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Aphidicolin-induced topological and recombinational events in simian virus 40

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ABSTRACT

Highly compacted (40S) SV40 DNA replication intermediates formed *in vivo* during aphidicolin exposure and immediately broke down in two stages. In the rapid initial stage, single strand DNA breaks caused loss of superhelicity in the 40S replication intermediates. This DNA breakage was accompanied by the formation of strong, permanent protein-DNA crosslinks which reached a maximum as nicking of the aberrant DNA replication intermediates was completed. These protein-associated DNA strand breaks were not repaired. In the slower second stage of breakdown, the aberrant DNA replication intermediates remained nicked and strongly associated with protein as they underwent DNA replication fork breakage and recombinational changes to produce high molecular weight forms.

INTRODUCTION

Aphidicolin specifically inhibits DNA polymerases alpha and delta by competitive inhibition with dCTP (1–3). These two polymerases replicate chromosomal DNA in mammalian cells (4,5). Inhibition of DNA synthesis by aphidicolin causes a number of secondary effects in mammalian cells, including endoreduplication (6), increased frequency of resistance to methotrexate (7) and sister chromatid exchange (8). The main group of human chromosome fragile sites is defined by aphidicolin inducibility (9). These fragile sites are important because of their role in chromosomal rearrangements associated with tumor progression (10).

Aphidicolin has also been shown to cause dramatic changes in replicating SV40 chromosomes. Viral chromosomes which are replicating at the time of aphidicolin exposure accumulate short nascent chains of about 40 nucleotides (11), and are irreversibly impaired (12). As purified DNA, these aphidicolin-inactivated SV40 chromosomes are highly torsionally stressed, and sediment collectively at about 40S (13).

In this report, we have used two-dimensional gel analysis to study the formation and breakdown of aphidicolin-induced 40S intermediates. The newly formed 40S viral chromosomes immediately underwent spontaneous *in vivo* breakdown in two phases. In the first (rapid) phase, 40S intermediates became strongly protein-associated as the parental DNA strands were nicked. In the second phase (lasting several hours), the protein-associated aberrant intermediates underwent DNA replication fork breakage and recombinational changes to yield aberrant forms of progressively higher molecular weight. Since the SV40 chromosome is often regarded as a model mammalian replicon, these observations may provide clues to the basis of recombinational events and genetic instability in mammalian chromosomes.

METHODS

Cell culture and virus infection

African green monkey kidney cells (CV-1) were grown in Eagle's minimal essential medium (Gibco) supplemented with 14 mM Hepes buffer, pH 7.2, and 4 mM $NaHCO_3$. Cells were infected with plaque purified SV40 strain 777 at a multiplicity of 10 plaque forming units per cell, and experiments were carried out at 36 hours post infection.

Radiolabeling and drug exposure

Aphidicolin (Sigma) was dissolved in dimethylsulfoxide at a concentration of 1.0 mg/ml. Replicating SV40 DNA was labeled with 50–250 μCi/ml [methyl-^{3}H]thymidine for 15 minutes before addition of aphidicolin or dimethylsulfoxide (unless otherwise stated). In some experiments, pulse label was chased with medium containing 10 μM unlabeled thymidine with or without aphidicolin.

Glass filter assay and purification of protein-DNA complexes

The GF/C filter binding assay for protein-DNA complexes stable in SDS and 0.4 M guanidinium chloride (GuHCl) has been described (14). Samples for filter assay of protein-DNA crosslinks were taken from Hirt extract supernatants without protease digestion and solvent extraction. Only proteins and protein-DNA complexes bind glass in 0.4 M GuHCl. All polynucleotides bind glass in 4 M GuHCl, thus binding at this concentration is equivalent to acid precipitable nucleic acid. The level of protein-

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DNA complexes in the Hirt extract supernatant (bound at 0.4 M GuHCl) is expressed as a percentage of total labeled DNA (bound to filters in 4.0 M GuHCl). Viral DNA replication intermediates strongly bound to protein are selectively retained on GF/C filters in 0.4 M GuHCl and can be eluted and analyzed by gel electrophoresis (15). The elution buffer for protein-DNA complexes was 0.1% SDS, 10 mM Tris HCl, pH 7.5, 1 mM NaEDTA, 100 mM NaCl. Eluted samples were treated with proteinase K before preparation for gel electrophoresis.

Extraction and electrophoresis of viral DNA

Experiments were stopped by removal of the medium and addition of Hirt lysing solution (16). The Hirt supernatant was digested with proteinase K (0.1 mg/ml, 45°C) for four hours, extracted with chloroform-isopropanol (24:1), precipitated with 2.5 volumes of ice cold ethanol, dried briefly and taken up in gel loading buffer. Two-dimensional neutral-alkaline gels for analysis of SV40 DNA replication have been described in detail (17–19) and are the subject of a recent review (20). Briefly, for neutral-alkaline gels, each sample was divided into two lanes for the first dimension (neutral agarose) gel electrophoresis. One lane, containing approximately one fourth of the sample, was processed for fluorography after the first dimension run, and the other was equilibrated in second dimension (alkaline) gel buffer, imbedded in an alkaline gel, and run at a right angle relative to the first dimension. In the figures shown here, the fluorographic images of the one and two dimensional gels were combined, with the first dimension gel being shown above the corresponding two dimensional gel.

For two-dimensional neutral-chloroquine gels, the first dimension agarose gel was equilibrated for four hours in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM NaEDTA), then in TBE buffer with chloroquine (15 μg/ml) overnight. The gel was then rotated 90 degrees, and electrophoresed at 2.4 volts/cm for 10 hours. All gels were processed for fluorography as described (19). For figures of neutral-chloroquine gels, the separate one-dimensional gel image was placed to the left of the corresponding two-dimensional gel image.

High resolution electrophoretic separation of SV40 DNA replication intermediates

The gel behavior of viral forms relevant to this report are briefly described here. The DNA replication intermediates are visualized by gel fluorography after pulse-labeling, extraction and electrophoresis. Thus the forms seen were replicating during the pulse-labeling period. Figure 1A depicts normal SV40 replication intermediates separated by one dimensional neutral agarose electrophoresis (across the top, left to right) and as separated by two dimensional neutral-alkaline gel electrophoresis. The second dimension alkaline gel pattern is shown below the one-dimensional pattern from which it is derived.

Form I (superhelical) genomes, the most compact forms, show the highest electrophoretic mobility. Form II (nicked circular) genomes are much less compact and show greatly reduced mobility. Form III (linear) genomes are less compact than form I, but migrate faster than form II due to their ability to reptate through the gel matrix. Replication intermediates (intermediate Cairns structures, or IC) are formed by the opening of replication 'bubbles' on form I DNA. As replication progresses, the replication bubbles become larger, the intermediate Cairns structures become progressively less compact, and their gel mobility is progressively reduced. The unreplicated region remains superhelical in intermediate Cairns structures (21,22). The late Cairns structure (LC) is a 'figure eight' form with an unreplicated region of only a few hundred base pairs. A kinetic pause causes an accumulation of intermediates at this stage (23). Thus, in the first dimension, intermediate Cairns structures appear as a continuous smear extending from the form I band to the

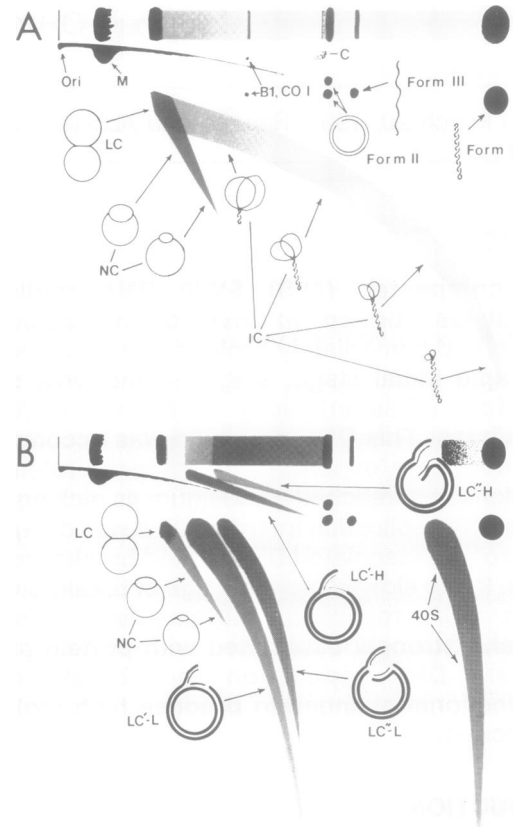

Figure 1. Separations of normal (A) and abnormal (B) replication intermediates on two-dimensional neutral-alkaline gels. The abnormal intermediates in B represent a composite. First dimension neutral gels are shown at the top of A and B, with the direction of migration from left to right. Second (alkaline) dimensions are shown as run from top to bottom. For clarity, intermediates are only labeled and diagrammed in the second dimension patterns. Note that only pulse-labeled nascent strands contribute to the fluorographic images diagrammed, so the arcs of replicating structures in alkaline second dimension gels are actually composed only of nascent strands derived from the indicated structures. This is also true of completed forms with single or double strand breaks, for instance forms II and III. Abbreviations: Ori, origin of electrophoresis; M, mitochondrial DNA; I, form I (unit length superhelical circular viral genomes); II, form II nicked circle; III, form III linear; IC, intermediate Cairns replication structures; NC, nicked Cairns structures; LC, late Cairns structure; B1, superhelical-relaxed catenated dimer with catenation linking number of one; CO I, form I of the third member of the SV40 circular head-to-tail oligomer series; C, unresolved circular head-to-tail dimer and C-family (superhelical-superhelical) catenated dimers; LC'-L, low molecular weight arc of nascent strands derived from 'sigma' forms with single broken DNA replication forks (ranging from a few nucleotides to unit SV40 genome length); LC'-H, high molecular weight arc of nascent strands derived from sigma forms (arising by covalent attachment of a nascent strand to a parental strand, and ranging from approximately unit length to twice unit length); LC''-L, low molecular weight arc of nascent strands derived from aberrant linear replication intermediates with two fork breaks; LC''-H, high molecular weight arc of nascent strands derived from linear forms with two fork breaks (again due to covalent attachment of nascent strands to parental strands); 40S, nascent strands from compacted (40S) replication intermediates.

position of the LC (some distance behind form II). In the denaturing second (alkaline) dimension, form III SV40 DNA gives rise to a single spot (full genome length single strand linear DNA), and form II DNA gives two spots (single strand linear and single strand circular DNAs). Form I (superhelical) viral genomes migrate as a single spot in the alkaline dimension since the pulse-labeled nascent strand is topologically linked to the parental strand. Nascent strands from intermediate Cairns structures form an arc which extends from ahead of the dye front to the level of full length linear single strands in the alkaline second dimension. A minor class of Cairns intermediates with nicks in the unreplicated region (nicked Cairns structures or NC) is also seen on neutral-alkaline gels. In the first (neutral) dimension, the nicked Cairns structures extend from the form II band to the LC band. In the alkaline second dimension, the nascent strands from nicked Cairns structures form a compact arc extending from ahead of the dye front to the level of full length linear single strands. The LC is shared by the normal and nicked families of replication intermediates.

One and two-dimensional gel separations of aberrant replication forms relevant to this report are diagrammed in Fig. 1B. Replication intermediates with single broken replication forks (sigma forms) produce an smear which extends from the form II band to a position just in front of the late Cairns structure on one-dimension gels. Intermediates with two broken replication forks produce an overlapping smear. In the alkaline second dimension, each smear produces an arc, termed LC′ for the sigma forms and LC″ for the linear forms with two broken replication forks (19). Forms with broken replication forks can be produced by *in vitro* digestion of normal replication intermediates with S1 nuclease (24) or by *in vivo* exposure to the topoisomerase I poison camptothecin (19). Camptothecin traps topoisomerase I in a covalent complex with DNA at the site of a DNA strand break (25). Thus, SV40 forms with broken replication forks resulting from camptothecin treatment are strongly protein-associated (15). In the case of camptothecin treatment, alkaline second dimension electrophoresis resolves the LC′ and LC″ smears into upper and lower arcs (Fig. 2B). The upper arcs are due to a recombinational event in which a pulse-labeled daughter strand becomes covalently linked to an unlabeled full length parental strand at the site of a fork break (19). When the arcs of nicked Cairns structures and the LC′ and LC″ families of intermediates are heavily labeled, they tend to overlap. As will be shown below, the highly compacted 40S intermediates migrate as a short smear just behind the form I band in the first dimension and produce a steep arc in the alkaline second dimension.

RESULTS

Formation of the 40S intermediate: two-dimensional, neutral-alkaline gel analysis

Normal SV40 DNA replication intermediates are shown in Fig. 2 (upper left, no aphidicolin). Exposure to aphidicolin caused a rapid mobility shift in the intermediate Cairns structures (Fig. 2). The shift was complete after two minutes of aphidicolin treatment, and the altered Cairns arc extended from the form I band to a position approximately half way between the form I and form II bands in the first dimension. Aphidicolin-induced 40S replication intermediates are known to migrate collectively at approximately this position (13). The neutral-alkaline gel analysis showed that the shift in first dimension gel mobility was due almost entirely to compaction of the most extensively replicated intermediates. The later the stage of replication, the greater was the shift in mobility. The earliest DNA replication intermediates did not appear to change their mobility in the neutral first dimension gel electrophoresis. The band of 40S intermediates, though compressed, still gave rise to an arc in the alkaline second dimension. This shows that the more extensively replicated members of this family were still less compact than the early members (due to the presence of larger replication bubbles).

Spontaneous *in vivo* relaxation of 40S intermediates

The sequence shown in Fig. 3 begins with a fully formed family of 40S intermediates similar to that seen at the end of the formation sequence in Fig. 2. During the following 10 minutes, the 40S intermediates were progressively converted to nicked Cairns structures *in vivo*. No intermediates were detected, suggesting that the conversion was due to a single event: the introduction of a nick in the unreplicated region. Relaxation of purified 40S intermediates *in vitro* with topoisomerase I converts

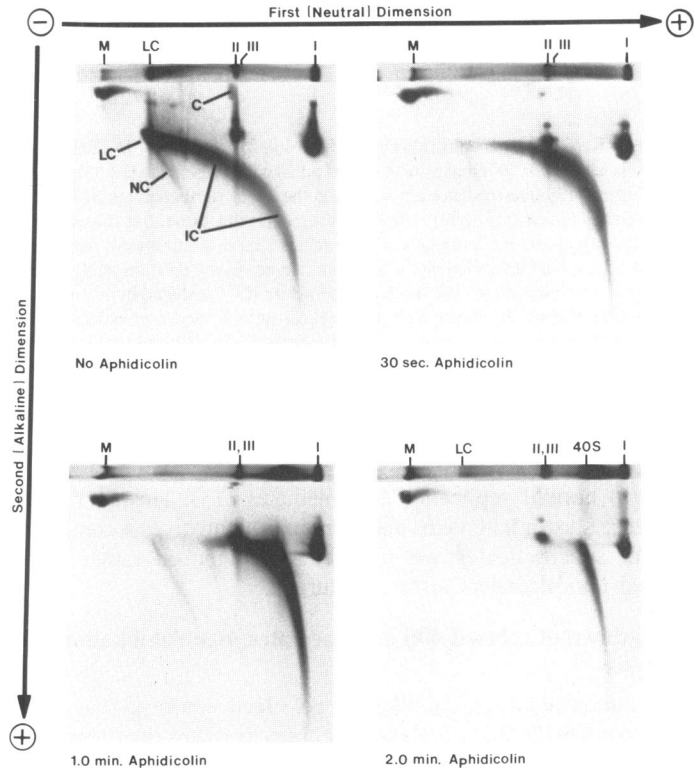

Figure 2. Formation of the 40S Intermediate. Two dimensional neutral-alkaline gels were used to study structural changes in DNA replication intermediates as a function of time of exposure to aphidicolin. No Aphidicolin (top left): cells labeled for 30 minutes, then extracted. Normal DNA replication intermediates and completely replicated forms were present: I, form I (superhelical) SV40 DNA; II, form II (relaxed circular) viral DNA; III, form III (unit length linear) viral DNA; IC, arc of nascent strands from intermediate Cairns structures; NC, nicked Cairns structures (Θ-form DNA replication intermediate in which the unreplicated portion is nicked or relaxed); LC, late Cairns structure (a Θ-form DNA replication intermediate that is approximately 95% completed); C, unresolved C-family (fully superhelical) catenated dimers and superhelical circular (head-to-tail) dimers; M, mitochondrial DNA. Top right; cells were labeled for 15 minutes, and the labeling media was brought to 60 μM aphidicolin for 30 seconds before Hirt extraction. Bottom left; cells were labeled for 16 minutes with 60 μM aphidicolin being present the last minute. Bottom right; cells were labeled for 17 minutes, with aphidicolin being present for the last two minutes. 40S, the aberrant 40S replication intermediate.

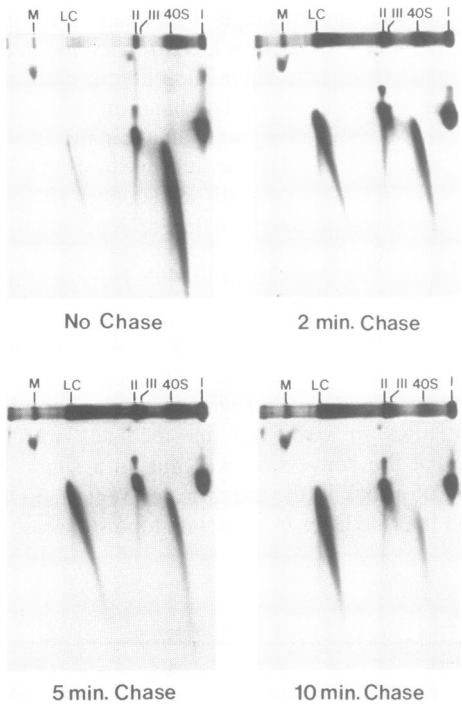

Figure 3. Rapid, spontaneous relaxation of the 40S intermediate. No Chase (top left); cells labeled for 20 minutes, with 60 μM aphidicolin present the last 5 minutes. Fully formed 40S intermediates are seen as in the lower right quadrant of Fig. 2. Two minutes Chase (top right); label and drug exposure same, but chased with unlabeled thymidine for 2 minutes. Five minutes Chase (bottom left); same but 5 minute chase with unlabeled thymidine. Ten minutes Chase (bottom right); same but with a 10 minute chase. The late Cairns structure (LC) is shared by IC (normal intermediate Cairns structures with superhelical unreplicated regions) and NC (intermediate Cairns structures with nicked or relaxed unreplicated regions). See Fig. 2, upper left quadrant for examples of all three. By one dimensional gel analysis, NC cannot be distinguished from normal late IC.

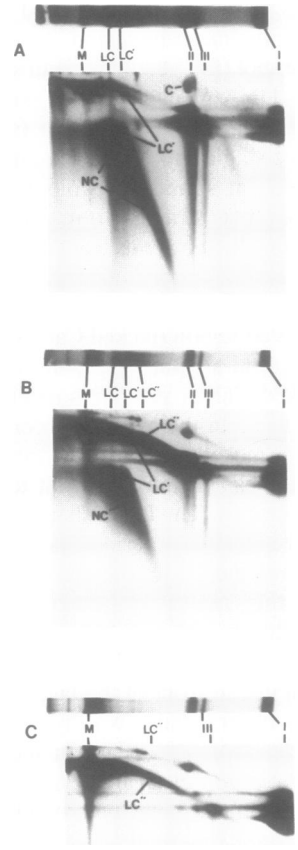

Figure 4. Progressive breakdown of relaxed 40S intermediates. A, Cells were labeled for 45 min, with aphidicolin (60 μM) being added to the labeling solution after 15 minutes. B, Cells labeled and aphidicolin-treated as in A, but chased with unlabeled thymidine for 2.5 hr. before extraction. C, cells labeled as in A, but chased for 12 hr. with unlabeled thymidine before extraction. Abbreviations same as in Fig. 1.

them to normal replication intermediates (13). However, the sequence shown here takes place *in vivo* and involves a complete loss of superhelical stress (conversion to nicked rather than normal intermediate Cairns structures).

Breakdown of relaxed 40S intermediates, neutral-alkaline gel analysis

Two-dimensional neutral-alkaline gel electrophoresis was also used to study the fate of relaxed 40S intermediates over a period of several hours. As shown in Fig. 4A, the arc of nicked Cairns structures appeared to broaden after 30 minutes of aphidicolin exposure. This apparent broadening was due to the appearance of a second arc (LC′) just ahead of the nicked Cairns arc and almost parallel to it. As discussed above, the LC′ family of intermediates is composed of relaxed circles with linear tails extending up to one genome length. These intermediates are the result of single replication fork breaks in normal intermediate Cairns structures. As in the case of camptothecin treatment (19,20), the LC′ intermediates formed during 40S breakdown were resolved into two arcs in the alkaline second dimension. The high molecular weight (upper) arc is due to covalent attachment of a nascent strand to a parental strand (19).

Additional changes could be seen two and a half hours later (Fig. 4B). At this point the lower LC′ arc was slightly more distinct, and the upper LC′ arc had become very heavy. The upper LC′ arc also appeared to 'tail' in the direction of the first dimension loading slot. Other forms seen at this time included a spot above the mitochondrial DNA, twin spots above the LC′ arcs, and an apparent LC″ upper arc. LC″ arcs, composed of linear forms, are also seen in camptothecin-treated SV40-infected cells where they result from double replication fork breaks (19,24). In the first dimension, the LC″ family extends from the form III band (the earliest member is unit length linear SV40 plus a few nucleotides of newly synthesized daughter strand) to a position just ahead of LC′ (Fig. 1B). Like the LC′ arc, the LC″ arc in camptothecin-treated cells is resolved into upper and lower arcs in alkaline second dimension gels (Fig. 1B). The upper LC″ arc extends from the level of full length linear single strands to the level of twice full length linear in the alkaline second dimension. Again, this is due to covalent attachment of a nascent strand to a parental strand in a fraction of the LC″ intermediates (19,20). S1 nuclease digestion of purified intermediate Cairns structures can produce lower LC′ and LC″ arcs without the upper arcs (24). Here (Fig. 4B), however, an upper LC″ arc was present without a lower arc.

After 12 hours of chase, the nicked Cairns arc and both upper and lower LC′ arcs were gone (Fig. 4C). The upper LC″ arc was still quite heavy at this point, and a heavy wedge of labeled DNA was seen to extend up into the mitochondrial DNA region. The unidentified spots above this arc and above the mitochondrial DNA remained. The form II DNA band had weakened substantially at this point, but the band identified as 'C' remained. Form I (superhelical) circular dimers and C-family (superhelical-superhelical) catenated dimers both appear in this area of two-dimensional neutral-alkaline gels.

2-Dimensional neutral-chloroquine gel analysis of 40S breakdown products

Two-dimensional neutral-chloroquine gel analysis of SV40 DNA replication intermediates has been described in detail (20). In this gel system, the first dimension is identical to the first dimension in neutral-alkaline gels: a neutral agarose gel separation based on compactness of the viral forms. In the second dimension, a strong intercalator (chloroquine) was present. Chloroquine unwinds the Watson-Crick helix and titrates out negative supercoils in covalently closed DNA (26,27). This causes supercoiled forms to become less compact and slows their rate of migration through the gel. Covalently closed, relaxed DNA and DNA with low levels of supercoiling becomes positively supercoiled and more compact in chloroquine at the concentration used here (28). These low superhelicity forms show increased mobility in the chloroquine dimension. Linear and nicked circular forms do not show relative mobility changes in chloroquine, and thus fall along diagonals.

Figure 5A shows the appearance of normal viral intermediates on a two-dimensional neutral-chloroquine gel. In the second dimension, the nicked circular form II and linear form III bands remain distinct. The form I band, however, is elongated in the second dimension since it is normally composed of covalently closed circles with approximately 12−24 supercoils. These topoisomers are partially resolved in the second (chloroquine) dimension so that the forms with lower levels of supercoiling are on the right of the elongated form I spot. DNA replication intermediates are distributed in an arc extending from the high superhelicity end of the form I oval, to the left, then up vertically to the late Cairns structure. Linear double-stranded DNA is located on a diagonal with a shallow reverse 'S' shape. This diagonal of linear forms extends from the origin slot, through the mitochondrial DNA then down through the form III DNA band. In Fig. 5A, only mitochondrial and form III DNA appear on this diagonal. The form I circular head-to-tail dimers and C-family (superhelical-superhelical) catenated dimers with low to moderate catenation linking numbers are resolved from form II by this type of gel. However, form I circular dimers and C-family catenated dimers are not well resolved from one another.

Exposure to aphidicolin caused rapid conversion of normal intermediate Cairns structures to 40S intermediates. Two minutes after addition of aphidicolin (Fig. 5B), the 40S intermediates were still forming, but already undergoing spontaneous relaxation to form an arc of nicked Cairns structures (NC) and a much weaker arc of sigma structures with broken replication forks (LC′). Note that the earliest 40S intermediates connected to the elongated form I spot at the low superhelicity end. This was even more apparent when the 40S was fully formed (Fig. 5C). After complete relaxation of the 40S intermediates (Fig. 5D), the arc of nicked Cairns structures and the weaker LC′ arc were both prominent. Six hours after aphidicolin exposure (Fig. 5E), the NC arc was gone, and an LC″ smear could be seen on the diagonal of linear forms (in agreement with the neutral-alkaline gel results).

Kinetics of 40S intermediate breakdown and protein-DNA binding

When 60 μM aphidicolin was added to SV40 infected cells during labeling, a time-dependent increase in strong protein association with the labeled DNA was seen (Fig. 6A). The protein association with pulse-labeled, Hirt extracted DNA reached a maximum about 45 minutes after addition of aphidicolin. One dimensional gel electrophoresis was carried out on aliquots of the same samples to determine the distribution of SV40 DNA replication intermediates at each time point (Fig. 6B). The first three lanes show labeled SV40 replication intermediates from three plates of cells treated with the solvent, dimethylsulfoxide. Only normal replication intermediates were seen in these solvent controls.

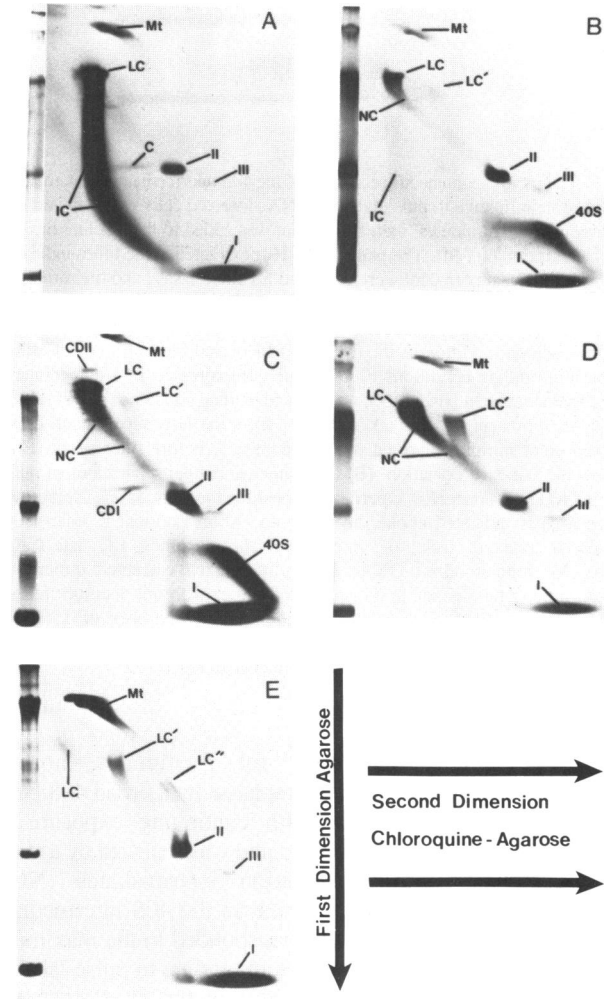

Figure 5. Two-Dimensional neutral-chloroquine gel analysis of 40S intermediate formation and breakdown. A, normal 15 minute pulse-labeled SV40 DNA replication intermediates; B, DNA replication intermediates after 2 minutes of exposure to 60 μM aphidicolin; C, DNA replication intermediates after 10 minutes of exposure to aphidicolin; D, DNA replication intermediates after 45 minutes of exposure to aphidicolin; E, Aberrant DNA replication intermediates chased with unlabeled thymidine (no aphidicolin) for six hours after a 45 minute exposure to aphidicolin. Abbreviations same as in Fig. 1.

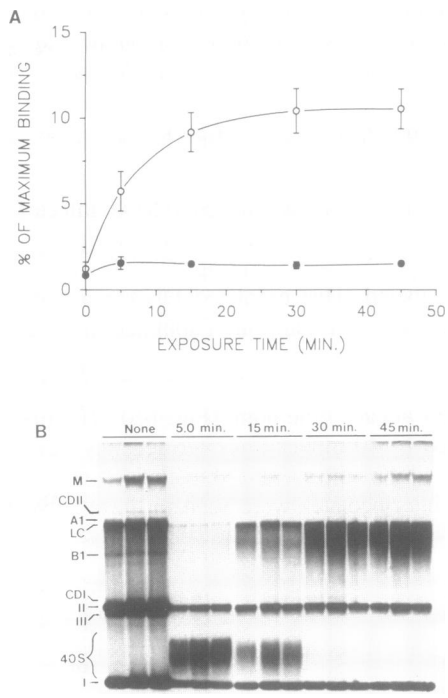

Figure 6. The rapid, spontaneous relaxation of the 40S intermediate is accompanied by strong protein linkage to pulse-labeled DNA. Infected cells were labeled with [^{3}H]thymidine for 15 minutes, then aphidicolin was added to the labeling medium (final concentration 60 μM). The plates were Hirt extracted at the times indicated, aliquots of the Hirt supernatant were removed for filter assay, and the remainder was processed for gel electrophoresis (proteinase K digestion, solvent extraction and ethanol precipitation). The 'zero exposure' samples were pulse labeled for 30 minutes without addition of the drug. (A) Increased binding to GF/C filters under protein binding conditions (0.4 M GuHCl) expressed as a percentage of the amount of label bound under total nucleic acid binding conditions (4 M GuHCl). Open circles represent aliquots taken directly from the Hirt supernatant, closed circles represent samples digested with proteinase K before filter assay. Error bars show the standard deviation. (B) One dimensional gel separations of pulse-labeled SV40 DNA from Hirt supernatants used for the filter assay. Aphidicolin exposure time is indicated above the gel lanes. Abbreviations: I, superhelical monomer; II, relaxed circle; III, linear viral chromosomes; LC, late Cairns structure; M, mitochondrial DNA; A1, fully relaxed catenated dimer with catenation linking number of 1; B1, catenated dimer with one member relaxed and one member superhelical, and catenation linking number of 1; CD I, superhelical circular head-to-tail dimer; CD II, relaxed circular head-to-tail dimer; 40S, broad band of compacted replication intermediates.

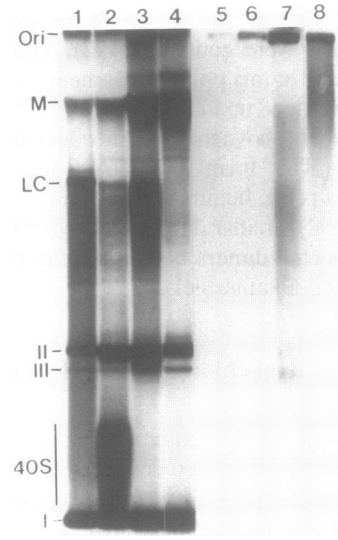

Figure 7. SV40 DNA replication intermediates associated with protein at different times after exposure to aphidicolin. Hirt supernatants from pulse-labeled infected cells were each divided into three aliquots. One aliquot from each sample was immediately processed for electrophoresis (proteinase K digestion, solvent extraction and ethanol precipitation), lanes 1–4, while another aliquot was adjusted to 0.4 M GuHCl, and filtered through a glass fiber filter. The filter bound material was eluted, treated with proteinase K, and separated by electrophoresis (lanes 5–8). The third aliquot was digested with proteinase K *before* filter binding. Lanes 1 and 5, cells were pulse labeled for 15 minutes and extracted; lanes 2 and 6, pulse labeled for 18 minutes with 60 μM aphidicolin present the last 3 minutes of labeling; lanes 3 and 7, labeled for 1 hour, with 60 μM aphidicolin present the last 45 minutes; lanes 4 and 8, labeled for 45 minutes, with aphidicolin present the last 30 minutes, then chased with unlabeled thymidine (10 μM) for 24 hours. Aliquots digested with proteinase K before filtration showed no filter-bound material (not shown). Abbreviations same as in Fig. 1.

When aphidicolin was added for 5 minutes, normal intermediate Cairns structures were replaced by a broad 40S band just above the form I band. With continuing exposure to aphidicolin, the 40S band disappeared and was replaced by a short smear of nicked Cairns replication intermediates (NC). Spontaneous loss of superhelical stress in the 40S intermediate was complete by 45 minutes and corresponded to the maximum protein association. The level of protein binding to pulse-labeled DNA did not decline significantly during the later stages of breakdown in which high molecular weight forms were produced (data not shown).

Aberrant replication intermediates strongly associated with protein during breakdown of the 40S intermediates

To find out which viral replication intermediates were strongly associated with protein, binding to GF/C filters was carried out in 0.4 M GuHCl and the bound forms were eluted, protease digested and analyzed by gel electrophoresis (Fig. 7). No labeled DNA bound to the filter in the control sample (solvent control, dimethylsulfoxide), indicating that normal replication intermediates are not protein-associated after Hirt lysis. In the sample treated with aphidicolin for 3 minutes, the 40S intermediate was at its maximum (Fig. 7, lane 2), but only a small amount of DNA bound to and eluted from the filter under protein binding conditions. This material did not enter the gel (lane 6). After 45 minutes of aphidicolin exposure, the spontaneous relaxation of the 40S structure was complete (lane 3). The filter eluate of this sample (lane 7) showed protein association with the relaxed 40S structures, forms II and III, some high molecular weight DNA, and a substantial amount of material unable to enter the gel. When aphidicolin-treated cells were chased with unlabeled thymidine for 24 hours (Fig. 7, lanes 4 and 8) the pulse-labeled, protein-associated DNA was of still higher molecular weight. The darkening of the mitochondrial DNA band (M) in lanes 1–4 may be due in part to the fact that mitochondrial DNA synthesis is not inhibited by aphidicolin (3). An additive exposure effect with aberrant SV40 forms in this region may also contribute to the darkening.

DISCUSSION

Two-dimensional gel analysis has given new insights into the nature and fate of the 40S intermediate. Electron microscopy has shown that the replicated daughter strands are more intertwined

in many 40S intermediates than in normal intermediate Cairns structures (13). The same report suggested that the 40S intermediates also had unusually high levels of negative superhelical stress. Our neutral-alkaline gel analysis indicated that the altered mobility in the first dimension gel was due to compaction of the later intermediate Cairns structures. However, neutral-chloroquine gel analysis clearly shows dramatically altered superhelicity in the earliest 40S intermediates. Since topoisomerase I digestion *in vitro* converts 40S intermediates to normal DNA replication intermediates (13), it is clear that altered parental strand linkage is the basis of the abnormal 40S structure. This is supported by our observation that nicked 40S intermediates and nicked Cairns structures are identical. Our findings indicate that most of the altered topology of late and intermediate 40S forms is distributed into the replicated region; probably as daughter strand intertwining. Altered superhelicity was seen in the earliest 40S intermediates, but the exact level of superhelicity is difficult to determine for DNA replication intermediates. We are presently attempting to determine the precise linkage of parental DNA strands in 40S intermediates.

Once formed, the 40S intermediates immediately underwent complete relaxation. The rapid *in vivo* loss of superhelical stress was surprising. According to the model of Dröge et al., the 40S intermediates are not under superhelical stress in the intact cell. Their model (13) is a variant of an earlier explanation for the higher than expected levels of negative superhelicity in normal intermediate Cairns structures (22). In this model, helicase continues to unwind parental DNA strands after polymerase movement is stopped by aphidicolin. Single strand binding protein prevents these extended forks from re-annealing *in vivo*. Extraction and deproteinization removes the single strand binding protein, allowing the parental strands to re-anneal. This re-annealing of the extended forks is proposed to cause high levels of negative superhelicity in the 40S intermediates. If, as the Dröge model suggests, the 40S intermediates are not abnormally stressed as chromatin, there is no reason for them to experience the rapid topological changes revealed by our gel analysis. Thus, our data indicate that the 40S intermediates are abnormal *in vivo*.

The negative superhelicity seen in normal DNA replication intermediates and form I DNA is due to chromatin structure (29). If the 40S intermediates were under excess negative superhelical stress *in vivo*, one would expect that excess negative superhelicity in viral chromatin would be quickly removed by intracellular topoisomerases, leaving just the superhelicity associated with normal chromatin structure. However, the 40S intermediates spontaneously lose *all* superhelical stress *in vivo*. We can envision only two possible explanations for this. The first possibility is that the 40S intermediates lose their chromatin structure *in vivo* and intracellular topoisomerases then completely relax the nucleosome-free viral DNA. The second possibility is that a permanent nick (single strand DNA break) is introduced into one of the parental DNA strands in the unreplicated region. Either of these unexpected events would imply that the 40S intermediates are, in fact, topologically abnormal *in vivo*. No such changes take place in normal intermediate Cairns structures or form I DNA.

The 'nick model' is the simplest and most likely of the two explanations for several reasons. First, relaxation of the 40S intermediates is an all-or-nothing event. The 40S intermediates disappeared and nicked Cairns structures appeared with no evidence of relaxation intermediates. This would be expected for nicking but not for normal topoisomerase action. Second, the relaxed 40S intermediates are indistinguishable from nicked Cairns structures by either neutral-alkaline or neutral-chloroquine gel analysis. Third, some replication fork breakage accompanies the spontaneous loss of superhelicity in the 40S intermediates. This fork breakage is due to parental DNA strand breaks, probably in double strand regions just ahead of replication forks.

However, there remains a problem concerning the nature of the nicks. If they were caused by an endonuclease, there is no reason that they could not be sealed by intracellular ligases. With chromatin structure intact, this would yield normal intermediate Cairns replication structures with the usual level of superhelicity in the unreplicated region. If the nick were caused by either topoisomerase I or II, the same result would be expected since these enzymes seal the DNA strand breaks as they complete their reaction cycles. However, topoisomerases can cause high levels of DNA strand breakage when their breakage-reunion cycles are disrupted (for instance, by topoisomerase poisons or denaturation). If the DNA nicking is caused by disruption of topoisomerase reactions, the relaxed 40S intermediates should be covalently associated with protein (the topoisomerase). GF/C filter binding assays showed that the nicking of the 40S intermediate was accompanied by the formation of protein-DNA binding that was stable in SDS and 0.4 M guanidinium chloride. The maximum level of protein binding to DNA was reached as nicking of the 40S structures was completed. In addition, elution of bound forms from GF/C filters showed that the nicked 40S intermediates (but not un-nicked 40S intermediates) were strongly linked to protein. Aphidicolin is not a topoisomerase poison, thus the 40S intermediate may be a suicide substrate for a cellular topoisomerase. Suicide substrates have been reported for both type I and type II topoisomerases (30,31).

The nicked 40S intermediates underwent additional breakdown over a period of several hours. This second phase breakdown included progressive breakage of replication forks and ligation of nascent strands to parental DNA strands at the sites of fork breaks. A similar recombination event occurs, in SV40-infected cells treated with the topoisomerase I poison camptothecin (19). When a moving replication fork encounters a camptothecin-stabilized topoisomerase I-DNA crosslink on either the leading or lagging strand side, a double strand break occurs (24). Single fork breaks produce sigma structures resembling rolling circles (LC′ family) while double fork breaks detach replication bubbles or produce linear forms (LC″ family). In a fraction of the LC′ and LC″ intermediates, a ligation of a nascent strand to a parental strand occurs. This is the origin of the upper LC′ and LC″ arcs which extend from the position of full length linear single strand DNA to the level of twice full length linear in the second dimension of neutral-alkaline gels. Such a ligation between a nascent strand and a parental strand at a broken replication fork can be viewed as a half-completed sister chromatid exchange (19). In camptothecin-treated cells, the replication fork breaks and ligations of parental strands to nascent strands occur very rapidly. The slower rate of LC″ formation and the absence of a lower LC″ arc during late breakdown of the relaxed 40S suggest that a somewhat different pathway may be followed. It seems likely that the upper LC″ intermediates are generated directly from the upper LC′, probably by a second fork break on the opposite side of the replication bubble. As the second phase of breakdown continued, high molecular weight forms were seen to extend into the region occupied by mitochondrial DNA. These high molecular weight forms were still strongly protein-associated. This progressive shift to higher molecular weight forms is due to continued recombinational change rather than DNA replication.

No DNA replication or incorporation of label occurs in 60 μM aphidicolin. These high molecular weight forms are the focus of current studies.

These observations also raise a question about the origin of nicked Cairns structures seen normally at low levels in Hirt extracts of untreated cells. These nicked Cairns structures may arise from a low, constant rate of polymerase failure similar to that caused in all viral replicons by aphidicolin treatment. If so, it is possible that similar replication failure can occur at low rates in cellular replicons. This might be the basis for the genetic instability seen in rapidly dividing populations of transformed cells.

SV40, of course, is a DNA tumor virus, and it is not certain that mammalian replicons will be similarly affected by aphidicolin treatment. However, SV40 DNA replication is considered a model for eukaryotic DNA replication. Thus, the aphidicolin-initiated topological and recombinational events reported here may have relevance for aphidicolin-induced recombinational events in mammalian cells.

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