Induction by Adozelesin and Hydroxyurea of Origin Recognition Complex-dependent DNA Damage and DNA Replication Checkpoints in Saccharomyces cerevisiae*

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DNA damaging agents induce a conserved intra-S-phase checkpoint that inhibits DNA replication in eukaryotic cells. To better understand this checkpoint and its role in determining the efficacy of antitumor drugs that damage DNA, we examined the effects of adozelesin, a DNA-alkylating antitumor agent that has a profound inhibitory effect on initiation of DNA replication in mammals, on the replication of Saccharomyces cerevisiae chromosomes. Adozelesin inhibited initiation of S. cerevisiae DNA replication by inducing an intra-S-phase DNA damage checkpoint. This inhibitory effect was abrogated in orc2–1 cells containing a temperature-sensitive mutation in a component of the origin recognition complex (ORC) that also causes a defect in initiation. The orc2–1 mutation also caused a defect in a checkpoint that regulates the activation of origins in late S phase in cells treated with hydroxyurea. Defects in both initiation and checkpoint regulation in the orc2–1 strain were suppressed by deletion of a gene encoding a putative acetyltransferase, SAS2. Adozelesin also induced a cellular response that requires a function of ORC in G1. A similar G1-specific response in mammals may contribute to the cytotoxic and antitumor properties of this and other DNA-damaging drugs.

Adozelesin is a member of the cyclopropylpyrroloindole (CPI)1 family of drugs, which interact with the minor groove of DNA and form covalent adducts with the N-3 of adenines (reviewed in Ref. 1). CPI drugs have potent antitumor activity in mice and are extremely cytotoxic to cultured cells at concentrations that are orders of magnitude lower than the cytotoxic doses of most DNA-damaging antitumor drugs. The nature of the cytotoxic and antitumor activity of adozelesin and other CPI drugs is not clear. However, several members of this class of drugs, including adozelesin, have been shown to exert profound inhibitory effects on DNA replication in the absence of apparent effects on RNA or protein metabolism (1). Like many other antitumor agents, the ability of CPI drugs to efficiently inhibit DNA replication may be responsible for their cytotoxic and antitumor effects.

To better understand the nature of adozelesin’s inhibitory effect on DNA replication, we recently analyzed the effects of this compound on the replication of viral genomes in cells infected with simian virus 40 (SV40) and determined that adozelesin inhibits SV40 viral DNA replication almost exclusively at the level of initiation, with little or no inhibitory effect on chain elongation (2). Adozelesin induces a similar initiation-inhibitory effect on the chromosomes of uninfected cells as well.2 The specific inhibition of initiation of SV40 DNA replication induced by adozelesin contrasts with the pronounced inhibitory effects on both the initiation and elongation of SV40 DNA replication induced by two more frequently studied DNA damaging agents, UV radiation and methylmethane sulfonate.3 In addition, complete inhibition of initiation of SV40 DNA replication is induced by adozelesin at much less than one lesion/replicating DNA molecule, indicating that it occurs in trans (2). The trans nature of the initiation-inhibitory effect suggests it is caused by a cellular response to DNA damage, such as the intra-S-phase checkpoint, which predominantly inhibits initiation of DNA replication (4).

Relatively little is known about the mammalian intra-S phase DNA damage checkpoint compared with checkpoints in other phases of the cell cycle. The paucity of information about this checkpoint is due to a number of factors. These include the failure to identify compounds that specifically inhibit initiation of DNA replication, the difficulty with which trans-inhibitory effects on initiation can be distinguished from cis-inhibitory effects on the elongation of nascent DNA chains caused by lesions that block replication fork progression, and the relative difficulty of performing genetic experiments in mammalian cells compared with less complex organisms such as yeast. In contrast, a number of gene products are known to be required for the intra-S-phase checkpoint in the budding yeast S. cerevisiae and the fission yeast Schizosaccharomyces pombe (reviewed in Refs. 5 and 6). These include the products of the MEC1 (S. cerevisiae) and rad3+ (S. pombe) genes, which are structurally and functionally homologous to a gene required for the mammalian intra-S-phase checkpoint, ATM (4). The homologous structure and function of these genes reflects the high degree of evolutionary conservation of the intra-S-phase checkpoint. Products of other genes that have close homologues in mammals, such as RAD53 (7), RFA1 (8), RFC5 (9), and PRR1 (10), are also required for the intra-S-phase checkpoint in S. cerevisiae.

The conservation of the intra-S-phase checkpoint and other

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1 The abbreviations used are: CPI, cyclopropylpyrroloindole; SV40, simian virus 40; ORC, origin recognition complex; HU, hydroxyurea; (N/N), neutral-neutral; FACS, fluorescence-activated cell sorting; RI, replication intermediate.

2 H. Miao, L. Silver, J. Seiler, and W. Burhans, manuscript in preparation.

3 H. Miao, L. Silver, T. Beerman, and W. Burhans, submitted for publication.
cancerous cellular pathways and the relative ease with which genetic manipulations can be performed makes yeast useful experimental models for addressing questions related to this checkpoint and drug mechanisms. In this study, we investigated the effects of adozolesin on cellular DNA replication in _S. cerevisiae_. One goal of our study was to learn more about the initiation-inhibitory effect of this drug and its relationship to the intra-S-phase checkpoint. A second goal was to understand how this inhibitory effect might be related to the cytotoxic and antitumor properties of this and related compounds that damage DNA.

Adozolesin inhibited initiation of _S. cerevisiae_ DNA replication, similar to its inhibitory effects on cellular and viral DNA replication in mammalian cells. This inhibitory effect was mediated by an intra-S-phase DNA damage checkpoint that required the function of the origin recognition complex (ORC), a highly conserved complex of proteins required for initiation of DNA replication in yeast and metazoans. ORC was also required for a related DNA replication checkpoint that suppressed initiation of DNA replication at normally late S phase-activated origins in cells blocked in early S phase with the ribonucleotide reductase inhibitor hydroxyurea. Adozolesin activated origins in cells blocked in early S phase with the inhibited initiation of DNA replication at normally late S phase—required for a related DNA replication checkpoint that is mediated by an intra-S-phase DNA damage checkpoint (10, 11). A second goal was to understand how this inhibitory effect might be related to the cytotoxic and antitumor properties of this and related DNA damaging drugs.

**EXPERIMENTAL PROCEDURES**

_Yeast Strains, Cell Cycle Synchronization, Flow Cytometry, and Adozolesin Treatment—Strains employed in this study are described in Table I. The orc2–1 sac2Δ strain was constructed from JRY5068 by replacing the ORC2 gene with orc2–1 from pJK1267 (gift from J. Rine) using standard cloning and gene replacement techniques (13). Tests of the viability of this strain at several temperatures confirmed that it is temperature-sensitive at 37 °C but not at 30 °C due to the partial suppression of the orc2–1 temperature sensitivity by sac2Δ (14). Cells were grown in YPD and exposed to cell cycle-inhibiting drugs to effect a cell cycle arrest in S or G2 phase. To determine whether or not this arrest corresponded to an inhibitory effect due to the defect in the DNA replication checkpoints in these strains (see text). However, their position in S phase remained earlier than the position in S of exponential cultures of cells treated for 4 h with HU to arrest them in S (data not shown). Therefore, to simplify the discussion, we continued to refer to them as “early S” cultures._

**RESULTS**

**Adozolesin Arrests the _S. cerevisiae_ Cell Cycle and Inhibits DNA Replication**—We first explored the possibility that adozolesin induces a cell cycle arrest in _S. cerevisiae_. Microscopic examination of cells from exponentially proliferating cultures of _S. cerevisiae_ treated for 4 h with 4 μM adozolesin revealed that adozolesin treatment caused them to accumulate as large-budded cells with single nuclei (Fig. 1A), indicating the presence of a cell cycle arrest in S or G2 phase. To determine whether or not this arrest was related to an inhibitory effect on DNA replication, we employed FACS to measure progression through S phase of untreated cells and cells treated with adozolesin (Fig. 1B). To avoid the potential effect of a transient late G1 checkpoint response to DNA damage that delays entry into S phase (19) cells were first collected in early S phase. Comparison of the DNA content of cells released from the early

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**Table I**

| Strain     | Genotype                        | Source     |
|------------|---------------------------------|------------|
| W303–1A    | MATa, ade2–1, ura3–1, his3–11, trp1–1, leu2–3, 112, can1–100 | B. Stillman|
| YB0057     | W303–1A, orc2–1                 | B. Stillman|
| Oay660     | MATa, ura3–1, trp1–1, his3–11,15, can1–100, ade2–1, bar3::hisG, leu2::ORC1 (LEU2) | S. Bell    |
| Oay661     | MATa, ura3–1, trp1–1, his3–11,15, can1–100, ade2–1, bar3::hisG, leu2::orc2–161, (LEU2) | S. Bell    |
| TWF98      | MATa, ura3 leu2 trp1 his3       | T. Weinart |
| DLY285     | MATa ura3 leu2 trp1 his3 mec1::HIS3 | T. Weinart |
| DLY284     | MATa ura3 leu2 trp1 his3 rad53::URA3 | T. Weinart |
| JRY5068    | MATa sac2::TRP1 ade2::his3 trp1 leu2 ura3 | J. Rine    |
| MLY1       | JRY5068, orc2–1                 | This study |

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4. Weinberger, M. Lu, and W. Burhans, unpublished observations.
S phase block (Fig. 1B, early S) for 3 h in the absence (Fig. 1B, HU release) or presence (Fig. 1B, HU release + adozelesin) of adozelesin showed that adozelesin inhibited DNA replication.

Inhibition of S. cerevisiae DNA Replication by Adozelesin Is Reduced in Strains with a Defective Intra-S-phase Checkpoint—To determine whether or not the inhibitory effect of adozelesin on S. cerevisiae DNA replication was mediated by the previously characterized intra-S-phase DNA damage checkpoint in this organism (7), we compared the effects of adozelesin on DNA replication in mec1 and rad53 strains that harbor defects in this checkpoint, as well as the G1 checkpoint (19), to its effects on DNA replication in an isogenic wild type strain. Wild type cells that were not treated with adozelesin rapidly progressed through S phase during 60 min after release of a G1 block and eventually cycled through G1 to the next S phase (Fig. 2, TWY98 (−)). In contrast, treatment of wild type cells with adozelesin after release of the G1 block resulted in much slower progression through S phase (Fig. 2, TWY98 (+)) due to the inhibitory effect of the drug on DNA replication.

Similar to untreated wild type cells, mec1 and rad53 cells released from a G1 block in the absence of drug moved through S phase rapidly (Fig. 2, mec1 (−) and rad53 (−)). However, both mec1 and rad53 cells also progressed rapidly through S phase in the presence of adozelesin (Fig. 2, mec1 (+) and rad53 (+)). The reduced inhibitory effect of adozelesin on DNA replication observed in mec1 and rad53 cells indicates that at least some of the inhibitory effect of this drug on DNA replication in isogenic wild type cells is caused by induction of the MEC1- and RAD53-dependent intra-S-phase DNA damage checkpoint.

Increased Sensitivity to Adozelesin of Strains with Mutations
tion, in orc1–161, orc2–1, and wild type cells. Neutral-neutral two-dimensional gels separate replicating DNA from nonreplicating DNA on the basis of size and shape (18). Replicating fragments of DNA that have initiated DNA replication contain two replication forks and migrate as a bubble arc (Fig. 3D, Bubble arc) in two-dimensional gels. Fragments that are replicated passively by a single replication fork migrate as a fork arc (Fig. 3D, Fork arc), and nonreplicated or fully replicated molecules migrate as a discrete spot (Fig. 3D, 1N DNA). In a population of cells, defective initiation is indicated by a reduction in the number of RIs in the bubble arc associated with an origin-containing fragment. RIs were isolated from wild type, orc2–1, and orc1–161 cells synchronized in early S phase with HU, fractionated on neutral-neutral two-dimensional gels, and the gels were probed with sequences from an early S phase-firing origin, ORI305 (Fig. 3E). Bubble arcs were observed in preparations of RIs from each strain, indicating that initiation of DNA replication was occurring at ORI305 in each case. As expected, the number of ORI305 initiation intermediates was reduced in orc2–1 cells compared with wild type cells, consistent with the fact that this strain contains a defect in initiation (21). Although reduced efficiency of initiation can cause passive replication of origin sequences (23), fork arcs were not observed in our experiments because HU blocked the progression of replication forks emanating from adjacent origins. Even fewer ORI305 RIs were observed in orc1–161 cells, indicating that orc1–161 has a more severe defect in initiation compared with orc2–1. Therefore, the decreased sensitivity to adozelesin of orc1–161 cells compared with orc2–1 cells does not occur because they are less defective in initiation of DNA replication. Furthermore, this result indicates that the greater sensitivity of orc2–1 cells cannot be related to a general toxic effect that is exacerbated by the decreased origin function in this strain.

**ORC2 Is Required for an Intra-S-phase DNA Damage Checkpoint That Inhibits Initiation of DNA Replication**—These results indicated that adozelesin induces a cellular response that requires a function of Orc2p. To determine whether or not this function is related to the intra-S-phase checkpoint, we asked whether the inhibitory effect of adozelesin on DNA replication was reduced in orc2–1 cells compared with wild type cells, similar to mec1 and rad53 cells treated with adozelesin (Fig. 2) or other DNA damaging agents (7).

As in Fig. 1B, to avoid the potential effects of the transient late G1 checkpoint response to DNA damage, which is defective in mec1 and rad53 strains (19), but might remain intact in ORC mutant strains, we synchronized cells in early S phase before addition of adozelesin. FACS analysis of DNA content showed that adozelesin retarded the progression through S phase of wild type cells (Fig. 4A, W303 ± adozelesin), similar to the results in Figs. 1 and 2. In contrast, adozelesin had little or no effect on progression of orc2–1 cells through S phase (Fig. 4A, orc2–1 ± adozelesin), similar to checkpoint-defective mec1 and rad53 strains (Fig. 2). The increased DNA content observed in orc2–1 cells treated with adozelesin was not observed in orc2–1 cells in which the a factor G1 block was maintained for similar lengths of time. Since cell growth and mitochondrial DNA replication are not blocked by a factor, the increased DNA content was not a consequence of mitochondrial DNA replication or an artifact of increased cell size. Instead, it was caused by a defective intra-S-phase DNA damage checkpoint that inhibits nuclear DNA replication in wild type cells and requires the function of Orc2p.

**orc1–161** cells, which are slightly resistant to adozelesin compared with wild type cells (Fig. 3C) behaved more like wild type cells in their response to adozelesin (Fig. 4A, orc1–161 ± adozelesin). Therefore, the intra-S-phase DNA damage checkpoint was either intact or less defective in orc1–161 cells. Since deletion of SAS2 suppresses the sensitivity of orc2–1 cells to adozelesin (Fig. 3B), we also asked whether or not deletion of SAS2 suppresses the checkpoint defect conferred by the orc2–1 mutation. In fact, significantly less DNA replication occurred in the orc2–1 sas2Δ strain treated with adozelesin compared with untreated orc2–1 sas2Δ cells (Fig. 4A, orc2–1 sas2Δ ± adozelesin). Therefore, in addition to suppressing the sensitiv-
FIG. 4. A, detection of a defective DNA damage checkpoint in orc2–1 cells treated with adozelesin. FACS measurements of the DNA content of wild type (W303) and orc2–1, orc1–161, sas2Δ, and orc2–1/sas2Δ mutant cells after release from an early S phase block for various lengths of time in the presence (+) or absence (−) of adozelesin at 27 °C. The dotted lines indicate the position of cells with a 1c or 2c content of DNA as determined by FACS analysis of control mid-log phase cells and cells synchronized in G1 and G2. Results for the parent wild type strain of orc1–161, which is closely related to W303, were similar to those of W303 (data not shown). B, detection of an initiation-inhibitory effect on initiation of DNA replication at ORI501 in wild type cells, which is abrogated by the mutation in orc2–1 cells. Phosphorimages of (N/N) two-dimensional gel blots show the status of replication intermediates at a mid to late S-firing origin, ORI501, in DNA harvested at various times after addition of adozelesin to cells released from a G1 block. FACS profiles above the (N/N) two-dimensional gels indicate the position in S phase of cells from which this DNA was harvested (see text for details).
ity of orc2–1 cells to higher temperatures and adozelesin treatment, deletion of SAS2 suppresses the intra-S-phase DNA damage checkpoint defect conferred by the orc2–1 mutation. The ability of sas2Δ cells to replicate DNA in the presence of adozelesin was also reduced compared with untreated control cells, similar to the behavior of adozelesin-treated wild type cells (Fig. 4A, sas2Δ + adozelesin). This indicates that the inhibitory effect of Sas2p on ORC function (14) is not related to a requirement for SAS2 in the intra-S-phase DNA damage checkpoint.

To determine whether or not the defect in the intra-S-phase checkpoint in orc2–1 cells corresponded to a reduced inhibitory effect on initiation at replication origins in this strain compared with wild type cells, we performed a neutral-neutral two-dimensional gel analysis of a fragment of replicating DNA associated with a mid to late S phase-firing chromosomal origin of replication (ORI501). Wild type (W303) and orc2–1 cells were treated with 4 μM adozelesin after release of an α-factor G1 block, and DNA was harvested 20 and 60 min later, when FACS profiles indicated they were in early or middle to late S phase, respectively (Fig. 4B). RIs were then separated from nonreplicating DNA in neutral-neutral two-dimensional gels, and blots of the gels were probed with ORI501 sequences. RIs were not observed at ORI501 in wild type cells 20 min after addition of adozelesin, when FACS analysis indicated that most cells were in early S phase (Fig. 4B). This reflects the fact that ORI501 is not activated until late S phase in wild type cells (24). However, 60 min after addition of adozelesin, when FACS profiles indicated the cells were in middle to late S phase, a prominent fork arc was observed in the absence of a bubble arc. This indicates that the fragment containing ORI501 was passively replicated by replication forks emanating from distal origins. Passive replication of normally active origins also occurs in orc2–1 cells when they are shifted to the nonpermissive temperature and reflects the reduced frequency of initiation at these origins (23). Since a fork arc is present, the failure to observe a bubble arc cannot be explained by a block to entry into S phase. Instead, it reflects an adozelesin-induced inhibitory effect on initiation of DNA replication at ORI501.

In contrast, 20 min after addition of adozelesin to orc2–1 cells, a bubble arc at ORI501 was observed in RIs from these cells in the absence of a fork arc (Fig. 4B, orc2–1, 20'). The presence of RIs in the ORI501 fragment from orc2–1 cells at an earlier time compared with wild type cells is consistent with the FACS data showing that adozelesin-treated orc2–1 cells are progressing through S phase faster than adozelesin-treated wild type cells. Detection of a bubble arc in the absence of a fork arc indicates that, unlike wild type cells, orc2–1 cells initiate replication at ORI501 in the presence of adozelesin. Therefore, the defective intra-S-phase DNA damage checkpoint in orc2–1 cells detected by FACS analysis corresponds to a reduced inhibitory effect on initiation of DNA replication.

**Fig. 5.** A, detection of an ORC-dependent replication checkpoint in S. cerevisiae cells treated with HU. FACS measurements of the DNA content of wild type (W303) and orc2–1, orc1–161, sas2Δ, and orc2–1/sas2Δ mutant cells incubated at 27 °C in the presence of 0.2 M HU for varying times after release of an α-factor G1 block. The dotted lines indicate the position of cells with a 1c or 2c content of DNA as determined by FACS analysis of control mid-log phase cells and cells synchronized in G1 and G2. B, a defect in the ORC-dependent replication checkpoint in orc2–1 cells causes the inappropriate activation of origins of replication (see text for details).
rad53 strains similarly blocked in early S phase with HU initiate DNA replication at origins of replication that normally are activated late in S phase (25). To determine whether or not the increased DNA replication observed by FACS in the orc2–1 strain was caused by a similar inappropriate firing of late S origins in early S phase, we examined the ability of the late S-activated ORI501 to initiate DNA replication in wild type, orc2–1, and orc2–1 sas2Δ cells synchronized in early S phase with HU.

FACS profiles of cells from each of these strains released into HU from an α-factor G1 block showed that most cells were in early S phase 90 min after addition of HU (Fig. 5B). RIs were isolated from these cells and separated on neutral-neutral two-dimensional gels, and the gels were blotted and probed with ORI501 sequences. Both bubble arcs and fork arcs were detected at ORI501 in DNA from orc2–1 cells, but not from wild type (W303) or orc2–1 sas2Δ cells (Fig. 5B). When the blots were stripped and reprobed with sequences from ORI306, an origin activated early in S phase, bubble arcs were observed in all three strains (Fig. 5B, ORI306), although the numbers of initiation intermediates associated with ORI306 in orc2–1 cells was reduced due to the initiation defect in this strain. The detection of large numbers of initiation intermediates at ORI306 in wild type and orc2–1 sas2Δ cells confirmed that the failure to observe RIs at ORI501 in these strains was not due to their loss during isolation or to a general failure to initiate DNA replication. In addition, the larger number of ORI306 RIs in the orc2–1 sas2Δ strain compared with orc2–1 indicated that deletion of SAS2 suppresses the orc2–1 initiation defect.

The detection of initiation intermediates at ORI501 in orc2–1 cells synchronized in early S phase with HU suggested that the mutation in orc2–1 caused ORI501 to be activated earlier in S phase than in wild type cells. However, orc2–1 cells were more difficult to synchronize than other strains due to a defect in transcriptional silencing at the HML mating type locus (26). Consequently, it remained possible that RIs observed at ORI501 in orc2–1 cells were produced in a small subpopulation of cells that were in late S phase due to the greater asynchrony of these cultures. ORI301 contains sequences that function as an origin of replication on plasmid molecules, but these sequences are normally passively replicated at their native chromosomal location (27) by replication forks emanating from distal origins that are activated earlier in S phase (28). However, chromosomal ORI301 sequences are activated in mec1 and rad53 strains with defective checkpoints when cells are blocked in early S phase (3) due to the altered timing of activation conferred by these mutations. Since ORI301 is never active in wild type cells, its activation is diagnostic of a defective checkpoint that regulates late S origin activation even in asynchronous cultures. To confirm that the mutation in orc2–1 causes inappropriate activation of late S origins in cells synchronized in early S phase with HU, we asked whether ORI301 is activated in orc2–1 cells blocked in early S phase with HU.

A faint bubble arc as well as a fork arc associated with passive DNA replication were detected in fragments of DNA containing ORI301 from orc2–1 cells synchronized in early S phase, indicating that the chromosomal copy of ORI301 is activated in the orc2–1 strain (Fig. 5B). In contrast, RIs were not observed at ORI301 in early S phase wild type or orc2–1 sas2Δ cells. When the blots were stripped and reprobed with sequences from ORI305, an early S-firing origin, bubble arcs were observed in DNA from both orc2–1 and wild type cells (Fig. 5B). Therefore, we conclude that orc2–1 cells contain a defective late S phase replication checkpoint resulting in the inappropriate activation of origins, which is suppressed by deletion of SAS2. Inappropriate origin activation is at least partly responsible for the increased DNA replication in the presence of HU detected by FACS in orc2–1 cells (Fig. 5A). This checkpoint is distinct from the replication checkpoint that blocks progression through G2/M when DNA replication is not complete (29, 30), because orc2–1 cells with defective late S phase replication checkpoints do not undergo mitosis or lose viability when treated with HU.

Adozelesin Also Affects ORC Function in G1—ORC is required for establishing and maintaining origin licensing in late mitosis and throughout G1 (20), and it also has an undefined function in G2 (31). We tested the possibility that the cellular response induced by adozelesin impinges on the function of ORC in these other compartments of the cell cycle. We first determined the cell cycle-specific effects of elevated temperature or adozelesin treatment on viability of wild type and ORC mutant cells. A, effect of shift to the nonpermissive temperature for 4 h on viability of orc2–1 cells synchronized in various stages of the cell cycle. B and C, effect on viability of adozelesin treatment for 4 h at 27 °C in synchronized populations of wild type and orc2–1 mutant cells. D, suppression of G2 sensitivity to adozelesin of orc2–1 cells by deletion of the SAS2 gene. E, effect on viability of adozelesin treatment for 4 h at 27 °C in synchronized populations of ORC1 wild type and orc1–161 mutant cells. Viability is expressed as percentage of total colony-forming units of synchronized cells held for 4 h (panels A–C and E) or for 0–3 h (panel D) at a semipermissive temperature (27 °C) in the absence of drug treatment in order to control for effects on viability of synchronization regimens. In panels C–E, viability of mutant strains was normalized to the viability of isogenic wild type parent strains.

**Fig. 6. Cell cycle-dependent effects of elevated temperature or adozelesin treatment on viability of wild type and ORC mutant cells.**

![Cell Cycle Experiment](image325x378 to 537x729)

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Y. Yang and D. Kowalski, personal communication.
Incubation of G1-synchronized orc2−1 cells at 37 °C for 4 h significantly reduced viability compared with incubation for the same length of time at the semipermissive temperature of 27 °C (Fig. 6A). Progressively smaller effects of elevated temperature on viability occurred in cells held for 4 h at 37 °C during an early S phase block, in cells blocked throughout S, and in cells blocked in G2/M. G0 cells were even less sensitive to the temperature shift (Fig. 6A).

A remarkably similar pattern of cell cycle-specific viability effects was induced by treating synchronized populations of both wild type and orc2−1 cells with adozelesin for 4 h at 27 °C (Fig. 6B). Furthermore, normalization of orc2−1 sensitivity to adozelesin to that of wild type cells at each cell cycle stage indicated that the increased sensitivity to adozelesin conferred by the orc2−1 mutation also occurred in a cell cycle-specific pattern that was similar to the pattern observed in the temperature shift experiments (Fig. 6, compare C with A). Therefore, similar to the reduced viability observed in the temperature shift experiments (Fig. 6A), the orc2−1-specific sensitivity to adozelesin is related to some aspect of ORC function that is critical for survival of adozelesin treatment at different stages of the cell cycle, especially G1, which is reduced in G0 cells.

The decreased sensitivity of orc2−1 cells to adozelesin when they were synchronized in G0, S, or G2/M raised the possibility that the increased viability of adozelesin-treated exponential populations of orc2−1 sas2Δ compared with orc2−1 cells (Fig. 3B) might be an artifact of their accumulation in G0, S, or G2/M. To rule out this out, we compared the sensitivity to adozelesin of orc2−1 and orc2−1 sas2Δ cells synchronized in G1. The results of this comparison indicated that deletion of SAS2 suppressed the G1-specific sensitivity of orc2−1 cells to adozelesin (Fig. 6D).

To determine whether the reduced adozelesin sensitivity conferred by the orc1−161 mutation (Fig. 3C) might also be related to alterations in a cellular response that requires ORC function in G1, we compared the sensitivity of orc1−161 and wild type cells when they were synchronized in G0, G1, S, and G2/M. Normalization of the sensitivity to adozelesin of orc1−161 to that of its isogenic wild type parent indicated that the reduced sensitivity of orc1−161 mostly occurred in G1 (Fig. 6E). These results indicate that ORC participates in a pathway leading to reduced survival of adozelesin-treated wild type cells in G1 phase, which is partially suppressed by the orc1−161 mutation.

**DISCUSSION**

**Adozelesin and Inhibition of Initiation of S. cerevisiae DNA Replication**—Aodozelesin is an experimental DNA-damaging antitumor agent that profoundly inhibits initiation of DNA replication in mammalian cells, with little or no effect on elongation (2). We are exploring the possibility that the potent cytotoxic and antitumor properties of adozelesin are related to this initiation-inhibitory effect. In this study, we show that adozelesin inhibits DNA replication in the budding yeast _S. cerevisiae_ (Fig. 1), and as in mammals, at least part of the inhibitory effect of this drug on DNA replication occurs at the level of initiation of new DNA chains (Fig. 4). This and a recent report of a similar effect induced by methylethene sulfonate (32) are the first reported examples of initiation-inhibitory effects on DNA replication induced by DNA-damaging agents in yeast. The conservation of the initiation-inhibitory response to DNA damage and the relative ease with which genetic manipulations can be performed in yeast should facilitate efforts to learn more about this response and its potential relationship to the cytotoxic and antitumor properties of drugs that damage DNA.

**Induction by Aodozelesin of an ORC-dependent Intra-S-phase DNA Damage Checkpoint**—Aodozelesin’s inhibitory effect on _S. cerevisiae_ DNA replication was caused in part by the induction of an ORC-dependent intra-S-phase DNA damage checkpoint that inhibits initiation of DNA replication (Fig. 4). Most of the previously identified components of this checkpoint function by generating or transducing DNA damage signals. One of the roles of checkpoint signals is to inhibit progression through the cell cycle by negatively regulating checkpoint targets. In contrast, the targets of these signals positively regulate progression through the cell cycle. Since the role ORC plays in initiation of DNA replication is required for progression through S phase, it may be a target of the intra-S-phase DNA damage checkpoint signaling pathway.

However, two other proteins that are required for initiation of DNA replication also play roles in this checkpoint: primase (10) and RP-A (8), and these proteins may function as targets instead of, or in addition to, ORC. ORC interacts with chromatin at origins of replication, and this interaction is altered in orc2−1 cells compared with wild type cells (33). The initiation defect of another ORC mutant, orc5−1, is associated with a loss of the structural integrity of ORC (15), and this mutant strain has a defective DNA damage checkpoint as well. 4 It is possible that, similar to its role in replication (34), the checkpoint role of ORC is a structural one that involves tethering a larger complex of proteins which includes both RP-A and polymerase α-prime to chromatin. Loss of the structural integrity of ORC and/or reduced interactions with chromatin associated with the orc2−1 mutation may alter the checkpoint and replication functions of proteins in the larger complex, such as primase and/or RP-A, which may be the ultimate targets of a signal transduction pathway that originates with DNA damage.

Interestingly, deletion of the SAS2 gene, which previously had been shown to partially suppress the temperature sensitivity of orc2−1 (14), also suppressed defects in initiation of DNA replication (Fig. 5), the DNA damage checkpoint (Fig. 4), and the related DNA replication checkpoint (Fig. 5; discussed below) harbored by this strain. Perhaps acetylation of chromatin proteins and/or ORC by the putative acetyltransferase encoded by this gene destabilizes interactions of ORC with chromatin that are important for ORC’s role in all three processes. Whatever the mechanism by which SAS2 alters ORC function, the recent discovery of interactions between human ORC and a histone acetyltransferase (35) suggests that this mechanism may be conserved.

**Existence of an ORC-dependent Late S Phase DNA Replication Checkpoint**—The increase in DNA content observed in orc2−1 cells compared with wild type cells when released from a G1 block in the presence of HU (Fig. 5A) indicates that ORC is also required for a checkpoint that inhibits DNA replication in the presence of HU. Activation of the normally late S-firing ORI501 and the inactive ORI301 in orc2−1 cells, but not wild type cells, synchronized with HU in early S phase (Fig. 5B), indicates that defects in this checkpoint cause the inappropriate activation of late S-firing origins of replication. The seemingly paradoxical activation of origins caused by a mutation that also reduces the efficiency of origin activation emphasizes the dual roles played by ORC in the positive and negative regulation of initiation of DNA replication.

Defects in a similar late S replication checkpoint are responsible for the increased synthesis of small nascent DNA strands at late S phase-activating origins, including ORI501, recently observed by Santocanale and Diffley (25) in mec1 and rad53 mutant strains synchronized in early S phase with HU. Presumably, Orc2p functions downstream of Mec1p and Rad53p in the same checkpoint. The existence of a late S replication
checkpoint that requires the function of Orc2p also is indicated by the altered timing of origin activation recently observed in orc2–1 cells in the absence of HU by Shirahige et al. (32). This latter observation indicates that the ORC-dependent late S phase replication checkpoint also operates in cells unperturbed by drugs. In fact, the existence of a constitutively active DNA replication checkpoint that suppresses origin firing was suggested by a recent report that the temperature sensitivity of orc2–1 is partially suppressed by a mutation in MECl that causes defective checkpoints (36). It is possible that the replication checkpoint remains partly intact in orc2–1 cells. If so, a partial inhibitory effect of Mec1p on origin activation may combine with the initiation defect in this strain to reduce the number of active origins below a critical threshold required for survival.

Adozesin Affects an ORC-dependent Process in G1 as Well as in S Phase—We expected that our screen for strains with altered sensitivity to adozesin (Fig. 2A) would identify mutations that caused a defective DNA damage checkpoint and thus allowed for the replication of adozesin-induced damage lesions. However, since many of the mutant strains we screened fail to initiate DNA replication at the nonpermissive temperature, we also anticipated that inhibition of initiation by a drug response might be synthetically lethal with these mutations. In principle, this would be similar to the synthetic lethality that can occur in strains containing two conditional mutant genes (37), except that transducers or effectors of the drug response would play the role of the second mutation.

In fact, most of the sensitivity of orc2–1 cells to adozesin occurs in G1 rather than in S phase (Fig. 6). This contrasts with the sensitivity of another intra-S-phase checkpoint mutant (pri1-M4) to UV radiation, which is clearly related to the replication of UV damage lesions in S phase (10). Although the G1 sensitivity to adozesin could be caused by cell cycle-specific differences in recombinational (38) or nucleotide excision repair (39) or altered drug uptake or DNA adduct formation in G1, the altered G1 sensitivity to adozesin conferred by the mutations in orc2–1 (Fig. 6C) and orc1–161 (Fig. 6E) clearly indicates that at least some of this sensitivity is related to ORC function, and ORC is not expected to play a direct role in any of these processes. Importantly, orc1–161 cells, which are resistant to adozesin in G1 compared with wild type cells (Fig. 6D), harbor a more severe initiation defect compared with orc2–1 cells (Fig. 3D). Consequently, the lethality of adozesin in orc2–1 cells cannot be an indirect effect on repair or other processes that is exacerbated by reduced origin function.

The similar cell cycle-specific pattern of sensitivity of orc2–1 cells to high temperature or adozesin (Fig. 6) and the fact that the initiation defect (Fig. 5B) and temperature (14) and adozesin (Figs. 3B and 6E) sensitivity are all suppressed by the deletion of SAS2 argues that the lethal effect of adozesin in these cells is directly related to temperature sensitivity and defective initiation. The precise relationship between defective initiation and temperature sensitivity has not been firmly established. The cell cycle-specific pattern of temperature sensitivity we observed in orc2–1 cells indicates that it must be related to a function of ORC that is most critical in G1, progressively less important as cells move through S phase, and essentially absent from cells in G2/M or G0. In fact, this is precisely the pattern expected if it involves the inactivation of origin licensing.

Origins are licensed for activation by the formation of prereplicative complexes ("pre-RCs") containing ORC in late mitosis or when cells re-enter the cell cycle from G0, where origins are not licensed (reviewed in Ref. 20). Licensing is maintained by ORC and other proteins throughout G1. Activation of origins occurs as cells proceed through S phase, and, to block re-initiation of DNA replication within the same cell cycle, they become unlicensed as they are activated. Relicensing of origins occurs once cells have finished replicating the genome at the end of S phase and proceeded to the next mitosis. The inability to relicense origins except in mitosis provides a "fail-safe" regulatory mechanism that protects cells from the loss of integrity of genetic information that would accompany multiple rounds of initiation within the same S phase.

Footprint analysis of protein-DNA interactions at origins of replication in orc2–1 cells shifted to the nonpermissive temperature indicates that origin licensing is disrupted at this temperature (33). Once disrupted, due to the fail-safe nature of this regulatory pathway, it may not be possible to restore the licensed state after return to the permissive temperature except by the normal processes that re-establish pre-RCs in mitosis or when cells re-enter the cell cycle from G0. This is suggested, for instance, by the failure of ORC5 expression to rescue orc5–1 cells in G1 from a permanent arrest associated with inactivation of ORC (31). In the absence of the capability to restore origin function in G1, the complete replication of the genome required for continued progression through the cell cycle to mitosis (where licensing could be re-established) cannot occur. Cells would become progressively less sensitive to the loss of origin licensing as they proceed through S phase because increasing numbers of replication forks will have been established before the temperature shift, thus increasing the capacity for replicating the genome. G2 and G0 cells would be insensitive to inactivation of licensing by the temperature shift because they are capable of relicensing origins in the absence of additional DNA replication once they are returned to a permissive temperature.

Based on these considerations, we consider it likely that inactivation of origin licensing is responsible for the permanent cell cycle arrest of orc2–1 cells when shifted to the nonpermissive temperature in G1. The similar pattern of temperature and adozesin sensitivity observed in orc2–1 cells staged at different points in the cell cycle and in G0 and the fact that both the temperature (14) and adozesin (Figs. 3 and 6) sensitivity are suppressed by the SAS2 deletion are consistent with the possibility that the lethal effect of adozesin in orc2–1 cells is also caused by the inactivation of origin licensing. The suppression of the G1 sensitivity of wild type cells by the orc1–161 mutation (Fig. 6E) suggests that a similar ORC-dependent mechanism underlies the sensitivity of wild type cells to this drug in a manner that is potentiated by the orc2–1 mutation.

Mechanisms Underlying the Cytotoxic and Antitumor Effects of Adozesin in Mammalian Cells—Whatever the underlying cause, the relationship between adozesin-induced lethality and ORC function in G1 defines a novel cytotoxic mechanism that has implications for understanding how this and other DNA damaging drugs kill tumor cells. The lethal effects of adozesin in mammals occur in a cell cycle-specific and proliferation-dependent pattern that is essentially identical to that observed in orc2–1 and wild type S. cerevisiae cells treated with this drug: very sensitive in G1, less sensitive in S phase, and least sensitive in G2/M and G0 (11, 12). The G1 resistance is particularly interesting, because a similar relationship between proliferative capacity and cytotoxic or apoptotic response has been observed with other DNA damaging agents and antitumor drugs (40, 41), at least one of which, like adozesin, has been shown to specifically inhibit initiation of DNA replication (42). In the context of the high degree of conservation of ORC proteins and other components of the origin licensing apparatus and the intra-S-phase DNA damage checkpoint, the G1-specific cytotoxic effects of adozesin and other antitumor
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drugs in mammals may be caused by a similar ORC-dependent response that inactivates origin licensing.

Tumor cells are frequently observed to proliferate slowly, particularly at early stages of tumor progression (40), which suggests they reside in an extended G1 state where they harbor licensed origins. In fact, the results of a recent study of origin licensing proteins as molecular markers for preneoplastic cells of the cervix suggest that a defining characteristic of these cells may be the presence of licensed origins in populations of cells that are only rarely synthesizing DNA (43). The sensitivity of these cells to a drug response that disrupts origin licensing (in contrast to normal cells in G0 that would be refractile to this response) may underlie the potent anticancer properties of adozelesin and related drugs.

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