Gene Replication in the Presence of Aphidicolin†

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ABSTRACT: DNA replication in the nucleus of eukaryotic cells is restricted to the S phase of the cell cycle, and different genes are duplicated at specific times, according to a well-defined temporal order. We have investigated whether activation of initiation sites, in proximity to genes that are replicated in different portions of the S phase, could be detected when synchronized 10T1/2 cells were maintained in aphidicolin (APC), an inhibitor of DNA polymerases α and δ. Cells released from confluence arrest into medium containing 2 μg/mL APC progressed into the S phase, and nascent DNA accumulated during incubations of 24 and 32 h. Exposure to APC for 40 or 48 h resulted in growth of the radiolabeled DNA into larger molecules. Replicating DNA was isolated in CsCl gradients and probed with ^{32}P-labeled gene probes for early-replicating genes (e.g., Ha-*ras*, *mos*, and *myc*) and a late-replicating gene (V_H Ig). DNA replicated during the 24-h incubation in APC was enriched in Ha-*ras* gene sequences. The V_H Ig gene did not replicate in cells incubated for as long as 56 h with APC. The *myc* and the *mos* genes were detected after 32 and 40 h in APC, respectively. The *myc* gene is replicated in 10T1/2 cells after Ha-*ras* but before *mos*. Therefore, the order of activation of these genes was conserved in the presence of APC. The delay in replication of *myc* and *mos* correlated well with the slowing of DNA replication by APC.

DNA replication in eukaryotes is a complex process and requires many different enzymatic activities. Among them, DNA polymerase α with its associated primase activity appears to be responsible for the bidirectional polymerization of DNA at replication origins and for the synthesis of Okazaki fragments in the lagging strand. Continuous DNA synthesis in the leading strand is proposed to be carried out by DNA polymerase δ (Prelich & Stillman, 1988; Tsurimoto & Stillman, 1989). Direct evidence for the simultaneous participation of these two DNA polymerases in replication complexes has been obtained in studies of SV40 DNA replication in vitro (Weinberg & Kelly, 1989; Tsurimoto et al., 1990). DNA polymerases α and δ are sensitive to inhibition by aphidicolin (APC),[1] and the treatment of cells with this drug reduces dramatically the level of DNA synthesis in the nucleus (Pedrali-Noy et al., 1980; Spadari et al., 1982, 1984). The predominant effect of APC is on DNA chain elongation (Cordeiro-Stone & Kaufman, 1985; Decker et al., 1986; Nethanel et al., 1988).

In this report, we describe experiments which were designed to measure initiation of gene replication in synchronized C3H 10T1/2 cells incubated in the presence of APC. Initiation of DNA synthesis at replicon origins occurs in a defined temporal order during the S phase [see review by Goldman et al. (1984)], and it must be under control mechanisms which assure that each sequence is replicated only once during each cell cycle. We have previously described the timing of replication of several genes in 10T1/2 cells (Doggett et al., 1988). Protooncogenes such as c-Ha-*ras*, c-*myc*, and c-*mos* are early-replicating genes, while other sequences such as those coding for the variable region of immunoglobulin heavy chains are late-replicating. We determined the relative recovery of early- and late-replicating sequences in DNA synthesized during continuous exposure of 10T1/2 cells to APC. We asked whether DNA replication could be initiated in chromosomal domains containing late-replicating genes when DNA strand growth was inhibited by APC.

Experimental Procedures

Cells and Culture Conditions. Stock cultures of C3H 10T1/2 clone 8 mouse embryo fibroblasts (passages 11–15) were grown in Eagle's basal medium containing 10% heat-inactivated fetal bovine serum (Hyclone, Grand Island, NY). Cell cultures were kept at subconfluent densities in a humidified atmosphere of 5% CO_2–95% air at 37 °C.

Cell Synchronization and Treatment of Cells with Aphidicolin. Cellular DNA was uniformly labeled by incubating cells with 5 or 10 nCi/mL [^{14}C]thymidine for 3 days. Synchronization was achieved by growing cells to confluence, thereby arresting cell division, followed by replating at a lower density (Grisham et al., 1980). In the experiments described in this report, cells were released from confluence arrest into medium containing 2 μg/mL APC. Under these conditions, cells enter the S phase and synthesize DNA, albeit at a slow rate (Cordeiro-Stone & Kaufman, 1985). Cell cultures were incubated for 24, 32, 40, 48, and 56 h in the presence of 2 μg/mL APC, 10 μM bromodeoxyuridine (BrdUrd), 10 μM bromodeoxycytidine (BrdCyt), 1 μM trifluorothymidine (TFT), and 5 μCi/mL [^{3}H]thymidine. In parallel plates, cells were seeded at the same density with the same concentrations of BrdUrd, BrdCyt, TFT, and [^{3}H]thymidine but in the absence of APC and incubated for 24, 32, and 48 h at 37 °C.

Velocity Sedimentation Analyses. The size distribution of DNA synthesized in the presence or absence of APC was

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[1] Abbreviations: APC, aphidicolin; BrdUrd, bromodeoxyuridine; BrdCyt, bromodeoxycytidine; TFT, trifluorothymidine; HBSS, Hanks' buffered saline solution; TE, 10 mM Tris/1 mM EDTA, pH 7.5; HL-DNA, DNA replicated in the presence of brominated precursors and displaying a hybrid density in CsCl gradients; LL-DNA, normal density DNA which did not replicate during the incubation of cell populations with brominated precursors of DNA synthesis.

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analyzed in alkaline sucrose gradients (Cordeiro-Stone & Kaufman, 1985). Briefly, cell cultures were washed 2 times with ice-cold Hanks' buffered saline solution (HBSS) and scraped from the plate into a total of 1 mL of 0.1 M NaCl/0.01 M EDTA, pH 8. Cells that were incubated in the presence or absence of APC for less than 48 h were stored on ice for 8–24 h before they were added to the sucrose gradients. We observed that the size distributions for DNA molecules labeled with [^{3}H]- or [^{14}C]thymidine were not affected by the incubation of intact cells in the ice-cold saline. Immediately before the samples were applied to the gradients, they were passed 5 times through a 22-gauge needle to dissociate clumps of cells. An aliquot of 0.5 mL of the cell suspension was added to 0.5 mL of 1 M NaOH/0.02 M EDTA which had already been added onto a 5–20% sucrose gradient containing 0.01 M EDTA, 2 M NaCl, and 0.4 M NaOH. Cell lysis and DNA unwinding proceeded for 1 h at room temperature under fluorescent lighting. These gradients were centrifuged at 25000 rpm for 5 h at 20 °C in a Beckman SW28 rotor. Equal-volume fractions were collected from the bottom of the tube, and acid-insoluble radioactivity was determined by liquid scintillation counting. In these double-labeling experiments, a correction was made for the spillover of ^{14}C radioactivity into the ^{3}H channel. The net ^{3}H radioactivity per fraction was also normalized to the number of cells added to the gradients, as measured by the total ^{14}C radioactivity recovered in all fractions (Kaufmann & Cleaver, 1981).

Isolation of Replicating DNA. The protocol described by Doggett et al. (1988) was used with minor modfications. Cells were washed 2 times with ice-cold HBSS, lysed in 100 mM NaCl/10 mM EDTA/0.5% sarkosyl/0.2 mg/mL proteinase K, and incubated for at least 2 h at 37 °C. Cell lysates were transferred to dialysis bags and were concentrated on a bed of sucrose at 4 °C for 2–4 h. Samples were then extensively dialyzed against 10 mM Tris/1 mM EDTA, pH 7.5 (TE). The NaCl concentration was increased to 0.5–1 M, and the samples were sheared 10 times through a 22-gauge needle. The DNA was again dialyzed against TE for 2–3 h at 4 °C and adjusted to 29.77 g with TE before adding to 38.64 g of CsCl.

Isopycnic centrifugation at 38000 rpm in a Beckman VTi50 vertical rotor separated the replicating (density-labeled) from the parental DNA. The gradient fractions containing ^{3}H-labeled, hybrid-density (HL) DNA and those containing the normal-density (LL) DNA, labeled only with [^{14}C]thymidine, were separately pooled and dialyzed against TE to eliminate the CsCl. After the volume was reduced under vacuum in a speedvac (Savant Instruments), the samples were extracted once with a 1:1 (v/v) mixture of phenol and chloroform/isoamyl alcohol (24:1) and twice with chloroform/isoamyl alcohol (24:1), and the DNA was precipitated with ethanol. After resuspending the pellet in 2 mL of TE, the radioactivity in HL- and LL-DNA was determined by liquid scintillation counting.

Hybridization of Replicating DNA with Gene Probes. Equal amounts (based on the ^{14}C radioactivity) of the purified HL- and LL-DNA were adsorbed onto nitrocellulose in a slot-blot apparatus, and ^{14}C autoradiographs were obtained as described previously (Doggett et al., 1988). Gene probes, hybridization conditions, and data analysis have also been described by Doggett et al. (1988).

Results

Growth of Nascent DNA in the Presence of APC. C3H 10T1/2 cell cultures were released from proliferation arrest at confluence by replating at lower density. We have reported that under these conditions, S-phase cells appeared in the population at 15–17 h after replating from confluence and reached their maximum at 24 h (Grisham et al., 1980; Cordeiro-Stone & Kaufman, 1985). When APC was added to the medium at 2 μg/mL, the cycling cells (91 ± 2% of the population) entered the S phase at about the same time as those in cultures free of inhibitor, but the rate of DNA chain growth was decreased by 93% (Cordeiro-Stone & Kaufman, 1985). We have also observed that during incubations of 24–31 h in APC, single-stranded DNA molecules of approximately 30 kb appeared to accumulate in the nucleus but elongation to larger molecules was not evident (Cordeiro-Stone & Kaufman, 1985). In Figure 1, we depicted the distribution in alkaline sucrose gradients of DNA from cells labeled during continuous incubation with [^{3}H]thymidine for 24, 32, 40, or 48 h in the presence of APC. Note that the net ^{3}H radioactivity per fraction was normalized for the number of cells added in each gradient (^{14}C radioactivity) in order to display the relative levels of DNA synthesis during these incubations. The bulk of the ^{3}H incorporation after 24 h stayed in the top fractions of the gradient, and it is predominantly the result of mitochondrial DNA synthesis (Cordeiro-Stone & Kaufman, 1985). When the same results are graphed by using the percent distribution of the total radioactivity recovered from each gradient, one can discern a small shoulder in the 24-h profile at the same position as the DNA peak from the 32-h sample [graph not shown here; for similar results, see Figure 4 in Cordeiro-Stone and Kaufman (1985)]. After 40 h, the distribution of DNA molecules synthesized in the presence of APC shifted slightly toward higher molecular weights, indicating that DNA strand growth had occurred. DNA strand growth was most evident by 48 h (Figure 1).

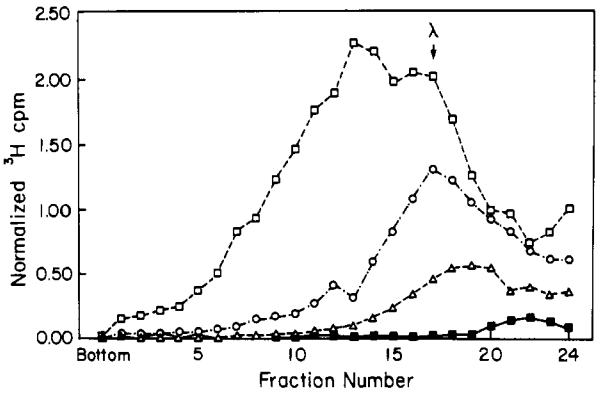

FIGURE 1: Size distribution of DNA replicated in the presence of APC. Confluent C3H 10T1/2 cells were replated in the presence of 2 μg/mL APC and 5 μCi/mL [^{3}H]thymidine for 24 (■), 32 (△), 40 (○), or 48 h (□). The size distribution of nascent DNA synthesized during these incubations was determined in alkaline sucrose gradients, as described under Experimental Procedures. Sedimentation was from right to left. The arrow indicates the peak fraction for the sedimentation of full-length λ DNA labeled with [^{14}C]thymidine and centrifuged under identical conditions.

The level of DNA synthesis in cells incubated in APC, as measured by the specific activity of incorporation of [^{3}H]-thymidine in DNA (^{3}H/^{14}C), increased exponentially with time. It changed from about 3 ± 1% (24 h) to 4 ± 1% (32 h) and 12 ± 3% (48 h) of the DNA synthesis observed in control cells labeled in the absence of APC. This increase in residual DNA synthesis appears to be the result of an increase in the number of cells in S phase (from 70% to 90%) between 24 and 32 h (Cordeiro-Stone & Kaufman, 1985) and an increase in DNA synthesis per S-phase cell, especially between 32 and 48 h in the presence of APC (Figure 1).

The inhibition of replication by APC was readily reversible. DNA molecules synthesized during exposure to APC for 32

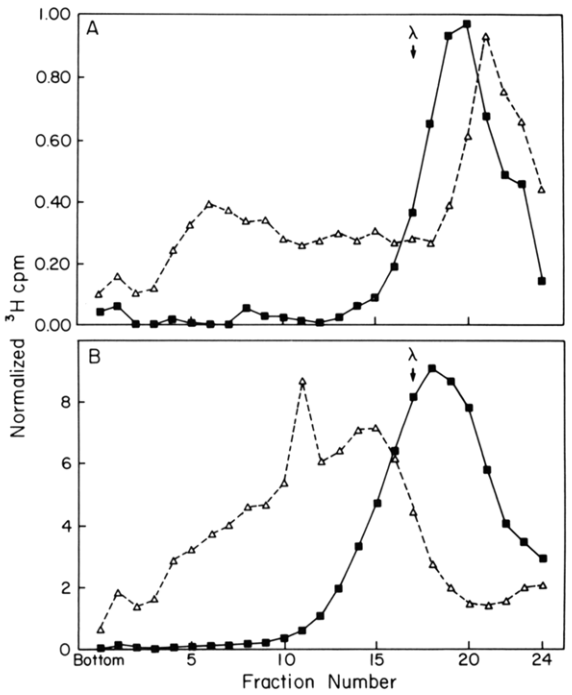

FIGURE 2: DNA strand growth in the presence of APC. Confluent C3H 10T1/2 cells were replated in the presence of 2 μg/mL APC. Cells were pulse-labeled with [^{3}H]thymidine at (A) 23–24 or (B) 39–40 h in the presence of APC. Cells were harvested immediately after the pulse, or incubated further for (A) 24 or (B) 8 h in the presence of APC, but without [^{3}H]thymidine. The size distribution of nascent DNA synthesized during the 1-h pulse (■—■) or after the chase in the presence of APC (Δ---Δ) was then determined as described under Experimental Procedures and in the legend of Figure 1.

or 40 h could be chased to higher average molecular weights once APC was removed (results not shown). In addition, the data in Figure 2 demonstrate that DNA molecules pulse-labeled at 23–24 h or 39–40 h could be chased to higher molecular weights in the continuous presence of APC. After a 24- or 8-h chase, respectively, the size distributions of the pulse-labeled DNA synthesized in the nucleus were similar to those observed during a 48-h incubation in APC (Figure 1). Mitochondrial DNA synthesis is not inhibited by aphidicolin (Zimmerman et al., 1980), and no significant changes were expected in the sedimentation of the labeled mitochondrial DNA upon chase of the radiolabeled precursor. Accordingly, a significant fraction of the total [^{3}H]thymidine incorporation during the 23–24-h pulse remained at the top of the gradient after the 24-h chase (Figure 2A). Because of the increased DNA synthesis activity in the nucleus at the time of the 39–40-h pulse, the contribution of [^{3}H]thymidine incorporation from mitochondrial DNA synthesis is relatively insignificant and less visible in the distribution profile shown in Figure 2B. We have also determined that the relative colony-forming efficiency of cells incubated in the presence of APC was 104, 78, 59, and 42% of control after 24, 32, 40, and 48 h in APC, respectively. Evidently, the colony-forming efficiency in APC was inversely proportional to the length of time that cells remained arrested in the S phase (Tyrrell, 1983).

Replication of Genes in the Presence of APC. The apparent accumulation of replicon-sized intermediates of DNA replication between 24 and 32 h of incubation in the presence of APC (Figure 1) could represent the uninhibited initiation of new replicons, in spite of the slower rate of DNA strand growth (Cordeiro-Stone & Kaufman, 1985). Therefore, we became interested in whether banks of replicons, which were programmed to replicate during mid- or late-S phase, were activated in the presence of aphidicolin. We knew from previous studies (Doggett et al., 1988) that the replication of the Ha-*ras* gene in 10T1/2 cells occurs at the onset of the S phase; it is therefore contained in replicons which are activated very early. The replication of *myc* and *mos* genes has been detected during the first and second hours of the S phase, respectively. The V_H Ig gene, however, is late-replicating in these same cells. We used these molecular probes to determine the temporal order and the kinetics of replication of genes in the presence of APC.

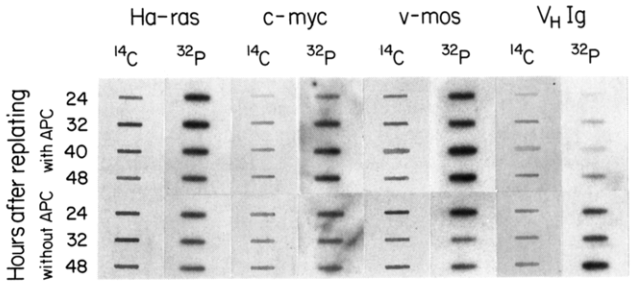

FIGURE 3: Replication of genes in the presence of APC. Replicated DNA (HL-DNA) was isolated by isopycnic CsCl gradient centrifugation, pooled, purified, and adsorbed onto nitrocellulose filters as described under Experimental Procedures. After the ^{14}C autoradiograph was obtained, filters were hybridized with ^{32}P-labeled gene probes. The ^{32}P signal was obtained by autoradiography after shielding the X-ray film from the ^{14}C irradiation with two sheets of paper.

Table I: Relative Enrichment of Gene Sequences in Replicating DNA[a]

h after release from confluence arrest	normalized ^{32}P/^{14}C			
	Ha-*ras*	c-*myc*	c-*mos*	V_H Ig
	(A) In the Presence of APC			
24	4.8	2.6	2.7	0.14
32	2.4	2.9	1.9	0.13
40	2.5	2.2	4.2	0.10
48	2.4	1.9	3.8	0.25
	(B) In the Absence of APC			
24	1.4	1.3	1.4	0.43
32	1.0	0.8	0.8	0.61
48	1.0	1.0	1.0	1.0

[a] The ratio between ^{32}P and ^{14}C peak areas was determined from densitometric scans of ^{32}P and ^{14}C autoradiographs for each slot blot shown in Figure 3. These ratios were then normalized to yield 1.0 for the ^{32}P/^{14}C ratio observed in the replicating DNA from cells incubated for 48 h in the absence of APC. See text for details.

Replicating DNA was purified, adsorbed onto nitrocellulose, and hybridized with ^{32}P-labeled gene probes (Figure 3). The ^{32}P signal for a particular gene in each slot blot was quantified by densitometry (Table I) and then normalized to the total amount of DNA in the slot (i.e., ^{14}C signal). We observed in control cell cultures incubated with brominated DNA precursors, but in the absence of APC, that all of the actively replicating cells had replicated completely their nuclear DNA between 32 and 48 h after replating from confluence arrest. Virtually all of the DNA prelabeled with [^{14}C]thymidine was associated with DNA molecules containing BrdUrd and [^{3}H]thymidine and banded in the CsCl gradients at the density expected for hybrid DNA molecules (results not shown). Therefore, the hybridization signal detected for any gene in replicating DNA purified from cells incubated for 48 h in the absence of APC represents the expected level of hybridization in total nuclear DNA. The ^{32}P/^{14}C values obtained for the hybridization of a given gene with the different DNA samples used (Figure 3) were normalized to the ^{32}P/^{14}C value observed

in total nuclear DNA (i.e., in replicating DNA from the 48-h-without-APC sample). This calculation provides a measurement of the level of enrichment of specified gene sequences in replicating DNA after a defined incubation period in the presence or absence of APC (Table I).

Our results show that late-replicating sequences were not significantly represented in hybrid DNA after 48 h (Figure 3), or even after a 56-h incubation in the presence of APC (result not shown). Strong hybridization signals were observed with replicating DNA from cells incubated for 24, 32, or 48 h without APC. The signal for the V_H Ig gene during incubations in the presence of APC was at most 10–25% of that observed after 48 h without APC (Figure 3, Table I). However, replication of early-replicating genes was readily observed in cells arrested by APC. The Ha-*ras* gene was enriched 5-fold in replicating DNA from cells incubated for 24 h in the presence of APC, as compared to longer incubations in the presence or absence of APC (Figure 3, Table I). Because these experiments were done at equal amounts of DNA per slot, the signal for the Ha-*ras* gene in replicating DNA diminished in samples corresponding to longer incubations with or without APC. This result reflected the dilution of the early-replicating sequences by the appearance of other sequences in replicating DNA as progressively more and more of the genome was replicated.

The replication of the c-*myc* gene has been detected during the first hour of the S phase, and the c-*mos* gene has been reported to replicate between hours 1 and 2 in 10T1/2 cells, or about 1 h after the replication of c-Ha-*ras* (Doggett et al., 1988). In the experiments presented here, a 32-h incubation in APC was required before the strongest hybridization signal with replicating DNA was observed for c-*myc*. The c-*mos* gene was preferentially detected in replicating DNA only after 40 h in the presence of APC (Figure 3, Table I).

Discussion

The data presented in this paper support the notion that mammalian cells exert stringent control over the temporal activation of replicons during the S phase. Aphidicolin does not interfere with the rate of entry of cells into the S phase and the initiation of DNA replication, although DNA elongation proceeds very slowly (Cordeiro-Stone & Kaufman, 1985). Consequently, the S phase is lengthened, but the sequential order of gene replication is preserved.

The results shown in Figures 1 and 2 indicate that nuclear DNA of low molecular weight was synthesized after 24 and 32 h (replicon initiation was not inhibited) but significant DNA strand growth was not evident until the incubation in APC was extended to 40 and 48 h. It has been reported that initiation of replication in the *ori* region of SV40 is resistant to the effects of APC. Nascent molecules up to 40 bp in size accumulated when SV40 DNA replicated in the presence of APC in isolated nuclei of CV-1 cells (Nethanel et al., 1988). The effect of APC on DNA synthesis in replicating SV40 chromosomes was to inhibit DNA chain elongation and synthesis of Okazaki fragments (Decker et al., 1986; Nethanel et al., 1988). However, there may be some important differences between the effects of APC on the replication of the SV40 genome and the duplication of mammalian chromosomes. Although APC is a reversible inhibitor of the polymerases α and δ (Pedrali-Noy & Spadari, 1979; Decker et al., 1987), the inhibition of replication in initiated SV40 molecules by APC was irreversible (Dinter-Gottlieb & Kaufmann, 1983; Nethanel et al., 1988). APC has been shown to destabilize nascent precursors of Okazaki fragments during replication of SV40 DNA in vitro (Nethanel et al., 1988). As a result, these precursors were not extended into mature Okazaki fragments. This destabilization by APC may also occur in vivo and is thought to be the putative secondary lesion induced by APC in replicating SV40 DNA molecules (Nethanel et al., 1988).

Our data indicate that APC does not irreversibly inhibit DNA replication in 10T1/2 cells. Once APC was removed, DNA strand growth resumed at near-normal rates (results not shown). In the presence of APC, DNA strand growth was also observed when incubations were extended to 40 and 48 h (Figures 1 and 2). The data in Figure 2 suggest that the inhibitory effect of APC on DNA strand growth was relaxed with time, as pulse-labeled nascent DNA grew to higher molecular weight during the chase in the presence of the inhibitor. APC inhibits DNA synthesis by competing with dCTP for the DNA polymerases (Spadari et al., 1984). The pool of dCTP increases as cells progress through the S phase, especially in the presence of APC (Pedrali-Noy et al., 1980). It is conceivable that as cells accumulated in the S phase after longer incubations (40 or 48 h), dCTP competed more effectively with APC for the DNA polymerases α and δ, thereby allowing for DNA strand growth. This conclusion is also supported by the observation that the total incorporation of [^{3}H]thymidine in DNA increased exponentially with the length of the incubation period in aphidicolin.

Our results suggest that the ordered activation of replicons is maintained but it is delayed in the presence of APC. For example, early-replicating sequences such as the c-Ha-*ras* and c-*mos* genes, but not the late-replicating V_H Ig gene, were detected among DNA sequences synthesized in the presence of APC. Furthermore, the sequence in which the replication of the early-replicating genes c-Ha-*ras*, c-*myc*, and c-*mos* was detected in cells maintained in the presence of APC was the same as their order of replication in the normal S phase. The delay in replication of genes was roughly proportional to the degree of inhibition of total DNA synthesis by APC. The level of DNA synthesis in cells incubated in APC for 32 or 48 h, as measured by the total incorporation of [^{3}H]thymidine in DNA, was 4% and 12%, respectively, of the total DNA synthesis activity in control cells labeled in the absence of APC. These values suggest that the overall rate of DNA synthesis was on average 12–13 times slower in the presence than in the absence of APC. This estimate is in agreement with a previous measurement of the rate of DNA strand growth by alkaline sucrose sedimentation (Cordeiro-Stone & Kaufman, 1985), which revealed a 93% inhibition (13–14-fold reduction) of this rate by APC, under conditions similar to those described here. Accordingly, the replication of the c-*mos* gene, which is seen 1 h after the c-Ha-*ras* gene in the absence of APC, was detected approximately 16 h later in the presence of APC (Figure 3, Table I). Although early-replicating and late-replicating genes can be distinguished during replication in the absence of APC (Brown et al., 1987; Doggett et al., 1988), it is difficult to determine subtle differences in the timing of replication and the correct order of replication of early-replicating genes. However, when replication is attenuated by APC, it is possible to clearly define the temporal order of gene replication. Replication in the presence of APC may be exploited to determine whether particular genes are contained within banks of replicons activated at the same time during S phase.

Since initiation of the SV40 replicon occurs by an APC-resistant mechanism, but DNA elongation in replication intermediates is inhibited, careful measurements of events at the *ori* region are facilitated when replication in vitro is initiated in the presence of APC (Decker et al., 1986, 1987). Ac-

cordingly, studies of replication in the presence of APC may have applications for understanding the dynamics of events at *ori* and termination regions during replication of mammalian chromosomes. For example, putative termination and origin regions for DNA replication in mammalian cells have been described (Epner et al., 1988; Brown et al., 1987). Slowing the S phase by exposure of cells to APC could help in developing physical maps of replication origins and of termination sequences in a particular replicon (Decker et al., 1986; Heintz & Stillman, 1988).

The progression of DNA synthesis through the S phase requires successful activation of banks of replicons in an ordered manner (Taylor, 1977). On the basis of experiments in Chinese hamster ovary (CHO) cells, Hamlin (1978) has suggested a model in which the activation of subsequent banks of replicons depends on the successful replication of previously activated replicons. Selective damage to replicating DNA was introduced by UV photolysis of DNA containing BrdUrd. When early-replicating DNA in CHO cells was damaged, DNA synthesis in the remainder of the S phase was inhibited (Hamlin, 1978). However, when DNA sequences replicating during the mid- or late-S phase were damaged at the beginning of the next S phase, DNA synthesis progressed normally in early S phase and was inhibited only in mid- or late-S phase, respectively. In our experiments (Figure 3 and Table I), we have employed molecular probes for DNA contained in temporally distinct banks of replicons. Our results support and extend Hamlin's model by demonstrating that initiation of new replicons could not be uncoupled from the completion of DNA replication in replicons initiated earlier. In the presence of APC, replication of c-*myc* and c-*mos* was delayed, and replication of the late-replicating V_H Ig gene sequences was not detected even 36 h after the first cells in the population approached the S phase (about 12–15 h after release from confluence inhibition). Our results are consistent with a model in which initiation of DNA synthesis at replication origins requires the sequential assembly of different factors, including the replicative DNA polymerases, in order to generate active replication complexes (Tsurimoto & Stillman, 1989). Since replication is slowed down by APC, but not interrupted, these replication factors might not dissociate from the DNA template, thus limiting the initiation of DNA synthesis at other origins.

The gene probes that we have used in this study do not correspond to contiguous sequences in the mouse genome but are located in four different chromosomes. Because the mammalian cell genome must contain hundreds of replicons in order to duplicate the nuclear DNA within the 8-h S phase, we have assumed that our probes identify genes associated with replicons that are activated at different times of the S phase. However, some parts of the genome, such as that containing the immunoglobulin genes, are replicated by activating origins which appear to be at least 250 kb apart (Hatton et al., 1988). At a rate of 3 kb/min for the rate of DNA polymerization per replicon, it would take about 1.5 h to complete DNA synthesis in each unit. Under these circumstances, the temporal order of replication of sequences within a replicon depends on their relative distance from the origin of replication. Therefore, our results cannot rule out the possibility that the early-replicating genes Ha-*ras*, *myc*, and *mos* are part of large replicons which are activated at the same time at the beginning of the S phase. In this case, *mos* would be found at least 180 kb from the site where replication was initiated, while Ha-*ras* and *myc* would be much closer to their respective origins. In order to discern between these two possibilities, it becomes necessary to generate a distribution map of origins of replication in mammalian chromosomes.

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Registry No. DNA polymerase, 9012-90-2.

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