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Inhibiting the Expression of DNA Replication-Initiation Proteins Induces Apoptosis in Human Cancer Cells[1]

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ABSTRACT

DNA replication-initiation proteins are expressed in cancer cells, whereas some of these proteins are not expressed in nonproliferating normal cells. Therefore, replication-initiation proteins may present attractive targets for anticancer therapy. Using selected antisense oligodeoxynucleotides and small interfering RNA molecules targeted to the mRNA encoding the DNA replication-initiation proteins hCdc6p, hMcm2p, and hCdc45p, we show that the target genes could be effectively and specifically silenced and that, consequently, DNA replication and cell proliferation were inhibited in cultured human cells. In addition, silencing of these genes resulted in apoptosis in both p53-positive and -negative cancer cells but not in normal cells: cancer cells entered an abortive S-phase, whereas normal cells arrested mainly in G_1 phase. Our studies are the first to suggest that inhibiting the expression of selective replication-initiation proteins is a novel and effective anticancer strategy.

INTRODUCTION

DNA replication is one of the most fundamental cellular processes. To ensure proper genome duplication and inheritance, eukaryotic cells exert strict control over DNA replication by regulating a series of replication-initiation proteins. Although obligatorily expressed in cancer cells, a subset of initiation proteins for DNA replication, such as Cdc6p (1, 2), MCM proteins (2), and Cdc45p,[3] are not expressed in nonproliferating normal cells. Inhibiting the expression of these proteins should therefore effectively abate DNA replication, thus stopping cancer growth while leaving most normal cells in the body largely unaffected. Thus, these proteins may present attractive targets for development of effective anticancer drugs with few side effects.

To date, at least six groups of initiation proteins, including ORC (Orc1p through -6p; Refs. 3–7), Noc3p (8), Cdc6p (1, 9–12), Cdt1p (13–16), MCM (Mcm2p- through -7p; Refs. 17–20), and Cdc45p (21–23), are known to be required for eukaryotic DNA replication. These proteins are conserved in eukaryotes, and homologues of most of them have been identified in model organisms from yeast to humans. To test the idea that inhibiting the expression of replication-initiation proteins can stop cancer cell growth, we have designed, screened, and tested antisense ODNs[4] and siRNA molecules targeted to three human DNA replication-initiation genes, *hCdc6*, *hMcm2*, and *hCdc45*. We found that selective antisense ODNs and siRNAs not only significantly reduced the mRNA and protein levels of the target gene products, thus stopping DNA replication and cell proliferation, but also resulted in apoptosis of both p53-positive and -negative cancer cells. Furthermore, silencing of these genes does not cause death of cells derived from normal tissues.

MATERIALS AND METHODS

ODNs and siRNAs. Two strategies were used to run the mFold program (24) to predict the secondary structures of the target mRNA. The first was to input the entire sequence of a target gene but to limit the range of allowable base pairing to 250 or 500 nucleotides (in two separate runs). The second was to input 500–700 nucleotide segments of a target gene for each run with 200 nucleotides of overlapping sequences between two adjacent segments, without limiting the range of base pairing. The first 10–15 lowest free-energy structures from the output of each run were compared and used to design antisense ODNs to target mRNA areas of 14–22 nucleotides that were predicted to be at least 60% unpaired. Some of the 170 antisense ODNs were designed based on two other considerations: (*a*) 19 ODNs were designed to span areas containing the tetranucleotide GGGA sequence on the target mRNA, which has been suggested to be a favorable target motif for antisense ODNs (25); and (*b*), at least one ODN was designed to encompass the start codon of each gene. For these two classes of ODNs, the numbers of unpaired bases on the target sites were also maximized based on the predicted mRNA structures, but the unpaired nucleotides could be <60%. It is of note that two ODNs, M2-47-as and C45-30-as, that were targeted to GAAA-containing sites were among the nine highly active antisense ODNs, and none of the ODNs encompassing the start codon had high activity.

All ODNs were custom-synthesized and purified to ~99% (MGW Biotech, Ebersberg, Germany), and the 16 phosphorothioate-end-modified antisense ODNs and control ODNs were further ethanol-precipitated twice to remove small-molecule impurities. Synthetic siRNAs (Dharmacon, Lafayette, CO) were further ethanol-precipitated twice. Following are the regions of the genes targeted by the 16 end-modified antisense ODNs and 8 siRNAs, as specified by the nt numbers of cDNA sequence entries (*hCdc6*, U77949; *hMcm2*, NM_004526; and *hCdc45*, AF053074) in databases: The antisense ODNs (C6-, targeted to *hCdc6*; M2-, targeted to *hMcm2*; C45-, targeted to *hCdc45*) were as follows: C6-30 (nt 1006–1025), C6-33 (nt 1163–1181), C6-35 (nt 1231–1246), C6-39 (nt 1550–1567), M2-33 (nt 1686–1705), M2-34 (nt 1716–1731), M2-47 (nt 2330–2346), M2-61 (nt 3268–3283), C45-8 (nt 455–469), C45-18 (nt 874–893), C45-22 (nt 1040–1059), and C45-30 (nt 1200–1215). The siRNAs (siC6., targeted to *hCdc6*; siM2., targeted to *hMcm2*; siC45., targeted to *hCdc45*): siC6.1 (nt 1234–1254), siC6.2 (nt 1085–1105), siC6.3 (nt 2509–2529), siM2.1 (nt 643–663), siM2.2 (nt 2727–2747), siM2.3 (nt 2804–2824), siC45.1 (nt 1189–1209), and siC45.2 (nt 460–480). The sequences of the mismatched control ODNs and RNAs that appear in the figures are as follows: C6-35-mm, 5′-AAGATGGGTAGGTCAA-3′; M2-47-mm, 5′-TCCCTCAGGTGGAAGCG-3′; C45-30-mm, 5′-AAGGAGTTGTCTCTCC-3′; siC6.1 mm (the sense strand; same for other control RNAs), 5′-AATTTTCCGCCTTATACCAGA-3′; siC6.2 mm, 5′-AAGACCGGTAGGTTTAGCACA-3′; siM2.1 mm, 5′-AAGGAGCGCATCTCCGACATG-3′; and siC45.1 mm, 5′-AAGGATGGCTCGGGAACGGAC-3′.

Cell Culture, Transfection, and Cell Viability Assay. All culture media and transfection agents were from Invitrogen (Carlsbad, CA). Cells were grown in various media supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. HeLa (cervical adenocarcinoma), Hone1 (nasopharyngeal cancer), T-Tn (esophageal cancer), and HepG2 (hepatocellular carcinoma) cells were grown in DMEM. NCI-H446 (small cell lung carcinoma), NCI-H460 (large cell lung carcinoma), Chang (from liver; a gift from M. Lung, Hong Kong University of Science and Technology, Hong Kong, China, who obtained the cells from R. S. Chang, University of California at Davis, Davis, CA, who generated the line; the cells we used are

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[3] Our unpublished data.

[4] The abbreviations use are: ODN, oligodeoxynucleotide; siRNA, small interfering RNA; FBS, fetal bovine serum; WST-1, water-soluble tetrazolium {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio[-1,3-benzene disulfonate}. nt, nucleotide; as, antisense; mm, mismatched; GFP, green fluorescent protein; RT-PCR, reverse transcription-PCR; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyltransferase (Tdt)-mediated nick end labeling; 6-CFDA, 6-carboxyfluorescein diacetate.

tumorigenic, as determined by non-contact-inhibited growth and xenograft growth in nude mice; data not shown), BEL-7402 (hepatocellular carcinoma), and L-02 (normal human liver cells; Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Shanghai, China) were cultured in RPMI 1640. Hep3B (hepatocellular carcinoma; $p53^{-/-}$) were maintained in MEM. One day before transfection, cells were trypsinized, diluted in growth medium, and seeded in 96-well plates. At 70–80% confluence, cells were transfected with a complex of ODNs (final concentrations during transfection, 0.7 μM for HeLa, Hone1, T-Tn, and Chang cells and 1.0 μM for other cells) and 0.7 μl (for HeLa, T-Tn, and Chang cells) or 1.0 μl (for other cells) of Lipofectamine 2000 (Invitrogen) in 100 μl of OptiMEM medium for 4 h. Cells were then incubated in FBS-containing growth medium for 44 h, before WST-1 assays were performed as described previously (26). Counted numbers of cells were used to construct the standard curves. siRNAs were transfected into cells at 40–50% confluence with a complex of siRNA (final concentration during transfection, 100 nM) and 1.0 μl of Oligofectamine (Invitrogen) in 150 μl of OptiMEM medium for 18 h. The cells were then incubated in FBS-containing growth medium for 54 h before the WST-1 assay.

Rescue by the Silently Mutated *hCdc6* Gene. The sense sequence targeted by C6-35-as was changed from 5′-TTGAACTTCCCACCTT-3′ to 5′-CTCAATTTTCCGCCGT-3′. The silently mutated and wild-type *hCdc6* genes were cloned into pcDNA3.1/Zeo(−) (Invitrogen) to obtain pC6SM and pC6WT, respectively. Cells at 80–90% confluence were transfected with 0.9 μg of pcDNA3.1/Zeo(−), pC6WT, or pC6SM together with 0.2 μg of pEGFP-N1 (Clontech, Palo Alto, CA) in 6-well plates with use of Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Transfected cells were trypsinized, split, and reseeded in growth medium, incubated for 12 h, and then further treated with Lipofectamine 2000, C6-35-mm, or C6-35-as or left untreated for normalization. Cells were collected 48 h post-ODN transfection, and GFP-positive cells were counted under a fluorescence microscope.

RT-PCR and Western Blotting. Total RNA was isolated with use of TRIzol reagent (Invitrogen) at 0 h (for ODNs) or 6 h (for siRNAs) post-transfection. Total RNA (100 ng) was used for cDNA synthesis using First Strand cDNA Synthesis Kit (MBI, Ramat Hasharon, Israel). PCR coamplification of *β-actin* with *hCdc6*, *hMcm2*, or *hCdc45* was performed with 1 μl of cDNA and *Taq* polymerase in 25-μl reactions. The ratio of *β-actin* primers to *hCdc6*, *hMcm2*, or *hCdc45* primers was 1:10. The sequences of the RT-PCR primers were as follows: *β-actin*, 5′-GATATCGCCGCGCTCGTCG-3′ and 5′-GGGAGGAGCTGGAAGCAG-3′; *hCdc6*, 5′-GGCCAGGATGTATTG-TACAC-3′ and GGCCCGAATGTGTAAAGC-3′; *hMcm2*, 5′-TATTATCAC-TAGCCTCTCC-3′ and 5′-GGTAGCGGGCAAAAGCTTG-3′; and *hCdc45*, 5′-CCAGGAGTTCCTTGCAGAC-3′ and 5′-CAATCTAAATGAAAGCCA-GTT-3′. The PCR program was as follows: 1 cycle of 95°C for 3 min followed by 35 cycles of 94°C for 30 s, 58°C for 3 s, and 72°C for 45 s and 1 cycle of 72°C for 5 min. For Western blotting, total proteins (15–20 μg) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose. For *hCdc6*, cells were collected at 1 h (for ODNs) or 10 h (for siRNAs) post-transfection. For *hMcm2* and *hCdc45*, cells were collected at 2 h post-transfection (for ODNs only), and proteins were treated with *Lambda* phosphatase (New England Biolabs, Beverly, MA) before SDS-PAGE. Antitubulin immunoblotting (data not shown) and Ponceau S staining were used as loading controls. All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), except for anti-hMcm2, which was from Becton Dickinson (Palo Alto, CA).

BrdUrd Incorporation. Transfected cells grown on cover slips coated with poly-D-lysine (Sigma, St. Louis, MO) were incubated with 50 μM BrdUrd (Sigma) for 1 h at 37°C at 1 h (for ODNs) or 12 h (for siRNA) post-transfection. Cells were then fixed in 20 mM glycine (pH 2.0)–70% ethanol at room temperature for 45 min and incubated sequentially with anti-BrdUrd and antimouse IgG-FITC conjugates (Sigma), each for 1 h at 37°C, with three washes in PBS after each antibody incubation. BrdUrd incorporation was observed for at least 200 cells/sample under a fluorescence microscope.

Apoptosis Assays. Apoptosis was assayed by use of the In Situ Cell Death Detection Kit with Fluorescein (TUNEL assay; Roche, Palo Alto, CA), the Annexin V-Cy3 Apoptosis Detection Kit (plus 6-CFDA; Sigma), and the caspase inhibitor Z-VAD-fmk (Enzyme Systems, Livermore, CA). For TUNEL assays, transfected cells grown on cover slips for 2 h (for ODNs) or 16 h (for siRNAs) post-transfection were fixed for 1 h in fresh 4% paraformaldehyde, rinsed in PBS, permeabilized for 2 min on ice in 0.1% Triton X-100–0.1% sodium citrate, and then labeled with FITC-dUTP and terminal transferase for 30 min. TUNEL-positive cells were observed under a fluorescence microscope. Annexin V assays were done according to the manufacturer's instructions with some modifications. Cells on cover slips were stained with a solution of 6-CFDA and Annexin V–Cy3 at the same time points as in the TUNEL assays. Z-VAD-fmk (final concentration, 1 μM) was added to growth medium immediately after transfection, and the cells were incubated for another 48 h before the WST-1 assay.

Flow Cytometry. Both floating and attached cells were collected and washed once with PBS. Cells were fixed in 70% ethanol for 1 h to overnight at −20°C, washed thoroughly with PBS, and then stained in 50 μg/ml RNase A, 0.1% Triton X-100, 0.1 mM EDTA (pH 7.4), and 50 μg/ml propidium iodide for 30 min at 4°C. Samples were analyzed with an FACSort instrument (Becton Dickinson).

RESULTS

Design and Initial Screening of Antisense ODNs. The primary action of antisense ODNs to inhibit gene expression is to bind to the complementary target mRNA and activate the endogenous RNase H to cleave the mRNA. We first applied the computer program mFold (24) to predict the secondary structures of the mRNAs of the target genes. The areas on the mRNA predicted to have 60–100% unpaired nucleotides were chosen as potential target sites for antisense ODNs. A total of 170 antisense ODNs of 14–22 nucleotides each were designed, of which 66 (named C6-1-as through C6-66-as) were targeted to *hCdc6*, 64 (M2-1-as through M2-64-as) to *hMcm2*, and 40 (C45-1-as through C45-40-as) to *hCdc45*. Some of the 170 antisense ODNs were designed based on other considerations, including targeting areas containing the tetranucleotide GGGA sequence (25) and the AUG start codon on the target mRNA (see "Materials and Methods" for more details).

Because silencing of replication-initiation genes was expected to result in inhibition of DNA replication and thus of cell proliferation, the 170 antisense ODNs were first individually subjected to initial screening for their abilities to inhibit cell proliferation in human cell lines derived from liver, esophageal, and cervical cancers. Sixteen of the antisense ODNs were initially considered active and chosen for further analysis (see below) because each inhibited cell growth by 50–70% (*i.e.*, viable cell numbers were 30–50% compared with untreated cells) in all of the cell lines tested, whereas the transfection agent alone inhibited cell growth by ~20% (data not shown), as measured by the WST-1 cell viability assay (26).

Selected Antisense ODNs and siRNAs Can Inhibit Target Gene Expression. To investigate the effects on inhibition of target gene expression, DNA replication and cell proliferation, the 16 ODNs that showed antiproliferation activities in the initial screening were modified with phosphorothioate linkages at both the 5′ and 3′ ends to increase resistance to exonucleases. Similarly modified sense and mismatched ODNs were used as the controls. The mismatched ODNs had the same base compositions of the corresponding antisense ODNs, but one in every five nucleotides on average in each ODN was changed. Possible inhibition of target gene expression was analyzed in several human cancer cell lines (Chang, HepG2, Hep3B, HeLa, and Hone1) and normal human liver L-02 cells (27, 28); we also confirmed that growth of L-02 cells is contact-inhibited (data not shown). Similar levels of transfection (~90% as measured by using an FITC-labeled ODN, C6-35-as; data not shown) for different cell lines were obtained by optimizing the amounts of the transfection agent and ODNs. Total RNA and proteins from the untreated cells and cells treated with the transfection agent alone or transfected with the antisense or control ODNs were analyzed by RT-PCR (Fig. 1, *A–C*) and Western blotting (Fig. 1, *D–F*), respectively. For RT-PCR, each RNA sample was analyzed for the mRNA of two other replication-

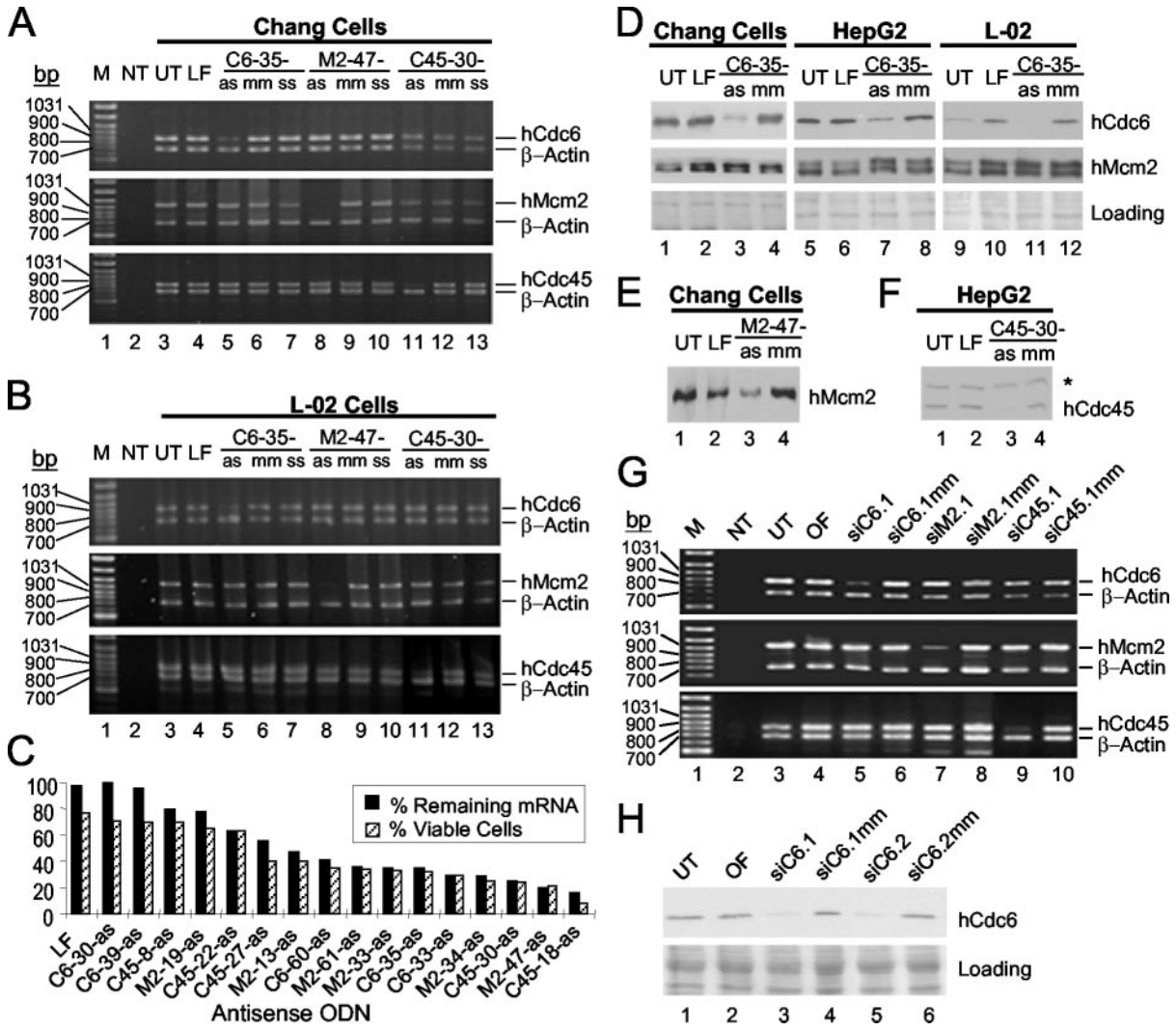

Fig. 1. Selected antisense ODNs and siRNAs inhibit target gene expression. *A* and *B*, RT-PCR analysis of the mRNA levels of *hCdc6*, *hMcm2*, or *hCdc45*, coamplified with the *β-actin* gene in Chang (*A*) and L-02 (*B*) cells treated with different ODNs as indicated. *Lane NT*, no template (negative control for RT-PCR); *Lane UT*, untreated cells; *Lane LF* (for Lipofectamine 2000), cells treated with the transfection agent alone; *Lane M*, molecular markers. *C*, quantitation of remaining mRNA levels for the target genes, as determined by densitometry scanning of RT-PCR products and by the number of viable cells measured by the WST-1 assay in Chang cells treated with different antisense ODNs. Data were normalized to the untreated cells. *D*, Western blot analysis of hCdc6 and hMcm2 proteins in Chang, HepG2, and L-02 cells treated with an antisense (C6-35-as) or mismatched (C6-35-mm) ODN. Note that hMcm2p appears as a doublet in these protein samples, which were not treated with phosphatase. Ponceau S staining of the blots was used as the loading control. *E*, Western blot analysis of hMcm2p in Chang cells treated with M2-47-as or M2-47-mm. Protein extracts were treated with phosphatase. *F*, Western blot analysis of hCdc45p in HepG2 cells treated with C45-30-as or C45-30-mm. *, band cross-reactive with the anti-Cdc45 antibodies. *G*, RT-PCR analysis of the mRNA levels for *hCdc6*, *hMcm2*, or *hCdc45*, coamplified with the *β-actin* gene in HeLa cells treated with different siRNAs and mismatched control RNAs as indicated. *Lane OF* (for Oligofectamine), transfection agent; *Lane M*, molecular markers. *H*, Western blot analysis of hCdc6 protein in HeLa cells treated with siRNAs or mismatched RNAs as indicated.

initiation proteins in addition to the target gene and the internal control *β-actin* gene was coamplified with each of the three replication-initiation genes.

The results show that the mRNA and protein product levels of the target genes were significantly and specifically reduced by several antisense ODNs in all cell lines tested. In Chang cells (Fig. 1*A*) and L-02 cells (Fig. 1*B*), for example, C6-35-as targeted to *hCdc6* lowered the level of *hCdc6* mRNA (Fig. 1, *A* and *B*, *Lane 5*, *top panels*), but not the mRNA levels of two other replication-initiation proteins, hMcm2p and hCdc45p, in the same cells transfected with C6-35-as (Fig. 1, *A* and *B*, *Lane 5*, *middle* and *bottom panels*). Similarly, M2-47-as targeted to *hMcm2* diminished the level of *hMcm2* mRNA (Fig. 1, *A* and *B*, *Lane 8*, *middle panels*) without affecting the levels of the mRNAs encoding hCdc6p and hCdc45p (Fig. 1, *A* and *B*, *Lane 8*, *top* and *bottom panels*), and C45-30-as targeted to *hCdc45* reduced the level of *hCdc45* mRNA (Fig. 1, *A* and *B*, *Lane 11*, *bottom panels*) but did not affect the levels of *hCdc6* and *hMcm2* mRNA (Fig. 1, *A* and *B*, *Lane 11*, *top* and *middle panels*). The transfection agent (*Lane LF*) alone and the sense (*Lane ss*) and mismatched (*Lane mm*) control ODNs did not noticeably alter the expression levels of the genes analyzed (Fig. 1, *A* and *B*). Similar results were obtained for all other cell lines tested (data not shown). In addition, six other antisense ODNs, C6-60-as, M2-61-as, M2-33-as, C6-33-as, M2-34-as, and C45-18-as, also significantly reduced (by ≥60%) the mRNA levels of the corresponding target genes (Fig. 1*C*).

As the mRNA levels were specifically reduced by the antisense ODNs, the protein levels of the target genes were also correspondingly lowered (Fig. 1, *D–F*). For example, as shown in Fig. 1*D*, the level of hCdc6p (*top panels*), but not of hMcm2p *(middle panels*) were reduced by C6-35-as (*Lanes 3*, *7*, and *11*) in three cell lines, whereas the transfection agent (*Lanes 2*, *6*, and *10*) and C6-35-mm (*Lanes 4*, *8*, and *12*) had no effect. Similarly, M2-47-as, but not the transfection agent or M2-47-mm, reduced the hMcm2p level (Fig. 1*E*), and C45-30-as, but not the transfection agent or C45-30-mm,

lowered the hCdc45p level (Fig. 1*F*). Together, these data suggest that the antisense ODNs effectively and specifically inhibited the expression of the corresponding target genes.

We also used a different gene-silencing strategy, RNA interference. Three siRNAs (siC6.1, siC6.2, and siC6.3) targeted to *hCdc6*, three (siM2.1, siM2.2, and siM2.3) to *hMcm2*, and two (siC45.1 and siC45.2) to *hCdc45* were designed based on general strategies (29), except that siC6.1 and siC45.1 were targeted to areas overlapping with the target sites of two active antisense ODNs, C6-35-as and C45-30-as, respectively. These two siRNAs still obeyed the general siRNA selection guidelines (29). All eight siRNAs were able to reduce target gene expression by 60–90% as measured by RT-PCR (Fig. 1*G*) and immunoblotting (Fig. 1*H*) experiments (data for some of the siRNAs not shown), whereas the transfection agent (*Lane OF*) and the control RNAs (*Lane mm*), with two to four mismatched bases, did not significantly affect the mRNA or protein levels of the target genes (Fig. 1, *G* and *H*).

DNA Replication Is Inhibited by the Antisense ODNs and siRNAs That Can Silence the Target Genes. Using BrdUrd incorporation assays, we showed that silencing of replication-initiation genes by antisense ODNs (Fig. 2, *A* and *B*) and siRNA (Fig. 2*C*) led to specific inhibition of DNA replication. For example, C6-35-as significantly reduced the number of HepG2 cells that incorporated BrdUrd, whereas the transfection agent alone and C6-35-mm had no effect (Fig. 2*A*). Similar results were also obtained for several other cell lines tested, as shown quantitatively in Fig. 2*B*. Approximately 25–45% of untreated cells and of those treated with the transfection agent alone or C6-35-mm incorporated BrdUrd, indicating that these cells were in S phase during the labeling period, as expected for exponentially growing human cells in culture. In contrast, only ~5% of cells were able to incorporate BrdUrd after transfection with C6-35-as in all cell lines tested (Fig. 2*B*). Therefore, C6-35-as inhibited DNA replication by 70–90% when the results were normalized to those for the untreated cells. Similar results were also obtained for other antisense ODNs tested, including M2-47-as, C45-18-as, and C45-30-as (data not shown). siRNAs inhibited DNA replication to degrees similar to those for the antisense ODNs (Fig. 2*C*). The results from both antisense ODNs and siRNAs are consistent with hCdc6p, hMcm2p, and hCdc45p being required for DNA replication in human cells.

Cell Proliferation Is Inhibited by the Antisense ODNs and siRNAs That Can Silence the Target Genes. The effects of the antisense and control ODNs on the proliferation of cancer and normal cells were measured by use of the WST-1 assay. The antisense ODNs C6-35-as, M2-47-as, and C45-30-as inhibited the proliferation of cancer cells (HepG2, Hep3B, and Chang cells) by 60–90% (*i.e.*, the

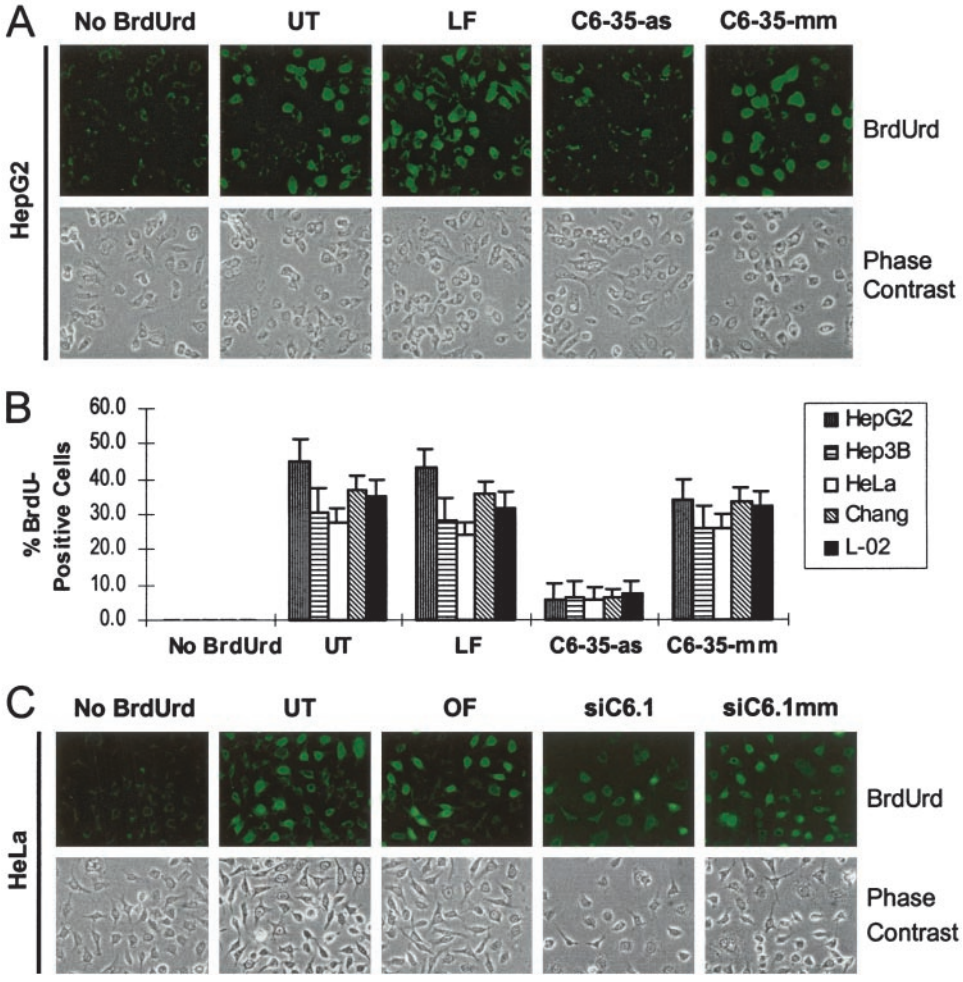

Fig. 2. Antisense ODNs and siRNAs inhibit DNA replication. *A*, BrdUrd incorporation in HepG2 cells transfected with the antisense ODN C6-35-as or the mismatched control ODN C6-35-mm. *Top row*, fluorescence micrographs after BrdUrd labeling and detection; *bottom row*, phase-contrast micrographs. Note that there was nonspecific cytoplasmic staining by the antibodies; therefore, only cells with nuclear fluorescence were scored as BrdUrd-positive cells. *B*, average percentages (±SD; *bars*) of cells incorporating BrdUrd for HepG2, Hep3B, HeLa, Chang, and L-02 cells transfected with C6-35-as or C6-35-mm. *C*, BrdUrd incorporation in HeLa cells transfected with the siRNA siC6.1 or the mismatched control RNA siC6.1 mm. Note that most cells treated with siC6.1 had only nonspecific cytoplasmic staining. *No BrdU*, negative control without BrdUrd labeling.

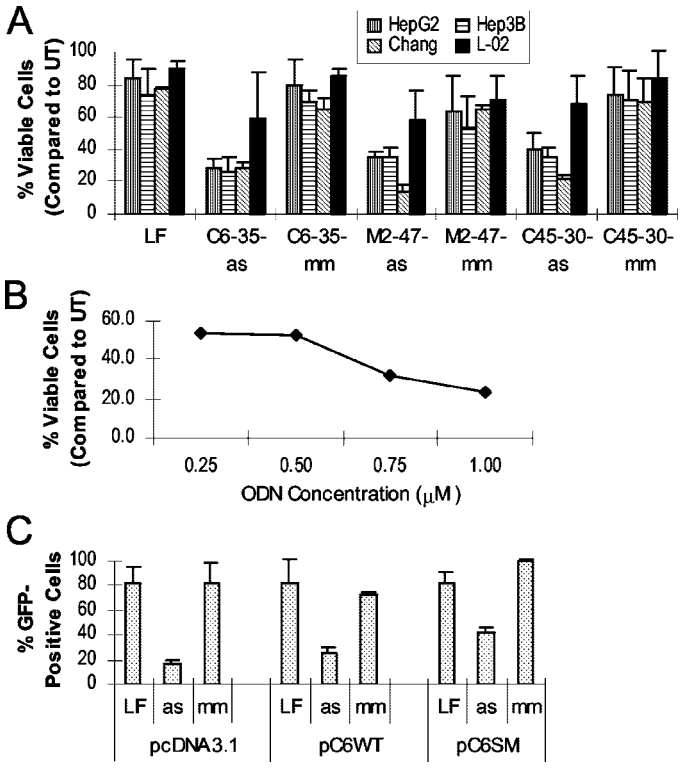

Fig. 3. Antisense ODNs inhibit cell proliferation and a silently mutated gene lessens the effects of the antisense ODN. *A*, cell viability measured by the WST-1 assay after transfection with different antisense or mismatched ODNs in HepG2, Hep3B, Chang, and L-02 cells. Data are average percentages (±SD; *bars*) of viable cells compared with untreated cells from three or more experiments. *B*, HepG2 cells were treated with different concentrations of M2-47-as, and the viability of the cells was measured by the WST-1 assay. *C*, the silently mutated *hCdc6* gene (pC6SM) lessens the inhibition of cell proliferation by C6-35-as in HeLa cells. Cells were pretransfected with pEGFP-N1 together with the vector (pcDNA3.1), the wild-type hCdc6 (*pC6WT*) or pC6SM, split, and reseeded, and aliquots of the cells were further treated with the transfection agent alone (*LF*), C6-35-as (*as*), or C6-35-mm (*mm*) or left untreated for normalization. The data are average percentages (±SD; *bars*) of GFP-positive cells treated with LF, C6-35-as, or C6-35-mm normalized to cells not further treated.

numbers of residual viable cells were 10–40% of the values for untreated cells), whereas inhibition of the normal L-02 cells was significantly less, at ~30–40% (Fig. 3*A*). This was consistent with our finding that although cell proliferation was impeded in both cancer and normal cells when DNA replication was inhibited, only cancer cells underwent apoptosis (see below). The residual viable cancer cells after the first transfection with the antisense ODNs were those that were un- or undertransfected cells because they could be inhibited to the same extent after regrowth, reseeding, and a second transfection by the same ODNs (data not shown).

As controls, the inhibition of cell proliferation was ~20% by the transfection agent alone and ~30% by the mismatched control ODNs (Fig. 3*A*). These values were significantly lower than the 60–90% inhibition afforded by the antisense ODNs (Fig. 3*A*), consistent with sequence-specific inhibition of target gene expression by the antisense ODNs. The antisense ODNs also inhibited cell proliferation in other cancer cell lines: BEL-7402, T-Tn, NCI-H446, and NCI-H460 (data not shown). Six other antisense ODNs (C6-60-as, M2-61-as, M2-33-as, C6-33-as, M2-34-as, and C45-18-as) that could reduce expression of the target genes by ≥60% also inhibited cell proliferation by ≥60% (Fig. 1*C*). In addition, the activities of the antisense ODNs were dose dependent (see Fig. 3*B* for an example), consistent with specific effects of the antisense ODNs in target gene silencing. Three siRNAs, siC6.1, siM2.1, and siC45.1, that were tested also inhibited proliferation of HeLa cells by 70–75% (data not shown).

Connection between Silencing of the Target Genes and Inhibition of Cell Proliferation. As described above, selected antisense ODNs specifically inhibited target gene expression (Fig. 1, *A–F*), DNA replication (Fig. 2, *A* and *B*), and cell proliferation (Figs. 3*A* and 1*C*). We then asked whether the observed effects on cell proliferation and viability were the consequence of silencing of the target genes, as opposed to unintended cellular components, by the antisense ODNs. We first compared the inhibition of target gene expression with that of cell proliferation for all 16 antisense ODNs analyzed (Fig. 1*C*). A general trend was found: the greater the reduction of target gene expression (as reflected in lower amounts of residual mRNA), the more the inhibition of cell proliferation (as reflected in lower cell viability), suggesting that inhibition of cell proliferation was consequently linked to silencing of the target genes.

The above inference was supported by rescue experiments using a silently mutated *hCdc6* gene whose sequence was changed in every wobble base on the target site for an antisense ODN, C6-35-as, without altering the encoded amino acids. Plasmid pC6SM (where SM denotes silent mutant), which expressed the silent mutant gene and was pretransfected into the cells, should have rescued the cells from the effects of C6-35-as if inhibition of cell proliferation resulted from silencing of the *hCdc6* gene by C6-35-as. Similar rescue strategies have been used previously to examine specificities of antisense ODNs (30) and siRNAs (31).

We first confirmed that hCdc6p was expressed from both HA-tagged and untagged versions of pC6SM and pC6WT (the wild-type *hCdc6* gene) after transient transfection by immunoblotting with anti-HA and anti-hCdc6 antibodies (data not shown). After transfection with C6-35-as, the level of hCdc6p expressed from pC6WT, as well as that from the endogenous hCdc6p, was much reduced as expected, whereas that from pC6SM was only moderately reduced (data not shown). However, possible rescue of cell viability could not be easily observed by measuring the cell number of the entire cell population because the transfection efficiency for plasmid DNA (~20%, as measured in a plasmid expressing GFP; data not shown) was much lower than that for ODNs. To overcome this problem, we cotransfected pC6SM with a plasmid expressing GFP (at a 4:1 molar ratio in favor of pC6SM or pC6WT) and monitored the percentage of GFP-positive cells in the population as the indication of cell viability after further transfection with C6-35-as. If the expected rescue occurred, the percentage of GFP-positive cells transfected with C6-35-as (normalized to cells not further treated) should have been higher for cells pretransfected with pC6SM than for those pretransfected with the vector control (pcDNA3.1) and those pretransfected with pC6WT.

As expected, pretransfection of the vector pcDNA3.1 did not rescue the cells from the effects of C6-35-as (Fig. 3*C*; ~18% GFP-positive cells, or ~82% growth inhibition). On the other hand, pretransfection with pC6SM increased the number of GFP-positive cells to ~45%, whereas pC6WT rescue was much lower, with ~25% GFP-positive cells (Fig. 3*C*). The reason that pC6SM did not fully rescue the cells (in which case the GFP-positive cells would be ~80%, as for the cells treated with the transfection agent instead of C6-35-as) is probably because some GFP-positive cells were under- or untransfected by pC6SM. As controls, the percentages of GFP-positive cells treated with the transfection agent alone or the mismatched control ODN (C6-35-mm) were not significantly affected by pretransfection with pC6SM, pC6WT, or pcDNA3.1 (Fig. 3*C*). These results strongly suggest that the inhibition of cell proliferation was the result of target gene silencing by the antisense ODN.

Apoptosis Occurs in Both p53-Positive and -Negative Cancer Cells But Not in Normal Cells When the Target Genes Are Silenced. Not only was cell proliferation inhibited, but most cancer cells transfected with the antisense ODNs and siRNAs died, as ob-

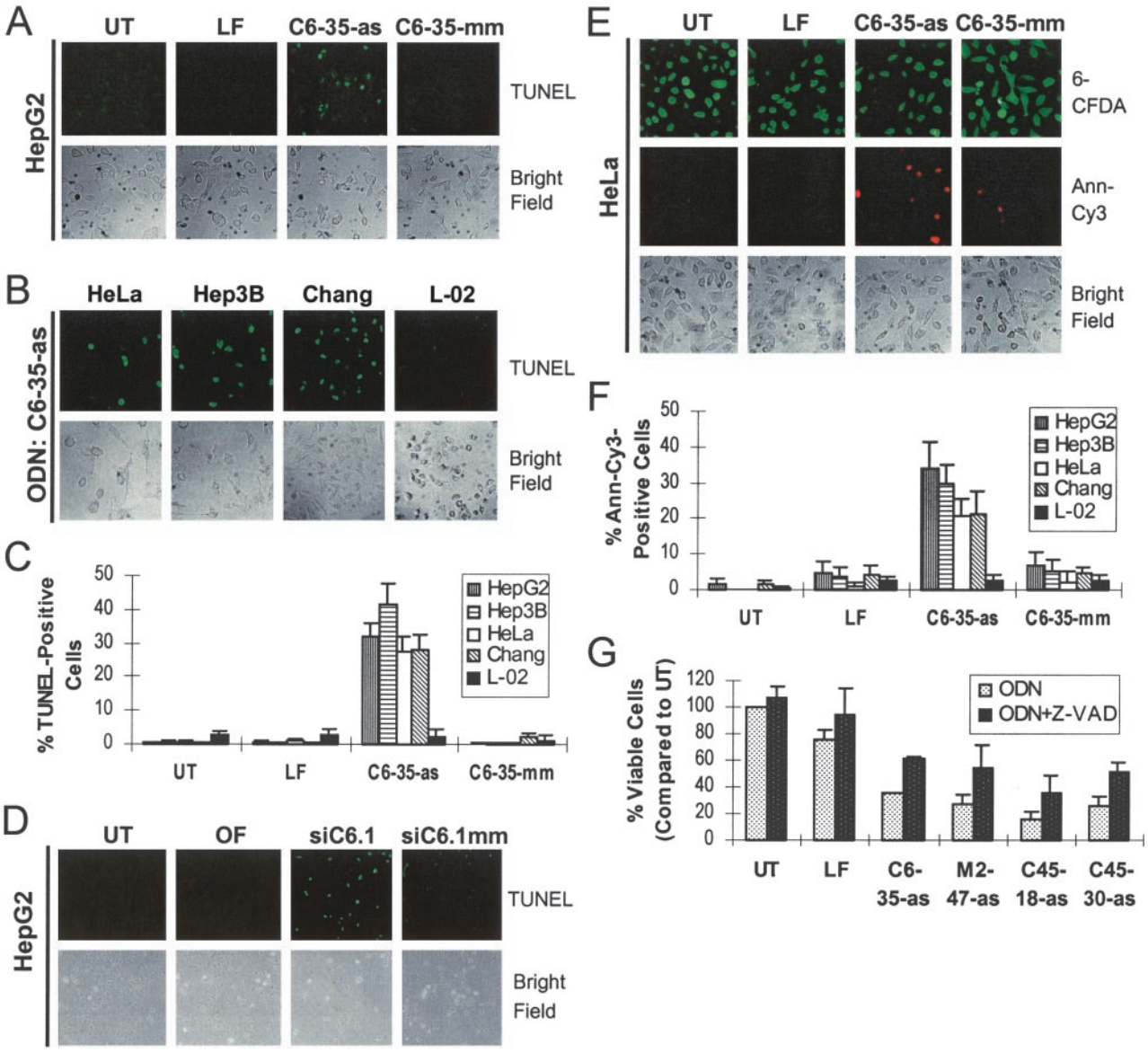

Fig. 4. Antisense ODNs and siRNA induce apoptosis in cancer cells. *A*, TUNEL assays were performed for untreated (*UT*) HepG2 cells and those treated with the transfection agent (*LF*), C6-35-as, or C6-35-mm. *B*, TUNEL assays for HeLa, Hep3B, Chang, and L-02 cells transfected with C6-35-as. *C*, average percentages (±SD; *bars*) of TUNEL-positive cells in different cell lines transfected with C6-35-as. LF and C6-35-mm were used as controls. *D*, TUNEL assays in HeLa cells transfected with the transfection agent (*OF*), siC6.1, or siC6.1 mm. *E*, Annexin V (*Ann-Cy3*) staining plus esterase (*6-CFDA*) assays in HeLa cells. *Green fluorescence* represents live or early apoptotic cells and *red fluorescence* represents apoptotic or necrotic cells. Cells positive for both represent early apoptotic cells. *F*, average percentages (±SD; *bars*) of Annexin V–Cy3-positive cells in different cell lines. *G*, apoptosis induced by the antisense ODNs was attenuated by the caspase inhibitor, Z-VAD-fmk. Chang cells were transfected with different antisense ODNs as indicated or also treated with Z-VAD after transfection. Cell viability was measured by the WST-1 assay. *Bars*, SD.

served under the microscope (data not shown). We then determined that the mode of cell death was apoptosis by several assays (Figs. 4 and 5). We first detected apoptosis by the TUNEL assay (32) in HepG2 cells after transfection with C6-35-as but not with the transfection agent or C6-35-mm (Fig. 4*A*). Similar degrees of apoptosis were induced in all of the cancer cell lines tested (HepG2, Hep3B, HeLa, and Chang) by C6-35-as but not by the transfection agent or C6-35-mm, and normal cells (L-02) did not undergo apoptosis (Fig. 4, *B* and *C*). Similar results were obtained for other antisense ODNs analyzed, including M2-47-as, C45-18-as, and C45-30-as (data not shown). The siRNA siC6.1, but not the transfection agent alone or the mismatched RNA, also induced apoptosis in HeLa cells (Fig. 4*D*). We confirmed that similar levels of transfection (data not shown), of target gene silencing (Fig. 1), and of inhibition of DNA replication (Fig. 2) were achieved in different cell lines by use of transfection conditions optimized for different cell lines. Therefore, because apoptosis occurred in both p53-wild-type (HepG2) and p53-defective (Hep3B) liver cancer cell lines, but not in normal liver cells (L-02), the results suggest that silencing of replication-initiation genes induces apoptosis in cancer, but not normal, cells and that the apoptosis is p53-independent.

We further analyzed the apoptotic effects of the antisense ODNs with Annexin V staining (33) coupled with an esterase assay (34), and the results concurred with those from the TUNEL assays described above. Annexin V-positive HeLa cells were observed after transfection with C6-35-as, but not after transfection with the transfection agent or C6-35-mm (Fig. 4*E*, *middle row*). Similar percentages of Annexin V-positive cells were observed for all cancer cell lines tested, whereas normal L-02 cells had few Annexin V-positive cells (Fig. 4*F*). Similar results (not shown) were obtained with all other antisense ODNs that were also tested in the TUNEL assays. Therefore, Annexin V staining assays support the conclusion drawn from the TUNEL

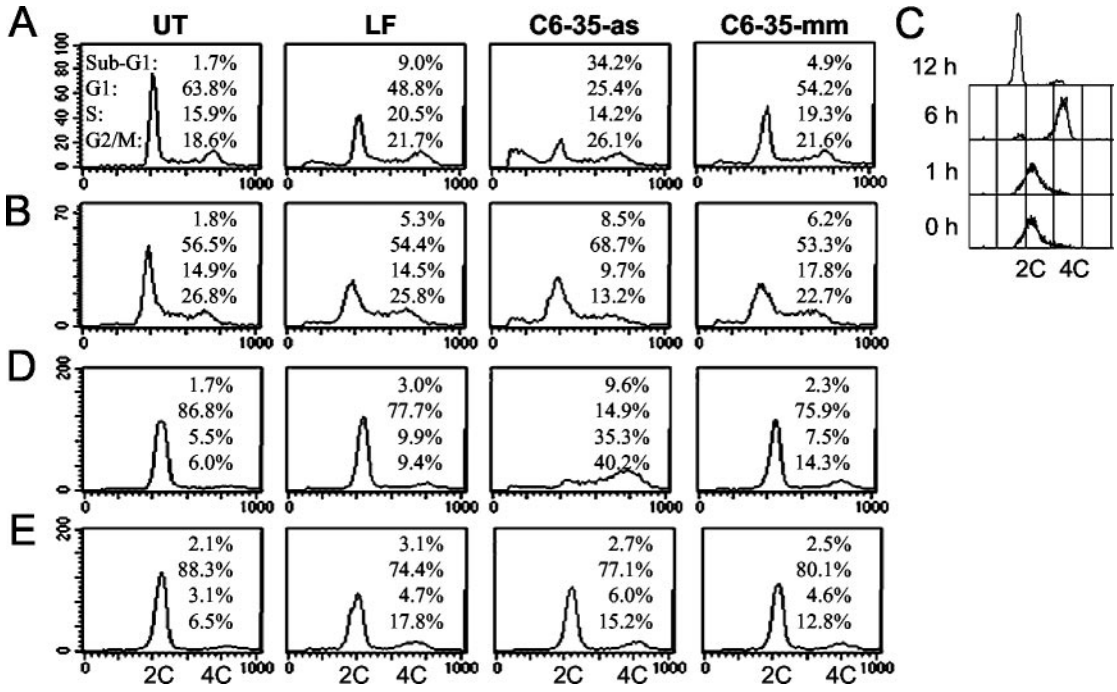

Fig. 5. Flow cytometry analysis of the DNA content of untreated (*UT*) cells and those treated with the transfection agent (*LF*), C6-35-as, or C6-35-mm in different cell lines. *A* and *B*, asynchronous HepG2 (*A*) or L-02 (*B*) cells were treated with the transfection agent, C6-35-as, or C6-35-mm and analyzed 20 h post-transfection. *C*, HeLa cells were synchronized by double thymidine G_1 block (0 h), released into fresh medium, and then analyzed at different time points after release as indicated. *D* and *E*, HeLa cells were treated with transfection agent, C6-35-as, or C6-35-mm at 1 h (*D*) or 6 h (*E*) after release from double thymidine block and then analyzed at 8 h post-transfection. The fractions of cells in different phases of the cell cycle are indicated.

assays. In addition, our data (Fig. 4*E*) also suggest that cell death induced by the antisense ODNs was not necrosis because the cells were still positive for 6-CFDA, which measures the activity of cellular esterases (which convert 6-CFDA to 6-carboxyfluorescein; Ref. 34) and should therefore be positive for early apoptotic cells, as well as for viable cells, but not for necrotic cells.

To determine whether apoptosis induced by the antisense ODNs proceeded via caspase activation, we added the caspase inhibitor Z-VAD to cell cultures immediately after transfection with the antisense ODNs and observed significantly reduced cell death as measured by the WST-1 assay (Fig. 4*G*) and microscopic observations (data not shown). Without Z-VAD, the numbers of viable cells after transfection with the four antisense ODNs tested were 18–30% of the numbers of untreated cells, whereas Z-VAD increased the percentages to 40–65% (Fig. 4*G*). These results suggest that apoptosis induced by the antisense ODNs was caspase dependent.

Apoptosis of cancer cells was also observed by flow cytometric analysis of DNA content. HepG2 cells had a sub-G_1 (<2C DNA) population (34.2%) after transfection with C6-35-as (Fig. 5*A*). On the other hand, most normal cells (L-02) showed G_1 arrest (68.7%) with a reduction of the S and G_2-M populations, and apoptosis was probably prevented as a consequence (Fig. 5*B*). As controls, the transfection agent and C6-35-mm did not significantly affect cell cycle distributions compared with the untreated cells in either the HepG2 or L-02 cell line (Fig. 5, *A* and *B*).

To examine the effects of inhibiting the expression of replication-initiation proteins in different phases of the cell cycle, we performed flow cytometry experiments with synchronized HeLa cells, which showed good synchrony with double thymidine block in G_1 and subsequent release (Fig. 5*C*). Most cells had not entered the S phase 1 h after release, but most had completed the S phase by 6 h after release and then finished mitosis after 12 h. We selected the time points of 1 and 6 h after release from G_1 block to transfect the cells with C6-35-as, using the transfection agent alone and C6-35-mm as the controls, and then analyzed the cells 8 h after transfection (with a transfection time of 4 h, the cells at harvest were at 12 h after release from G_1). When the cells were treated with the transfection agent or C6-35-mm at 1 h after release (when the cells had not entered S phase, according to the data shown in Fig. 5*C*), they were able to complete DNA replication and mitosis by the harvest time, as did the untreated cells (Fig. 5*D*), consistent with unimpeded cell cycle progression shown in Fig. 5*C*. In contrast, the cells transfected with C6-35-as at 1 h after release were blocked in the S and G_2-M phases (Fig. 5*D*; 35.3% in S and 40.2% in G_2-M phase), and apoptosis was observed at a later time point (20 h post-transfection; data not shown). On the other hand, when the cells were transfected with C6-35-as at 6 h after release from G_1 (when they had completed DNA replication, according to the results shown in Fig. 5*C*), they were able to undergo mitosis to become G_1 cells by the harvest time, as did the untreated cells and those treated with the transfection agent or C6-35-mm (Fig. 5*E*). Therefore, our data (Fig. 5, *C–E*) are consistent with the replication-initiation protein being required for DNA replication but not for mitosis, indicating that the antisense ODN did not have significant nonspecific effects on the cells, at least during mitosis.

DISCUSSION

We have shown that selected antisense ODNs and siRNAs targeted to three human DNA replication-initiation genes effectively and specifically inhibited target gene expression, DNA replication, and cell proliferation. Moreover, the antisense ODNs and siRNAs induced apoptosis in both p53-positive and -negative cancer cells but not in normal L-02 cells. Given that various antisense ODNs and siRNAs, targeted to different replication-initiation genes, brought about similar effects on a variety of cancer cell lines, we conclude that a novel and effective anticancer strategy would be to inhibit the expression of selective replication-initiation proteins with antisense ODNs and siRNAs.

Our data show that inhibition of cell proliferation and induction of apoptosis is consequently linked to silencing of the target genes by the antisense ODNs and siRNAs, as summarized below. (*a*) Several antisense ODNs and siRNAs largely silenced the corresponding target genes while leaving the expression of other functionally related replication-initiation proteins unaffected. (*b*) The 16 antisense ODNs targeted to three replication-initiation genes showed good agreement between their abilities to silence the target genes and to inhibit cell proliferation. (*c*) A silently mutated gene significantly lessened the effects of the antisense ODN, providing very strong evidence that the inhibition of cell proliferation and induction of apoptosis did not result from unintended inhibition of an unknown cellular target(s). (*d*) The antisense ODN did not block mitosis when synchronized cells were transfected after they had completed S phase, indicating that the antisense ODN did not cause any detectable harmful effect on cells in the G_2-M phase. (*e*) Inhibition of the expression of the target genes by two different gene-silencing strategies, antisense and RNA interference, had similar effects on DNA replication, cell proliferation, and cell viability.

The p53-independent nature of apoptosis induced by inhibiting the expression of replication-initiation proteins is highly desirable for cancer therapy, because approximately half of all cancers are p53 deficient. Therefore, and because DNA replication is required for the growth of all cancers, this anticancer strategy should be broadly applicable. Furthermore, it is of high significance that normal cells, at least the L-02 cells that we examined, do not die when the expression of replication-initiation proteins is inhibited. This is probably because normal cells possess intact checkpoint controls to arrest the cell cycle in G_1 phase to avoid cell death when DNA replication is prevented, whereas cancer cells usually lack normal checkpoint controls and will enter an abortive S phase, producing partially replicated chromosomes and resulting in chromosome damage and apoptosis.

Because the vast majority of human body cells are nonproliferating and thus do not express or need most replication-initiation proteins (1, 2),[3] antisense ODNs, siRNAs, and other agents that can silence these genes should not harm, or affect the cellular functions of, most normal cells in the body. This should provide the first level of selectivity of these agents as potential anticancer therapeutics. As for the small fraction of normal, proliferative body cells, they should still be able to perform their duties while arresting their cell cycles instead of entering into a fatal S phase when the expression of a replication-initiation protein is inhibited. This should provide another level of selectivity for cancer therapy: stopping proliferation of normal cells without interfering with their functions or killing them. We therefore suggest that inhibition of replication-initiation genes is an effective anticancer strategy that should not cause serious side effects.

It has been reported that gene silencing of Orc6p (a subunit of the initiator protein ORC, which is expressed in both stationary and cycling cells) by siRNAs results in deregulation of the cell cycle, including a block in mitosis and appearance of multinucleated cells (35). It has also recently been shown that a fragment of the geminin protein, which inhibits the activity of the replication-initiation protein Cdt1p, can inhibit DNA replication and cell proliferation and lead to apoptosis of cancer cells in culture (36). As proposed (36), although geminin or a fragment thereof cannot be targeted to body cells, small molecules that may inhibit the activities of replication-initiation proteins are potential anticancer drugs. On the other hand, antisense ODNs (reviewed in Refs. 37–40) and siRNAs (41–43) can inhibit gene expression and have been shown to exert expected biological actions in animals as well as in cultured cells. Therefore, antisense ODNs and siRNAs that can inhibit the expression of selective replication-initiation proteins may become effective anticancer agents. In addition, appropriate carriers (reviewed in Refs. 44 and 45) and/or chemical modifications may further increase the *in vivo* stability, delivery, cellular uptake, and thus efficacy of these potential anticancer drugs.

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