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Effects of 2'-Deoxy-2'-Azidocytidine on Polyoma Virus DNA Replication: Evidence for Rolling Circle-Type Mechanism

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Rolling circle-type molecules were found in polyoma virus-infected cells after inhibition of DNA synthesis with 2'-deoxy-2'-azidocytidine. The circular DNA molecules were always relaxed and of polyoma length. Most of the attached tails were less than two times the length of the polyoma genome, but tails with a length of up to 4.75 times the genome were also found. After cleavage of the total pool of replicating molecules with either endo R·*Eco*RI or endo R·*Bam*I, Y-shaped molecules with replicated portions of various lengths were generated from rolling circle-type molecules. Moreover, after cleavage, Y-shaped molecules with three unequal arms were found, which could be explained as derived from the tail in rolling circle-type molecules starting from the normal origin, i.e., 29% from the endo R·*Eco*RI cleavage site. Rolling circle-type molecules were also found during a normal, noninhibited infection cycle. In such cells, a relatively higher frequency of rolling circle-type molecules was observed late during infection. Compared with control cultures, cultures inhibited with 2'-deoxy-2'-azidocytidine showed a greater amount of rolling circle-type molecules relative to normal replicative intermediates. 2'-Deoxy-2'-azidocytidine has previously been shown to inhibit the initiation of new rounds of replication; thus, the result obtained here indicates that a rolling circle-type mechanism is independent of the reinitiation of DNA synthesis.

Polyoma virus is a small virus containing circular DNA with a molecular weight of 3.4×10^6 (4, 7). Infection of permissive cells leads to replication of the viral DNA molecule and subsequent production of new virus particles. The virus has been used as a model for DNA replication in eucaryotic cells, and the data obtained indicate similarities between the mechanism of replication of cellular DNA and that of viral DNA (2, 10, 12, 14, 15, 18). One such similarity is the process of bidirectional replication.

Polyoma DNA replication is initiated predominantly at a site that is situated 29% from the *Eco*RI cleavage site. From the origin, the two replication forks proceed in opposite directions (bidirectional synthesis), and termination of the replication cycle occurs at a site 180° from the origin of replication (2). These results are similar to those obtained with simian virus 40 (5) and were mainly obtained with restriction enzymes and electron microscopy. However, in studies with these techniques, it was also suggested that, in about 10% of the pool of replicating molecules, replication starts from an alternative origin near the *Eco*RI cleavage site and proceeds in only one direction (unidirectional synthesis) (16). Alternatively, these data could be reconciled in terms of the "rolling circle" model for DNA replication (6).

It has earlier been shown with polyoma virus-infected cells that the nucleoside analog 2'-deoxy-2'-azidocytidine (Cz) is an inhibitor of the initiation event (1, 17). In this study, advantage was taken of the observation that the main effect of the inhibition is a markedly decreased pool of replicating intermediates (RI). Because of this effect, a relatively higher frequency of circular polyoma DNA molecules with tails (rolling circle-type molecules) could be observed among the replicating molecules (1).

In the present paper, data will be presented that indicate that polyoma DNA also replicates according to a rolling circle-type mechanism. This replication process occurs both in Cz-inhibited and in normal, noninhibited polyoma-infected cells and starts from the normal origin, i.e., 29% from the *Eco*RI cleavage site.

MATERIALS AND METHODS

Preparative procedures. Mouse 3T6 cells were infected with plaque-purified polyoma virus as described previously (18), and, at different times after infection, the viral DNA was selectively extracted with a solution of 0.25% Triton X-100 (Packard), 25 mM Tris-hydrochloride (pH 7.6), 10 mM EDTA, and 1 M

NaCl (modification of procedure described by Hirt [9]). After precipitation of the cellular DNA (SW27 rotor [Beckman]; 25,000 rpm for 45 min at 4°C), the supernatant was collected and treated with Pronase followed by phenol-$CHCl_3$. After precipitation with ethanol, the polyoma DNA was partially purified on neutral sucrose gradients. A sample of this material was chromatographed on benzoylated-naphthoylated DEAE (BND)-cellulose (18) to enrich for replicating molecules. When added, Cz was used at a concentration of 2 mM in the culture medium. Cz was a gift from J. Hobbs and F. Eckstein, Max-Planck Institut für Experimentelle Medizin, Göttingen. Polyoma DNA was pulse-labeled with [^{3}H]thymidine (NEN Chemicals GmbH; 6.7 Ci/mmol) at a final medium concentration of 1.5 μM for 1 h.

Enzymes. Restriction endonucleases, endo R·*Bam*I and endo R·*Eco*RI were gifts from G. Magnusson, Karolinska Institute, Stockholm. Incubations were performed for 60 min at 37°C in a 50 mM Tris-hydrochloride (pH 7.6) buffer containing 10 mM $MgCl_2$ (endo R·*Eco*RI) or in 6 mM Tris-hydrochloride (pH 7.6)–6 mM $MgCl_2$–6 mM mercaptoethanol (endo R·*Bam* I). Reactions were stopped by the addition of EDTA.

Electron microscopy. DNA was visualized by the modified Kleinschmidt technique as described by Davis et al. (3). When stated, ethidium bromide was included in the spreading mixture to untwist supercoiled form I and RI DNA. Pictures were taken in a JEOL 100 S electron microscope, and length measurements were performed by optically projecting molecules onto a Hewlett-Packard digitizer with a partially smoothed length calculation program. Standard deviation was better than 1%.

RESULTS AND DISCUSSION

Rolling circle-type replicative intermediates in Cz-inhibited, infected mouse cells. Mouse 3T6 cells were infected with polyoma virus, and cell cultures were divided into two sets. One culture received Cz 22 h postinfection (p.i.), and the parallel culture served as control. At 25 h p.i., the cell cultures were pulse-labeled for 1 h with [^{3}H]thymidine, and, at 26 h p.i., viral DNA was selectively extracted and purified. Radioactivity measurements revealed that the Cz-inhibited cultures only incorporated about 10% of the label incorporated by the controls. The DNA was further purified on neutral sucrose gradients, and material sedimenting in a peak between about 20 to 30S was pooled, dialyzed, and mounted for analysis by electron microscopy with the formamid modification of the Kleinschmidt technique as described by Davis et al. (3). Ethidium bromide was included to untwist supercoiled form I and RI molecules.

In Cz-inhibited cultures, the following molecules could be seen: (i) circular polyoma DNA molecules, (ii) circular DNA molecules with a replication eye (RI), (iii) circular molecules with a tail (rcRI), (iv) linear DNA of various lengths, and (v) mitochondrial DNA. Of the total pool of viral DNA, 0.5 ± 0.2% was in the form of RI, and 0.2 ± 0.2% was in the form of rcRI. Compared with control cultures in which the pool of RI represented 4.5 ± 0.2%, a clear reduction of the pool of RI was again observed in Cz-inhibited cells (1). (A further analysis of replicating molecules in control cells will be presented below.) To enrich for replicating molecules, the material from Cz-inhibited cells was chromatographed on BND-cellulose (88% recovery), and material eluted with caffeine was collected and mounted for electron microscopy analysis. (Ethidium bromide was now omitted because the circular part of the rolling circle complex was not supercoiled.) In the DNA sample from Cz-inhibited cells, 77 molecules of the rolling circle type (Fig. 1) were analyzed according to the size of the circle and tail. The mean length of the circle was 1.71 μm ± 3% and thus represents the length of polyoma DNA circles. In Fig. 2 the relative number of tails are grouped into size classes according to the extent of their replication. About 80% of the molecules had a tail that was less than 1.4 times the length of the genome, but molecules with a tail up to 4.75 times the polyoma length were also detected (Fig. 2). This distribution of tails could be an effect either of the purification procedure (if the most-replicated molecules were discarded in the Hirt precipitate or after the neutral sucrose gradient centrifugation) or of the possible loss of a full copy by excision soon after its synthesis.

rcRI are also present in normal, noninhibited, infected cells. In the experiment described above, control cultures were also pulse-labeled for 1 h with [^{3}H]thymidine at 18, 25, and 32 h p.i. The pool of viral DNA in control cultures was purified and analyzed at different times after infection, i.e., 19, 26, (parallel to inhibited cells), and 33 h p.i., in the same way as described for Cz-inhibited cells. Analysis of the total pool of viral DNA by electron microscopy revealed that at 19 h p.i., 7.2 ± 0.2% were in the form of normal RI, and no rcRI were observed. At 26 h the corresponding figures were 4.5 ± 0.2% and 0.1 ± 0.05% for RI and rcRI, respectively. At 33 h p.i., the pool of normal RI corresponded to 1.6 ± 0.2% of total viral DNA, and 0.09 ± 0.05% of the molecules were in the form of rcRI. It is thus evident that the RI/form I ratio decreases with time from about 7% (19 h) to 2% (33 h) of the total pool. However, as a percentage of all replicating molecules, the ratio of rcRI to RI increases with time.

The tails were again of different lengths, ranging from 18 to 172% of the genome length (data

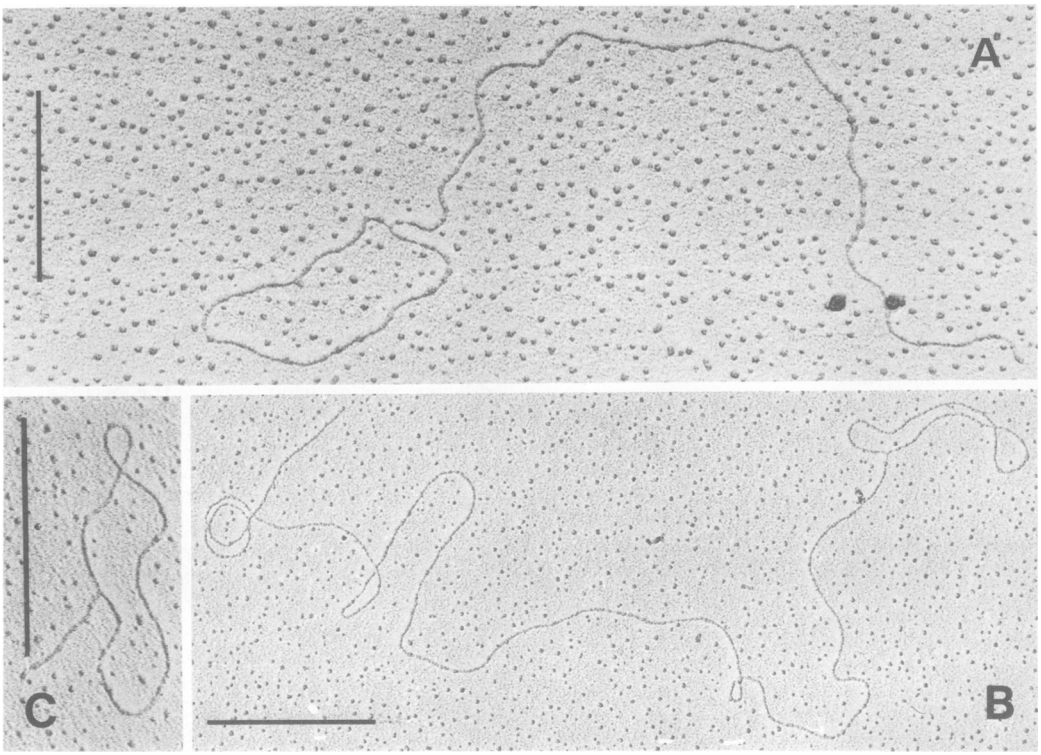

FIG. 1. *Electron micrographs of polyoma DNA replicating via a rolling circle mechanism. The molecule shown in (A) was found in material isolated from control cultures; those in (B) and (C) were isolated from Cz-inhibited cultures. Ethidium bromide was not included in the spreading mixture; thus, the micrographs show that the circular polyoma molecules replicating like a rolling circle lack supercoils. Bar, 0.5 μm.*

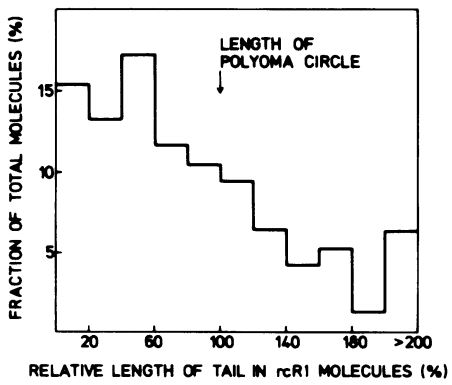

FIG. 2. *Histogram of the distribution of tail lengths in rolling circle-type molecules. Polyoma DNA was isolated from Cz-inhibited cultures and chromatographed on BND cellulose before analysis by electron microscopy. The length of a tail was correlated with the peripheral length of the corresponding polyoma circle, and the total length of a circle is equal to 100%. The number of molecules measured was 77; the mean length of the circles was 1.71 μm (standard deviation, 3%). Length measurements were performed as described in the text.*

not shown). An example of one such molecule is presented in Fig. 1A.

rcRI give rise to Y-shaped molecules by restriction enzyme digest. In Fig. 3, the different hypothetical *Eco*RI cleavage products from rcRI are depicted. Assuming that replication starts at the normal origin, going "towards" the *Eco*RI site, molecules in which the fork has not passed the *Eco*RI site will produce a Y-shaped molecule with three unequal arms (Fig. 3, line A). If the fork passes the *Eco*RI site, Y-shaped molecules with two equal arms will arise (Fig. 3, lines B and C). If replication starts in the other direction, Y-shaped molecules will arise. One type (Fig. 3, line D) will have three unequal arms; when the fork has passed the *Eco*RI site, two arms will be equal (Fig. 3, line E). If replication continues and passes the *Eco*RI site, such molecules will, of course, only give rise to Y-shaped molecules having two arms alike. Moreover, when polyoma DNA has replicated about 15% towards the *Eco*RI site or 35% in the other direction, such molecules will also be scored as molecules with two equal arms. Fig. 3 (line F) also demonstrates the result of cleaving a bidi-

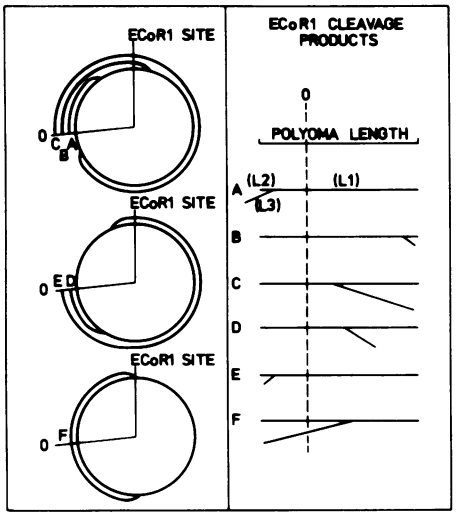

FIG. 3. *Different hypothetical EcoRI digestion products originating from rolling circle-type molecules. Replication is supposed to start in either direction from the origin (O). In (A), (B), and (C), replication proceeds "towards" the EcoRI site. In (A), the replication fork has not passed the cleavage site, and a Y-shaped molecule with three unequal arms will arise. As soon as the fork has passed the EcoRI site, as in (B) and (C), Y-shaped molecules with two arms of similar length will be generated. (D) and (E) represent replication going in the "other direction," and cleavage of such molecules will give rise either to Y-shaped molecules with three arms of unequal length (D) or to molecules with two arms of equal length (E). F demonstrates the result of cleavage of bidirectionally replicating molecules in which one fork, is close to the EcoRI site. Symbols (for Y-shaped molecule with three arms of unequal length): L1, the longer arm of the parental strand; L2, shorter arm of the parental strand; L3, "tail" arm. See Fig. 6 for analysis of arm lengths.*

rectionally replicating RI with one arm close to the cleavage site (58% replicated) and shows that Y-shaped molecules that are about 60% replicated will arise.

The total pool of replicating molecules (after BND-cellulose chromatography) from both Cz-inhibited and control cultures was treated with *Eco*RI. Analysis of the molecules by electron microscopy showed that treatment with *Eco*RI generated the following molecules: (i) linear molecules with polyoma length, (ii) linear molecules with a replication eye and double-Y molecules (resulting from normal bidirectional replication), (iii) Y-shaped molecules (Fig. 4),

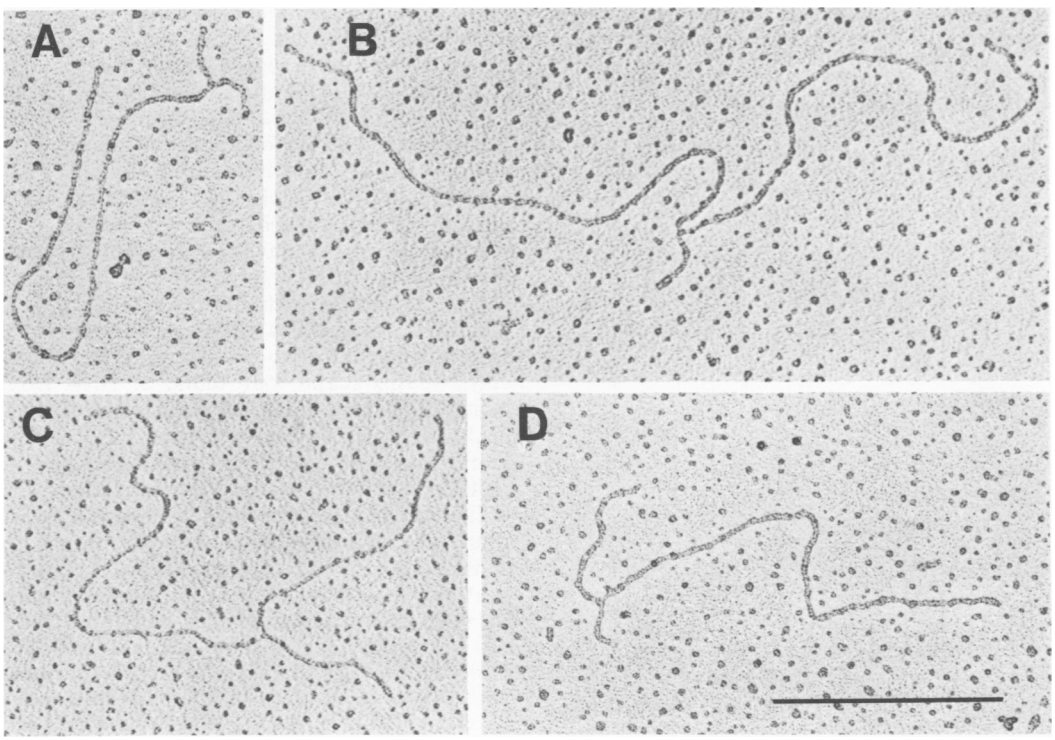

FIG. 4. *Electron micrographs of Y-shaped molecules resulting from EcoRI digestion of replicating molecules. The molecules shown were isolated from Cz-inhibited (A–C) and control (D) cultures. In all cases, the sum of one short and one long arm corresponds to the polyoma length. Bar, 0.5 μm.*

and (iv) other linear molecules. The Y-shaped molecules were of two kinds, either molecules with two of the arms of equal length (Fig. 4A and B) or molecules with three unequal arms (Fig. 4C and D). In both cases the sum of two arms is equal to the polyoma length. The latter molecules (unequal arms) constituted about 5 to 15% of the total number of Y-shaped molecules; the analysis of these molecules will be given below.

In Fig. 5, the proportions of Y-shaped molecules with two arms of equal length from Cz-inhibited and control cultures are divided into 10 classes, i.e., from 1 to 10% through 91 to 99% replicated (the extent of replication is calculated by dividing the mean of the two arms of similar length by the total length). In both samples, molecules were found at all stages of replication, with a peak at about 60% replicated. However, the proportion of this material was much smaller in Cz-inhibited cells (Fig. 5A) than in the controls (Fig. 5B). The explanation for this finding is that Y-shaped molecules that are about 60% replicated arise from normal bidirectionally replicating molecules (Fig. 3). Thus, the result of this experiment again indicates that there are relatively far fewer normal RI in Cz-inhibited infected cells than in the controls.

As mentioned above, it has previously been proposed that these Y-shaped molecules originate from molecules in which DNA synthesis is initiated at another origin close to the *Eco*RI site and goes in one direction (16). To further characterize viral DNA from Cz-inhibited and control cultures, portions of the pools of replicating molecules were treated with the restriction enzyme *Bam* I. This enzyme cuts at a site about 10% from the origin in the other direction from *Eco*RI (8). Thus, if another origin exists, linear molecules with a replication eye starting at about 40% (10% *Bam* I + 29% *Eco*RI) should be found. No such molecules were found in either sample. On the other hand, Y-shaped, polyoma-length molecules at different stages of replication and molecules arising from the cleavage of normal bidirectionally replicating molecules (linear molecules with a replication eye at about 10% from one of the ends and double-Y molecules) were found (data not shown). After *Hpa* II cleavage of simian virus 40 RI (16), molecules were found that could be explained by unidirectional synthesis from an alternative origin near the *Eco*RI site. The different results obtained here can be explained either by the fact that the data presented by Robberson et al. (16) do not distinguish between unidirectional synthesis and asymmetric bidirectional synthesis from a normal origin (5) or by the fact that the virus studied here does not contain defective molecules with an alternative origin. Y-shaped molecules have been reported after *Eco*RI digest of both polyoma (16) and simian virus 40 (5, 16) DNA molecules. Those data, together with the results from the restriction enzyme digests presented here, are thus interpreted to be a result of polyoma DNA molecules replicating via a rolling circle-type mechanism.

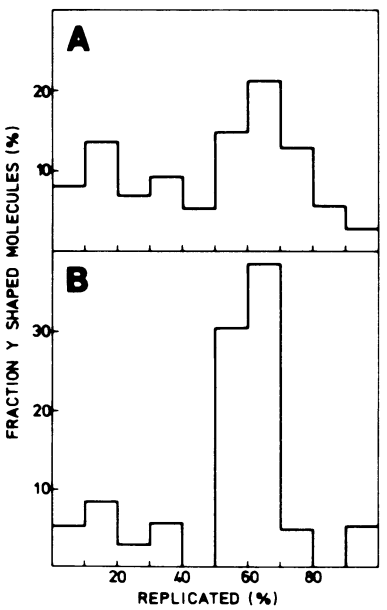

FIG. 5. *Histogram of Y-shaped molecules having two arms of equal length arising from EcoRI digestion of replicating molecules. (A) Results from Cz-inhibited cells; (B) those from controls. Length measurements were performed as described in the text.*

Rolling circle-type replication starts from the normal origin. To analyze the Y-shaped molecules with three arms of unequal length, the molecules from Cz-inhibited cells were divided into two classes (only two molecules of this shape were found in DNA from control cultures). Class A was composed of molecules in which the longest parental strand is more than 70%, and class B was composed of molecules with a parental strand less than 70% of the total length. When the "tail" arm was less than 30% replicated, class A was supposed to contain cleaved rcRI in which the direction of synthesis was towards the *Eco*RI site (cf. Fig. 3). Class B contained molecules generated from rcRI in which synthesis was in the opposite direction (cf. Fig. 3). Class A molecules corresponded to about 75% of the total molecules found and were analyzed in the following way: L1 represented the longer arm, and L2 represented the shorter arm of the parental strand. L3 was the third, "tail" arm. All lengths were

normalized to the total length (L1 + L2). In Fig. 6, the relative length of L3 is plotted versus L1. If L3 replicated unidirectionally, production of an L3 corresponding to 20% would cause a 20% increase in L1. If L3 were the cause of bidirectional replication (caused by a breakage of one parental strand at the fork), a length of L3 of 20% would only correspond to an increase in L1 of 10%. The molecules analyzed followed unidirectional replication (slope, 1.06; correlation coefficient, 0.95) and extrapolated towards the normal origin (L3 = 0 and L1 = 70.2%). The class B molecules (only five found) also followed unidirectional synthesis (data not shown). Moreover, the two Y-shaped molecules with three unequal arms found in DNA from control cultures (one shown in Fig. 4D) could also be explained by a rolling circle-type mechanism.

Cz has previously been shown to interfere mainly with the initiation event and less with the elongation process, thus giving rise to a decrease in the pool of RI (1). However, since the rolling circle mechanism does not require reinitiation, this process would be only marginally affected by Cz, and would thus lead to an increase of rcRI relative to normal RI.

The 10-fold increase of rcRI relative to normal RI in Cz-inhibited cells made this study possible, because at maximum virus replication no more than 2% of the total amount of RI in control cultures are rcRI. However, it is not known how many rcRI might be lost during isolation and purification procedures (e.g., that of Hirt [9]).

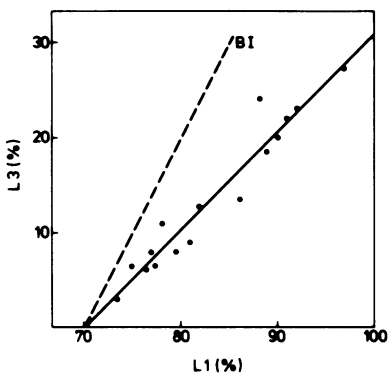

FIG. 6. *Relative length of arm L3 versus the relative length of arm L1 in a Y-shaped molecule with three unequal arms. Of 22 such molecules found in Cz-inhibited cultures, 17 had a longest parental arm (L1) that was more than 70% of the total length. Of these, 16 molecules had a "tail" arm (L3) that was shorter than 30%, of the total length, and those molecules are depicted here. (The other molecule, depicted in Fig. 4C, is explained by the rolling circle mechanism proceeding in the opposite direction; compare Fig. 3, line D). The theoretical line for bidirectional replication (BI; slope = 2) is included. The 16 molecules analyzed formed a regression line with a slope of 1.06 and a correlation coefficient of 0.95 (slope of theoretical line for unidirectional synthesis = 1).*

The occurrence of this replication process also tells us something about the structure and function of the polyoma DNA molecule. First, replication can probably start in different directions from the normal origin (producing either rcRI or RI); second, the origin probably also serves as an "excision site," since the progeny DNA circle seems to be cut out from origin to origin. Otherwise, other Y-shaped molecules would occur after *Eco*RI cleavage. The observation that the tails in rcRI are synthesized in both directions from the origin can be explained in two ways. Either both parental strands can be nicked at the origin, or rolling circle-type molecules are the result of molecules starting out on a bidirectional course with an immediate breakage of one strand at the fork. However, since most (75%) molecules synthesize in direction towards the *Eco*RI site, this is interpreted to mean that most rcRI are started by a nick in the L strand (11) at the origin. Moreover, in line with the suggestion by Lai and Nathans (13), the normal termination site does not seem to have a structure that forces a fork to stop.

The main observation in this report is that polyoma DNA also replicates via a rolling circle-type mechanism that can be found in both Cz-inhibited and control cultures. How general this process is among eucaryotic viruses (rcRI-like molecules were found also during the simian virus 40 infection cycle [5]), how much of total mature viral DNA is produced via rcRI, and whether virus-coded polypeptides are necessary for this replication process, constitute some challenging questions for future research.

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