Visualization of novel simian virus 40 DNA recombination intermediates induced by ultraviolet light irradiation

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ABSTRACT
Electron microscopic technique was used to examine the structures of SV40 DNA recombination intermediates induced by ultraviolet irradiation as an approach for understanding recombination mechanisms in animal cells. Putative recombination Intermediate with the characteristic Holliday junction was observed in both SV40 and CV-1 monkey kidney cell DNA. These results suggest that Holliday recombination Intermediate is a common Intermediate in eukaryotic as well as prokaryotic recombination pathways. In UV irradiated cells, putative SV40 DNA recombination intermediates with multiple recombining partners were observed. In addition, UV irradiation induced two types of novel joint molecules of SV40 DNA. The first type contains replication intermediates as one of the joint molecules with the putative recombination junction located in the newly replicated DNA arms. The second type of novel joint molecules is represented by of the 'dumbbell' structures with two circular SV40 DNA linked by a linear DNA of varying lengths. The structures of these novel recombination Intermediates suggest a strand-invasion mechanism for UV-induced DNA recombination.

INTRODUCTION
Study of recombination pathways in animal cells has been impeded by the lack of genetics. To circumvent this shortcoming, viral and plasmid DNA with well-characterized physical and genetic structures has been used to probe recombination mechanisms in animal cells. Whereas the majority of the studies analyzed the recombination products formed in vivo or in vitro as a means of inferring the recombination mechanisms (see review in ref. 1), we have focused our attention on the structures of in vivo recombination intermediates (2, 3). These intermediates can have complex structures, such as multiple cross-overs or strand invasion, so that direct visualization with the electron microscope is most suitable for studying them (2–9). Using this technique, we have demonstrated the presence of both the Holliday type (2) and the strand-invasion, Meselson-Radding type (3) recombination intermediates in human adenovirus DNA from infected HeLa cells. Those results provide the first direct evidence for the existence of both types of recombination intermediates in animal cells.

However, the adenovirus nucleoprotein complexes structure and the DNA replication mechanism differ from those of cellular chromatin (10, 11), so that the recombination intermediates observed in adenovirus DNA may not represent those in the recombination pathways of cellular DNA. In order to provide a better model for DNA recombination in animal cells, we analyzed the structures of in vivo recombination intermediates of simian virus 40 DNA. In infected cells SV40 genome is organized in a nucleosome structure similar to cellular chromatin, and the mechanism of viral DNA replication is also similar to that of cell DNA (12). SV40 DNA recombination, therefore, may closely resemble the recombination process in animal cells. Examination of the structures of SV40 DNA with the electron microscope shows the presence of joint molecules with Holliday junctions similar to those found in the recombination intermediates of human adenovirus DNA, and in bacterial plasmids and phages. Furthermore, Holliday structures can be observed in joint molecules between wild type and a mutant SV40 DNA in cells co-infected with the two viruses. These observations suggest that Holliday structures may represent recombination intermediates in animal cells.

UV irradiation has been shown to increase recombination frequency (see review in ref. 13). In order to understand the mechanisms of UV-induced recombination, we also investigated the structures of SV40 DNA recombination intermediates after irradiation of infected cells with 254 nm UV light. It was found that UV irradiation induced several types of complex recombination intermediates. The implications of these novel recombination intermediates for the study of UV-enhanced recombination are discussed below.

MATERIALS AND METHODS
CV-1 cells were grown in Dulbecco modified minimum essential medium (DMEM) plus 10% fetal calf serum and 50 μg/ml gentamicin. Cells were infected with SV40 virus at 10 pfu/cell. ln1412 mutant SV40 virus stock was a generous gift from T. Shenk. Intracellular SV40 DNA was extracted from the infected cells by the Hirt extraction method (14). Infected cells were washed with 10 ml of phosphate-buffered saline, and irradiated from the top with a short-wave UV lamp (model R-52, principal emission 254 nm, Ultraviolet Products Inc.) at a dose of 90 J/m². The UV lamp was calibrated using a J-225 black ray ultraviolet meter (Ultraviolet Products Inc.), and by D37 lethal
dose for phage Phi-X 174. After irradiation, the infected cells were incubated in DMEM supplemented with 2% fetal calf serum for 3 hours before extraction of SV40 DNA. Electron microscopy was performed as previously described (2) using a Zeiss EM10 microscope.

RESULTS

Observation of SV40 joint molecules with Holliday junctions in intracellular SV40 DNA

Examination of SV40 DNA extracted from cells at 28 hours postinfection revealed the presence of joint molecules, with two circular SV40 DNA linked at a single point. These could arise either by catenation or through recombination. Joint molecules formed through DNA recombination would have the structure of two circles linked at one point. And they were indeed found (Fig. 1). In general, these molecules could be distinguished from the catenated dimers by the non-overlapping of the two circular DNA at the joint. More specifically, recombination intermediates could be positively identified by the characteristic four-way Holliday junction, with four short single-stranded DNA at the joint (Fig. 1A and 1C). The Holliday junction in Figure 1C, showing four connected strands of DNA is enlarged in Figure 1-D.

However, in order to be certain that the molecules observed were not catenated dimers, we digested the DNA with a restriction endonuclease that cuts SV40 DNA once. This process would dissociate the catenated dimers into two individual linear molecules, whereas the two molecules joined by a Holliday junction would remain associated. Furthermore, after restriction endonuclease digestion two linear DNA molecules in a putative recombination intermediate with a Holliday junction would form the characteristic 'chi' structure that has been previously observed in bacterial phage and plasmid DNA (see review in ref. 15), as

Fig. 1. Electron micrographs of putative SV40 DNA recombination intermediates. Holliday recombination junctions with four connected single strands can be seen in panels A and C (arrows). Panel D is an enlarged view of the Holliday junction in panel C. Although not apparent, panel B was also found on high magnification to have a four-stranded Holliday junction.

Fig. 2. Electron micrographs of SV40 chi recombination intermediates generated by digestion of SV40 circular DNA with Kpnl, which cleaves SV40 DNA once at nucleotide 294. In panel A, the equal length branches are on the same side of the recombination junction; hence the chi structure is cis. The trans configuration, with two equal length branches on opposite sides of the recombination junction, is shown in panels B, C, and D. Holliday junctions with four connecting single strands are clearly visible in panels B-D (curved arrow). The molecule in panel C is undergoing double cross-over, with both recombination junctions in the trans configuration. Bar represents 0.5 μm.
well as in human adenovirus DNA (2). The chi structure has two pairs of equal length branches, and the sum of the lengths of the two unequal branches equals the length SV40 DNA.

To examine Holliday type recombination intermediates in intracellular SV40 DNA during lytic infection, viral DNA extracted at 28 hours post infection was cleaved with Kpnl endonuclease, which cuts SV40 DNA once at nucleotide 294. Examination with the electron microscope indeed revealed the presence of chi structures (Fig. 2) at a frequency of 0.08% (8/10,000). Chi structures with equal length branches on the same side, the cis configuration (Fig. 2A), or with equal length branches on opposite sides, the trans configuration (Fig. 2B, C, D), were both observed. Molecules with the trans configuration showed the characteristic Holliday recombination junction, which contains four partially melted single-stranded DNA branches linking the two DNA molecules (Fig. 2, B-D, arrow). In Figure 2C, SV40 DNA is seen undergoing double cross-over with both recombination junctions in the trans configuration. Holliday junctions with four connected single-stranded DNA linking four double-stranded DNA branches were also observed in CV-1 cell DNA extracted from UV-irradiated cells, although at a very low frequency (Fig. 3). These results clearly demonstrate the existence of Holliday structures in animal cells.

To provide further evidence that the joint molecules with Holliday junctions are indeed recombination intermediates, the analysis described above was also performed using SV40 DNA extracted from a mixed infection with two types of SV40 virus. CV-1 cells were co-infected with wild type SV40 virus and a viable mutant in1412 containing two origins of DNA replication (16). The DNA of the two viruses can be distinguished by digestion with BglII restriction endonuclease, which cuts SV40 DNA once at the origin of DNA replication. Wild type DNA is converted to full-length linear DNA, whereas mutant DNA is cleaved into two fragments of 2.8 and 2.4 kb. Electron microscope examination of BglII-cleaved viral DNA extracted from mixed infection showed the presence of chi recombination intermediates between the wild type and in1412 DNA. Figures 4A and 4B are joint molecules between wild type DNA and mutant DNA fragments in cis and trans configuration, respectively. This result shows that the Holliday structures observed indeed represent intermediates formed during DNA recombination in CV-1 cells.

**Observation of novel SV40 DNA joint molecules in UV-irradiated cells**

Ultraviolet light irradiation has been shown to induce mitotic recombination in both prokaryotic and eukaryotic systems (13). In order to facilitate the analysis of SV40 recombination intermediates, and to study the mechanisms of UV-induced recombination, SV40-infected CV-1 cells were irradiated at 24 hours postinfection with 254 nm light at a dosage of 90 J/m². After incubation of the irradiated cells for 3 hours postirradiation, SV40 DNA was extracted and examined with the electron microscope. Analysis of SV40 DNA with or without cleavage by Kpnl restriction enzyme showed the presence both of simple chi structures with Holliday junctions, and of complex joint molecules involving more than two SV40 DNA molecules. Figure 5A shows DNA without restriction endonuclease cleavage, while Figure 5B shows DNA cleaved with Kpnl endonuclease.

The structure of the simple chi molecules was indistinguishable from that found in the unirradiated cells, but they were found three times more frequently in the UV irradiated sample. The
complex joint molecules containing several SV40 DNA connected together were rarely observed in the DNA extracted from unirradiated cells. The examples shown in Figures 5A and 5B contain eleven and five SV40 DNA molecules respectively connected together by Holliday junctions. Up to 25 molecules were found to form a complex network after SV40 DNA was linearized with KpnI restriction endonuclease. In Figure 5B, the putative recombination intermediate contains two trans and three cis recombination junctions (a Holliday junction with four connecting single strands is present in the upper left). Twenty-three such complex joint molecules with an average of three to four molecules joined together were observed in a scan of about 10,000 molecules. The simple and complex joint molecules extracted from the UV-irradiated cells were frequently found to contain single-stranded DNA linking the two recombining partners, suggesting a strand-invasion-initiated recombination.

In addition to these putative recombination intermediates with multiple recombining partners, UV irradiation also induced two types of novel SV40 DNA joint molecules not found in the unirradiated control. The first of these contained SV40 replication intermediates among the recombining molecules. The recombination junction was located in the newly replicated arms of SV40 replication intermediates. In Figure 6, two replication intermediates are connected through the newly replicated parts of the DNA. Figures 6A and 6B show molecules without and with KpnI digestion, respectively. The interesting and unusual

Fig. 5. Electron micrographs of recombination intermediates with multiple recombining partners. In panel A, eleven SV40 DNA form a recombination network without restriction endonuclease digestion. In panel B, there are five recombining molecules with five recombination junctions (arrows), two of which are in trans configurations and three in cis configurations. The characteristic Holliday junction with four connecting single stranded DNA is clearly seen in one case. One of the cis junctions (the leftmost) is linked by a single stranded bridge.

Fig. 6. Electron micrographs of joint molecules containing two SV40 replication intermediates at the same stage of DNA replication. Recombination junctions and replication forks are indicated by large and small arrows, respectively. In both examples, the putative recombination junctions are located in the newly replicated arms. The tracings of the molecules are shown on the left panels. In panel A, the recombination junction is in the trans configuration, with the four single-stranded branch Holliday junction clearly visible. Single-stranded regions are present near the replication forks, as shown by dotted curves in the tracing on the left. Replication is 73% complete in both replication intermediates. The recombination junction is located at 26% genome length from the origin of replication, assuming bidirectional replication with equal rate at the two replication forks. In panel B, the DNA has been cleaved with KpnI. Replication in these molecules is 81% complete. The recombination junction is in the cis configuration, and is located at 24% genome length from the origin. Assuming that the shorter replication fork progresses from the origin towards the late region (because the KpnI site is located on the late side of origin), the recombination junction is located at coordinate 91 on the SV40 map. A short single-stranded DNA can be seen connecting the recombining DNA (large arrow). Bar represents 0.5 μm.

Fig. 7. Electron micrographs of novel dumbbell SV40 joint DNA molecules with two circular SV40 DNA linked by a linear DNA, having lengths ranging from 200 bp to 18 kb. The junctions between the interconnecting linear DNA and the circular SV40 DNA are indicated by large arrows. When the length of the interconnecting linear DNA is short, it is usually found to be single stranded. The lengths of the interconnecting linear DNA in panels A, B, C, and D are 83, 24, 37, and 300% of SV40 DNA length, respectively. Note that one of the circular DNA in panel D is undergoing DNA replication (the replication forks are marked by small arrows). see Figure 9 and text for interpretation.
feature here is that the two recombining molecules are at the same stage of DNA replication: 73% and 81% replicated in Figures 6A and 6B, respectively. Replication intermediates with many recombination partners are also present in the complex joint molecules; the recombination junction is located in the newly replicated arms (data not shown). These replication-recombination molecules, however, are rare compared with the simple joint molecules.

The second type of joint molecule found in UV-irradiated samples was the 'dumbbell' structure, containing two circular SV40 DNA linked by a linear DNA (Fig. 7). The linear DNA connecting two SV40 DNA circles varied from 0.2 to 18 kb in length. When the interconnecting linear DNA was short it was sometimes observed as single-stranded. Molecules with one circular SV40 DNA linked to a replication intermediate through a linear DNA (Fig. 8), or two replicating DNA linked by a linear DNA (data not shown) were also observed. The structures of DNA shown in Figure 8, in which a mature SV40 circular DNA was connected to a replicating intermediate at one of the replication forks by a single-stranded DNA, suggested a single-stranded-DNA-invasion-initiated DNA replication. Dumbbell structures were also observed in viral DNA extracted from cells co-infected with SV40 and in1412 viruses (Fig. 4C and 4D, interconnecting DNA marked by arrows). The mechanisms for the formation of the dumbbell molecules will be discussed below (Fig. 9).

DISCUSSION

We have previously presented electron microscope evidence of Holliday structures in intracellular human adenovirus DNA from virus infected HeLa cells (2). This provided the first evidence for the presence of Holliday structures in animal cells. In the present work, the presence of Holliday structures in intracellular SV40 DNA is further demonstrated. Furthermore, joint molecules with characteristic Holliday junctions have also been found in CV-1 cell DNA after UV irradiation (Fig. 3), as well as in DNA extracted from mitotic S. cerevisiae (data not shown). These results indicate that Holliday-type structures are a common

![Image]

Fig. 8. Electron micrographs of SV40 DNA connected to a replication intermediate at one of the replication forks through a short single-stranded DNA. The circular mature SV40 DNA is seen linked to one of the replication forks (arrows) by a short single-stranded DNA (the longer single-stranded DNA in panel B is marked by a thick arrow). In panel A, the circular SV40 DNA is seen connected to a newly-initiated replication intermediate. This structure is consistent with the invasion-initiated DNA replication in the recipient molecule as depicted in pathway A in Figure 9. Extension of the interconnecting strand leads to a structure shown in Figure 7D. Completion of DNA replication in the recipient molecules leads to the dumbbell structure shown in Figure 7A-C.

![Diagram]

Fig. 9. Models for the generation of dumbbell structure. In scheme A, single-stranded tail of a rolling-circle replication would invade a SV40 DNA, generating a replication fork that moves unidirectionally. Structures consistent with such a mechanism are shown in Figure 8. Completion of the replication of the recipient molecule would result in the dumbbell structure seen in Figure 7. Alternatively, a nick at the Holliday junction creates a 3' end that could be used to initiate a rolling-circle replication. If the nick is created on the outer strand, the result is the formation of the dumbbell (pathway B). Replication initiated at the complementary nick on the inner strand (pathway C) would result in a theta structure with three unequal arms, the replication being terminated by the formation of a dimer and a monomer circle.
recombination intermediate in both prokaryotic and eukaryotic cells.

It has been well established that Holliday structures represent a recombination intermediate in bacteria (15, 17). The observation of joint molecules with one DNA molecule derived from wild type SV40 virus and one from viable SV40 mutant inl412 strongly suggests that these joint molecules are indeed recombination intermediates.

The present work also shows that UV irradiation induces formation of several types of complex joint molecules. The mechanisms by which UV does this are still not understood. Electron microscope examination of SV40 DNA extracted from UV-irradiated cells showed that 0.5% of the viral DNA contained single-stranded gaps (data not shown). The presence of single-stranded gaps in UV-irradiated samples was also shown by BND-cellulose chromatography. These single-stranded regions could potentially initiate recombination by strand invasion of the homologous sequence in another molecule. Indeed, several recombination models like the Meselson-Radding model have suggested such a mechanism of DNA recombination (see review in ref. 17). In E. coli, filling of single-stranded gaps after UV damage has been found to be closely associated with DNA recombination (13). DNA replication-coupled strand invasion has been demonstrated in human adenovirus type 5 DNA (3) as well as phage T4 DNA (18). The observation of DNA molecules with one SV40 DNA connected to one of the replication forks of SV40 replication intermediates through a short single stranded DNA (Fig. 8) would be consistent with a strand-invasion-initiated DNA recombination (Fig. 9A).

One of the novel recombination intermediates induced by UV irradiation contains two recombination intermediates joined at the newly replicated arms (Fig. 6). The two recombining replication intermediates are always at the same stage of replication. Since replication intermediates represent only a small portion of the intracellular SV40 DNA (19), it is highly unlikely that two replication intermediates at the same stage of DNA replication collide to form recombination intermediates. A possible explanation for the formation of such molecules might be provided by initiating DNA replication by reciprocal invasion of the two recombining molecules. Strand-invasion-induced DNA replication has been observed in T4 phage DNA (18). If DNA replication in each of the recombining molecules is induced by reciprocal strand invasion, the two replication events would proceed to approximately the same extent, and the two replication intermediates would be joined at the replication arms as observed in Figure 6. Further experiments would be needed to prove this hypothesis.

The other type of novel recombination intermediate observed in SV40 DNA extracted from UV-irradiated cells is the dumbbell structure. This structure has been observed recently by Jenab and Johnson (20) in the products of in vitro cell-free SV40 replication. It is also present in SV40 DNA extracted from cells treated with hydroxyurea (unpublished observation). Models to account for the formation of these molecules are depicted in Figure 9. A rolling dumbbell molecule could be generated by a strand-invasion mechanism (Fig. 9A). In this model, a rolling circle with a single-stranded tail invades a mature SV40 DNA to initiate a replication fork in the recipient molecule, much like recombination-initiated DNA replication in T4 phage (18). A dumbbell molecule is formed upon the completion of replication in the recipient molecule. Evidence in favor of this model is that rolling-circle-type replication was observed in SV40 DNA extracted from UV-irradiated and hydroxyurea-treated cells (data not shown). Also, molecules consistent with the strand-invasion-induced DNA replication intermediates shown in Figure 9A were indeed observed (e.g. Fig. 8).

The single-stranded tail of the rolling circle is 5' ended, so that DNA replication initiated by this invasion mechanism must proceed unidirectionally, and the bridging DNA between two DNA circles must always linked to one of the replication forks (as observed in Figures 7D and 8). Another prediction of this model is that the length of bridging DNA will always be equal to the difference in lengths between two branches of the recombining SV40 DNA after cleavage by a single cut restriction endonuclease. This was indeed observed experimentally (Figs. 4C and 4D).

Another possible mechanism for the generation of dumbbell structures is shown in Figure 9B. In this model, a nick at the outer strand of the Holliday junction creates a free 3' end which could be used to initiate a rolling-circle-type of replication, generating the dumbbell structure. If the nick is in the inner circle as shown in Figure 9C, initiation of replication would generate a theta structure with three unequal branches. These molecules would look like normal SV40 DNA replication intermediates except that two of the branches in a normal replication intermediate should always be of equal length. However, unlike its counterpart of rolling-circle replication (Fig. 9B), the replication intermediates shown in figure 9C would not accumulate. Completion of the replication would result in one circular dimer and one monomer. Although this model for the formation of dumbbell structures cannot presently be excluded, the observation of molecules shown in Figure 8 tends to favor the strand-invasion model described earlier (Fig. 9A).

In summary, the data presented here indicate that Holliday-type recombination junctions can be observed in SV40 and CV-1 DNA organized in nucleosome structures. Analysis of the structures of complex SV40 recombination intermediates by the electron microscope also suggests a strand-invasion-initiated recombination mechanism. The asymmetric transfer of DNA strands by such a mechanism, as shown in Figure 7 and 8, would generate non-reciprocal genetic exchanges. The present work suggests that asymmetric recombination may well be initiated by a rolling-circle-type mechanism.

ACKNOWLEDGEMENT

This article is dedicated to Paul Berg for the celebration of his 65th birthday. I thank him for giving me the opportunity to learn animal virology and biochemistry in his laboratory.

REFERENCES
