. Short nascent DNA chain fractions of regimens A, C and D (Fig. 4, fractions under bar) were hybridized to Atu_I-restricted SV40 DNA blotted on nitrocellulose filter, as described in METHODS. Radioactive bands were excised from the filters and counted. WT - wild type SV40 short nascent chains, A, C, D-SV40 tsA 209 short nascent chains from the corresponding regimens. Relative hybridization efficiency is expressed in normalized cpm/bp values

proximal fragments. In deed, these chains hybridized symmetrically to both template strands of a unidirectional probe (the separated strands of Hpa_I+Bgl_I restricted SV40 DNA, ref. 16). However, even SV40 tsA 209 Okazaki pieces which were synthesized in the nonrestricted control (regimen A) exhibited a poor asymmetry of hybridization to the unidirectional probe (not shown), in contrast to SV40 wt Okazaki pieces which hybridize almost exclusively to the retrograde template strands (15,16).

DISCUSSION

The purpose of this work was to synchronize SV40 replicons at an early stage, explicitly, after the activation of the origin by T-antigen but before DNA chain elongation has occurred. It was expected that temperature-shifts and aphidicolin arrest would uncouple these processes in SV40 tsA replicons. Indeed, the presence of aphidicolin during the recovery period at 32^{o}, at concentrations which almost completely block SV40 DNA synthesis (19), did not block the incorporation of DNA precursors into replicating viral DNA in sub-

sequent pulses without the drug, either in vivo at 40° (Fig. 1, Table 1) or in isolated nuclei (Table 2, Fig. 2). Since ts T-antigen does not activate the origin at 40° (5, 8) and since the nuclei system supports only ongoing replication and not replicon initiation (22), it can be concluded that DNA synthesized during the labeling period was in replicons activated in the presence of aphidicolin. Thus, T-antigen activated the origin of replication while DNA chain elongation was severely inhibited (at least elongation catalyzed by DNA polymerase α, the only known replicative polymerase in cell nuclei, c.f. ref. 28). While this result does not offer a clue as to the actual steps induced by T-antigen prior to the onset of DNA synthesis, similar investigations with additional inhibitors and drugs may provide a solution to this problem.

Steady state SV40 replicons become progressively inactivated during incubation with aphidicolin with about a $t_{1/2}$ of 30 min, at both 37° and 32°C and at 2 μg/ml of the drug (our unpublished results). Yet the DNA synthesized during the in vivo pulse following regimen D matured into F1 DNA almost to the extent seen with the control recovered without aphidicolin (Table 1, Fig. 1). Hence, the arrest of DNA chain elongation by aphidicolin did not lead to the massive secondary damage observed with steady-state replicons. This discrepancy could be due to the inherently greater resistance of newly activated replicons to the secondary effects (for example, a lesser exposure of single stranded regions in replication forks to nucleases), or alternatively, due to a shorter effective exposure to the drug. Further investigation is needed, however, to clarify this point.

It was expected that after the uncoupled replicon activation and upon removal of aphidicolin, DNA precursors would be incorporated first into short leading chains and origin-proximal Okazaki pieces. The properties of (RI) DNA molecules which were pulse-labeled in vitro following the temperature-shifts and aphidicolin arrest agree with this expectation. Thus, their nascent DNA was not distributed between comparable fractions of long chains and Okazaki pieces, as is the case with steady-state (RI) DNA, but consisted largely of short, "Okazaki-like" chains (Fig. 3), presumably a mixture of bona fide Okazaki pieces and short leading chains whose lengths reflected the distance traversed by the replication forks during the brief in vitro pulse. The ability to subsequently chase these short chains into longer ones (Fig. 4) supports this idea, as it demonstrates that they were not artefactual degradation products. Moreover, comparative hybridization of the short-chain fractions from the various (RI) DNAs to the Atu I-restri-

cted SV40 DNA probe (Fig. 5) indicated a preferential incorporation of label into the origin-proximal region of regimen-D (RI) DNA with a ca. 10:1 efficiency ratio (cpm/bp) between origin and terminus. The control preparations showed about a 2 fold higher hybridization efficiency of origin over other fragments, a result which can be accounted for by the normal occurrence of origin specific short nascent chains of both fork polarities in steady-state (RI) DNA. The non-origin fragments hybridize, however, only to short lagging chains (Okazaki pieces), since the corresponding leading portions are in the long chain fraction which was removed in this experiment by the Sepharose 6B step.

Despite the preferred hybridization of regimen D-nascent DNA to origin-proximal fragments, there existed in it a substantial fraction which hybridized to genomic regions removed away from the origin, in apparent conflict with the idea that all the SV40 replicons became synchronized and that the progress of the replication forks from the origin was limited. Explanations may be offered, however, to resolve this contradiction. Thus, during SV40 tsA infection, defective DNA molecules with displaced or multiple replication origins may accumulate (29, 30). Second, during high temperature restriction of SV40 tsA mutants, nondescript origins are activated (24). It is possible that such abnormal origins are activated, albeit more difficult to detect, even at the permissive temperature. The observation that SV40 tsA 209 Okazaki pieces exhibited a poor asymmetry of hybridization to the unidirectional probe (our unpublished results), unlike their wt-counterparts (15, 16), is consistent with these explanations of misplaced and nondescript replication origins during SV40 tsA infection. Finally, residual DNA synthesis is expected during the aphidicolin-arrest, resulting in a certain background of more advanced replicons.

Despite these complications, it is possible that the synchronization of SV40 tsA replicons by temperature-shifts and aphidicolin-arrest will provide young leading chains containing the original 5'-termini and serve for the study of their priming mechanism.

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