Antisense Inhibition of CAS, the Human Homologue of the Yeast Chromosome Segregation Gene CSE1, Interferes with Mitosis in HeLa Cells

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ABSTRACT: We have analyzed the effects on HeLa cells of reduction of the CAS protein, the human homologue to yeast chromosome segregation protein CSE1. Expression of CAS antisense cDNA decreases the amount of CAS protein in HeLa cells and perturbs progression from G2 (retards transition from G2) to G1 in the cell cycle. Increased levels of cyclin B in CAS antisense transfected cells correlated with an arrest in G2 phase or mitosis. This arrest upon CAS attenuation is consistent with observations that yeast with CSE1 mutations are defective in mitosis and cyclin B degradation.

CAS[1] is the human homologue of the yeast chromosome segregation gene CSE1 (*1*). CSE1 plays a role in cell proliferation. The CSE1 gene was originally found because CSE1 mutations result in a defect in chromosome segregation (*2*). More recently, using a genetic screen in yeast, Irniger *et al.* (*3*) have found that three proteins, CDC16, CDC23, and also CSE1, are necessary for cyclin B degradation. The functions of CDC16 and CDC23 in cyclin B degradation could be explained by their presence in the ubiquitinylation/degradation complex (*3*−*5*). However, the function of CSE1 remained elusive.

The human homologue of yeast CSE1, CAS, was originally isolated as a gene related to apoptosis; CAS antisense rendered cells resistant to apoptosis induced by TNF and *Pseudomonas* and diphtheria toxins (*1, 6, 7*). But CAS, like its yeast counterpart, appears to also have a role in cell proliferation, because it is expressed at low levels in resting cells and at high levels in proliferating normal and tumor cells (*1*). A possible connection between CAS expression and cell proliferation in cancer was suggested by the finding that the chromosomal region that contains the CAS gene (20q13) is often amplified in breast and colon cancer, and that CAS gene amplification was found in BT474 breast cancer cells (*8*). Finally, we have found that CAS is associated with microtubules and the mitotic spindle (*9*). This distribution (on microtubuli and the spindle) is the same as the localization of the anaphase promoting complex that ubiquitinylates cyclin B for degradation which is necessary for initiation of mitosis (*5*).

Our hypothesis is that CAS has the same or a similar function in cell cycle regulation of mammalian cells as CSE1 in yeast; i.e., CAS is necessary for mitosis. To evaluate this, we decreased the amount of CAS protein in HeLa cells by expression of CAS antisense cDNA and analyzed the effect on the cell cycle by flow cytometry. We found that antisense inhibition of CAS expression negatively affects progression of cells from G2 to G1. This indicates a function of CAS in cell cycle progression (G2 to G1) in higher eukaryotes.

METHODS

Plasmids. The plasmid for expression of CAS antisense RNA, pcDNA3/CASR5, contains full-length CAS cDNA (*1*) without poly(A) in the antisense direction behind the CMV promoter of pcDNA3 (Invitrogen). Vector without insert, pcDNA3, was used as control for transfection studies. Double cesium chloride banded plasmid DNA was used for transfections.

(Co)Transfections and Magnetic Affinity Cell Sorting. Electroporation was performed according to Goldstein *et al.* (*10*) and Harper *et al.* (*11*). For each transfection, 1 μg of sorting vector pCMV.IL2R and 10 μg of expression plasmid were used per 1.5×10^6 cells. Cells were pulse-labeled with 10 mM BrdU for 30 min 12 h after transfection, washed twice with PBS, and put in chase medium (DMEM, 10% FBS, 100 mM thymidine, 10 mM deoxycytidine). We have found that most of the HeLa cells were transfected either in G1 or in G2, while replicating (S phase) HeLa cells are practically refractory to transfection (V.V.O., unpublished). This facilitates the analysis of BrdU labeling experiments since most DNA synthesizing cells in the transfected population appear as a result of progression of cells from G1 to S phase after transfection. Magnetic affinity cell sorting was performed as described by Sakamoto *et al.* (*12*).

Immunoblot Analyses. CAS protein in total cell extracts of transfected and sorted HeLa cells was visualized by immunoblot analysis with rabbit anti-CAS polyclonal antibody (protein-A-purified), goat anti-rabbit IgG−biotin, and avidin−peroxidase (Vector Labs ABC kit) (*9*). The same conditions were used for detection and comparison of the amount of cyclin B, except that we used mouse anti-cyclin B (Santa Cruz, clone H 20) and goat anti-mouse biotin, and

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[1] Abbreviations: FACS, fluorescence-activated cell sorting; CSE1, yeast chromosome segregation gene 1; CAS, human homologue of CSE1; PI, propidium iodide; HU, hydroxyurea; BrdU, bromodeoxyuridine.

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the ECL chemiluminescence detection system from Amersham. The intensities of the CAS or cyclin B specific bands were quantitated with an Ambis (Quantprobe) image analyzer.

FACS Analysis. For flow cytometry, we prepared nuclei from sorted cells (*13*). Nuclei either were stained directly with propidium iodide or were fixed with ethanol for 20 min, denatured using 30 min incubation in 2 N HCl, neutralized, and stained for 45 min with FITC-conjugated anti-bromodeoxyuridine antibody. Then the nuclei were stained for 5 min with propidium iodide according to the manufacturer's protocol (Becton-Dickinson). Flow cytometry was performed on a Becton-Dickinson FACscan flow cytometer with a doublet-discrimination module. The gating of BrdU positive cells with either G1 or G2 DNA content is shown in Figure 3B, and the percentage of cells in these gates was obtained with the Lysis II software. Using these scan parameters on isolated nuclei, the distinct cell cycle populations separated nicely and could be gated unambiguous. Major adjustments of the gates due to slight shifts of the 3D-peaks were not necessary. The preparation and PI stain of nuclei result in a clearer separation of the different cell cycle populations (compared to PI stain of whole cells) which facilitates the analysis of differences in cell cycle progression. The analysis of nuclei has the disadvantage that it will eliminate cells from our analysis that are in mitosis just at the moment of harvest (at that stage of the cell cycle, there is no nucleus to be isolated). Mitosis in HeLa is short (~1 h) compared to G2 (several hours), and we feel that the potential loss of mitotic cells does not significantly affect our analysis. If there is any effect, it would cause an underestimation of the cell cycle effect of CAS antisense. Therefore, our analysis of nuclei provides a conservative estimation of the effect of CAS antisense on the cell cycle. The statistical deviation of the gated cell population was calculated as the square root of the number of cells in the gate for each experiment and presented as percentage deviation (see Table 2). Usually, $(3-5) \times 10^3$ events were collected and analyzed using the Lysis II software (Becton-Dickinson).

RESULTS

CAS Antisense cDNA Reduces the Level of CAS Protein. Proliferation-dependent variations in CAS expression levels as well as evidence for CAS gene amplifications in some tumor cells (*1*, *8*) suggested that proliferation (and thus possibly cell cycle regulation) might be sensitive to the availability of the CAS gene product. One possibility to test this hypothesis is to lower the amount of CAS protein in cells and analyze the effects of that on the cell cycle. It is well established that antisense cDNA can reduce the expression of the corresponding cellular gene (*14*, *15*). To reduce the amount of CAS protein in cells and to analyze effects that it might have, we cotransfected HeLa cells with a plasmid for expression of the α subunit of the IL2 receptor and either CAS antisense or control plasmids. Cotransfection with the IL2R plasmid allows sorting of transfected cells with anti-IL2R-coated magnetic beads. Such sorted populations contain >90% cells transfected with both plasmids so that the effects of the other nonselected plasmids, CAS antisense or control, can be easily analyzed (*12*, *13*). Figure 1 shows a scheme and details of the transfection, sorting,

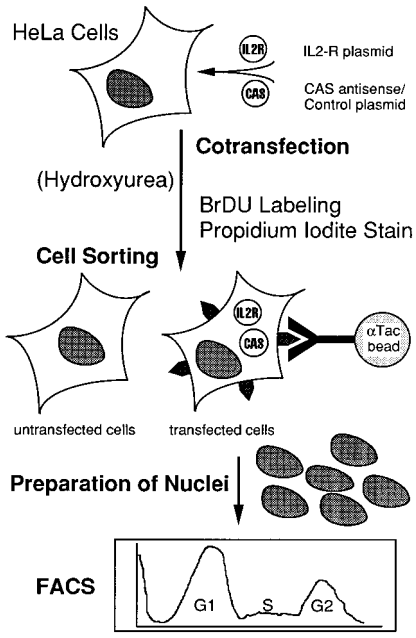

FIGURE 1: Cotransfection and FACS analysis of CAS antisense effects on the cell cycle. HeLa cells are cotransfected with CAS antisense and the sorting vector pCMV IL2R expressing surface marker, then are pulse-labeled with BrdU 12 h after transfection, and chased for different times. Hydroxyurea or nocodazole are added optionally to block replication or mitosis, respectively. The transfected cells, expressing surface marker IL2R, are then affinity-sorted using magnetic beads coupled with anti-IL2R antibodies. Nuclei from the sorted cells are analyzed by FACS.

and analysis of the sorted cells. All our measurements (proteins, gels, FACS) were performed with such immunoseparated cell populations. To determine if CAS antisense cDNA reduces the expression of CAS, we compared the CAS protein content in sorted cell populations transfected with control or antisense plasmids at 12 h, 24 h, and 36 h after transfection. Figure 2 shows a Western blot with polyclonal anti-CAS antibodies of equal amounts of sorted total cell extracts of antisense and control cells 12 h after transfection. Cells that were transfected with the CAS antisense plasmid contain significantly less CAS protein. Densitometric quantification of the immunoblot indicates a 60 ± 10% reduction of CAS protein in antisense cDNA containing cells compared to control cells 12 h after tranfection. Samples taken 24 h after transfection also showed a 60% ± 10% reduction of CAS protein, and samples taken 36 h after tranfection still showed a 45 ± 10% reduction in CAS protein (data not shown). This shows that transfection of cells with CAS antisense plasmids reduces the levels of CAS protein.

CAS Antisense Perturbs Cell Cycle Progression from G2 to G1. Mutations in CSE1, the yeast homologue of CAS, cause yeast cells to become arrested in mitosis (*2*). Does down-regulation of CAS also affect the cell cycle in mammalian cells? To address this issue, we pulse-labeled replicating cells with the thymidine analogue bromodeoxyuridine (BrdU), allowed them to proceed through the cell cycle, and then analyzed them by two-parameter flow cytometry with anti-BrdU antibodies and PI (see Methods). This protocol (BrdU pulse−chase) shows us how BrdU-labeled cells, "synchronized" in S phase on the moment of labeling, move through the cell cycle according to their DNA content measured by PI staining.

HeLa cells were transfected by electroporation with CAS antisense or control plasmids, labeled 12 h later for 30 min

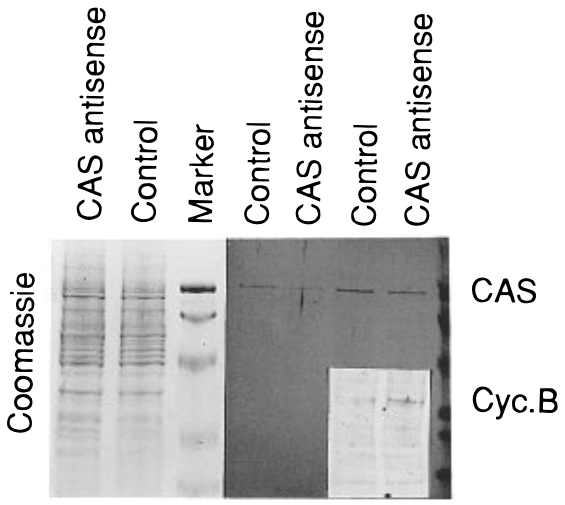

FIGURE 2: Reduction of CAS protein expression by CAS antisense. Equal amounts of total cell extracts of transfected and sorted HeLa cells (12 h after transfection) were separated on 12.5% reducing SDS−PAGE and either stained with Coomassie blue to demonstrate equal loading or transferred to nitrocellulose to detect CAS and cyclin B protein by Western analysis with rabbit anti-CAS polyclonal antibody and mouse anti-cyclin B monoclonal antibody as described under Methods. Left lanes, Coomassie blue stain of 15 μL cell extract each; middle lanes, Western with 5 μL of cell extract each; right lanes, Western with 15 μL cell extract each. The relative intensities of the CAS and cyclin B protein bands were quantified with an Ambis (Quantprobe) image analyses, which showed a 60 $\pm$ 10% reduction of CAS protein and a 2-fold increase of cyclin B in CAS antisense cells compared to control cells (see also Table 1).

with BrdU and chased for different times. Since HeLa cells are refractory to transfection in S phase (see Methods), the electroporation produces a "synchronized" population of transfected cells that are enriched in G1 and G2 which is shown in Figure 3. In both control and transfected cell populations, the labeled cells progress through the S phase and then reappear in G1 due to mitosis (compare Figure 3A,B immediately after labeling to Figure 3C,D after 8 h and Figure 4A,B after 12 h). At 12 h of chase, comparison of the DNA content of BrdU-labeled nuclei from control cells and CAS antisense cells reveals that the CAS antisense population is enriched in G2 cells, compared to control cells (Figure 4A vs 4B). In the BrdU-positive control cell population, the percentage of G1 cells is always 1.5-fold greater than G2 cells. In contrast, in CAS antisense cells, the percentages of G1 and G2 cells are nearly equal (Table 1). This significant enrichment of G2 cells vs G1 cells in CAS antisense cells was reproducible and is especially notable if one considers that the reduction of CAS expression by CAS antisense was not complete (see Figure 2, Table 1). Also, our experimental system (FACS of isolated nuclei) probably underestimates the number of cells that become arrested in mitosis (see Methods). Thus, any accumulation of CAS antisense cells in G2 and mitosis might actually even be more pronounced than what we see.

The observation that CAS depletion increases the number of G2 cells could be explained by inhibition of mitosis, in direct analogy with mitotic arrest of yeast CSE1 mutants. Another, albeit unlikely, explanation for an altered G2 to G1 ratio could be that G1−S transition is accelerated. To distinguish between these possibilities, the BrdU pulse−chase protocol was used with the following modification. After the 12 h chase period, DNA synthesis was blocked by addition of 2 mM hydroxyurea (HU), and the cells were sorted and analyzed 12 h later by FACS. In this setting, the supply of new G2 cells due to transit of cells from G1 through S phase is blocked by HU. Therefore, a decrease over time of G2 cells and an accumulation of G1 cells will reflect solely the rate of transition from G2 to G1 phase. In the absence of any effect of CAS antisense on this transition, control and CAS antisense cells should exhibit the same rate of disappearance of G2 and accumulation of G1 cells. If, however, CAS reduction inhibits the transit of cells from G2 to G1, we would expect that the labeled G2 population will be more slowly converted to G1 after HU treatment. The data in Figures 4 and 5 and Table 1 show that the latter is the case. Twelve hours after HU treatment, control cells have 90% of their BrdU-positive cell population in G1 and only 10% in G2, showing almost complete conversion of G2 cells to G1. In contrast, under the same conditions, CAS antisense transfected cells still have 31% of their population remaining in G2.

Another experiment that confirms the interference of CAS antisense with transition from G2 to G1 is shown in Figure 6 and Table 2. Cells were labeled with BrdU, transfected, and blocked by HU as before. Then the samples were analyzed at different times after the chase and HU block. The percentage of G2 cells in control transfectants declines over time in a roughly linear manner from ~50% (beginning of HU block) to ~15% after 12 h of HU block. In CAS antisense cells, we observed a significant reduction in G2/G1 transit, 30% of the cells still being in G2 12 h after the HU block. Because two G1 cells arise from one G2 cell, these data can be used to analyze the percentage of cells that have not divided in the 12 h after BrdU label and HU block. The calculation in Table 2 shows that after 6 h of HU block, 36% of the control cells have not undergone mitosis whereas 55% of the CAS antisense cells did not undergo mitosis. The difference is even more pronounced at 12 h after HU block: only 18% of the control cells have not undergone mitosis, but 47% of the CAS antisense cells have not undergone mitosis at that time interval. We conclude that depletion of CAS interferes with the transition from G2 to G1, possibly because it affects mitosis.

Increased Amounts of Cyclin B in CAS Antisense Transfected Cells. The degradation of cyclin B is a key step and necessary for mitosis in eukaryotic cells (ubiquitinylation, APC complex, see references *3*−*5*, *16*−*19*). It is known that CSE1 mutants are defective in cyclin B degradation in yeast, although it is not clear whether this is due to a direct involvement of CSE1 in the degradation process or merely due to inhibition of mitosis in the CSE1 mutants and cyclin accumulation is a secondary effect (see Discussion). If CAS has the same function in the mammalian cell cycle as the homologous yeast CSE1 protein in yeast (*2*), we would expect that CAS protein reduction might lead to reduced cyclin B degradation. We therefore analyzed whether the reduction of CAS protein in mammalian cells influences the cyclin B levels in CAS antisense cells. Transfected cells were affinity-sorted, and the cyclin B levels in cell extracts from CAS antisense- and control-transfected cells were compared by immunoblotting as described above for the comparison of CAS protein in these cells. Figure 2A (insert) shows the difference in cyclin B1 levels between control and CAS antisense cells 12 h after transfection (see Figure 4 for cell cycle distributions of the cells at that time point; 6% G2 cells in the control and 13% G2 cells in CAS antisense). Densitometric quantification of immunoblots containing

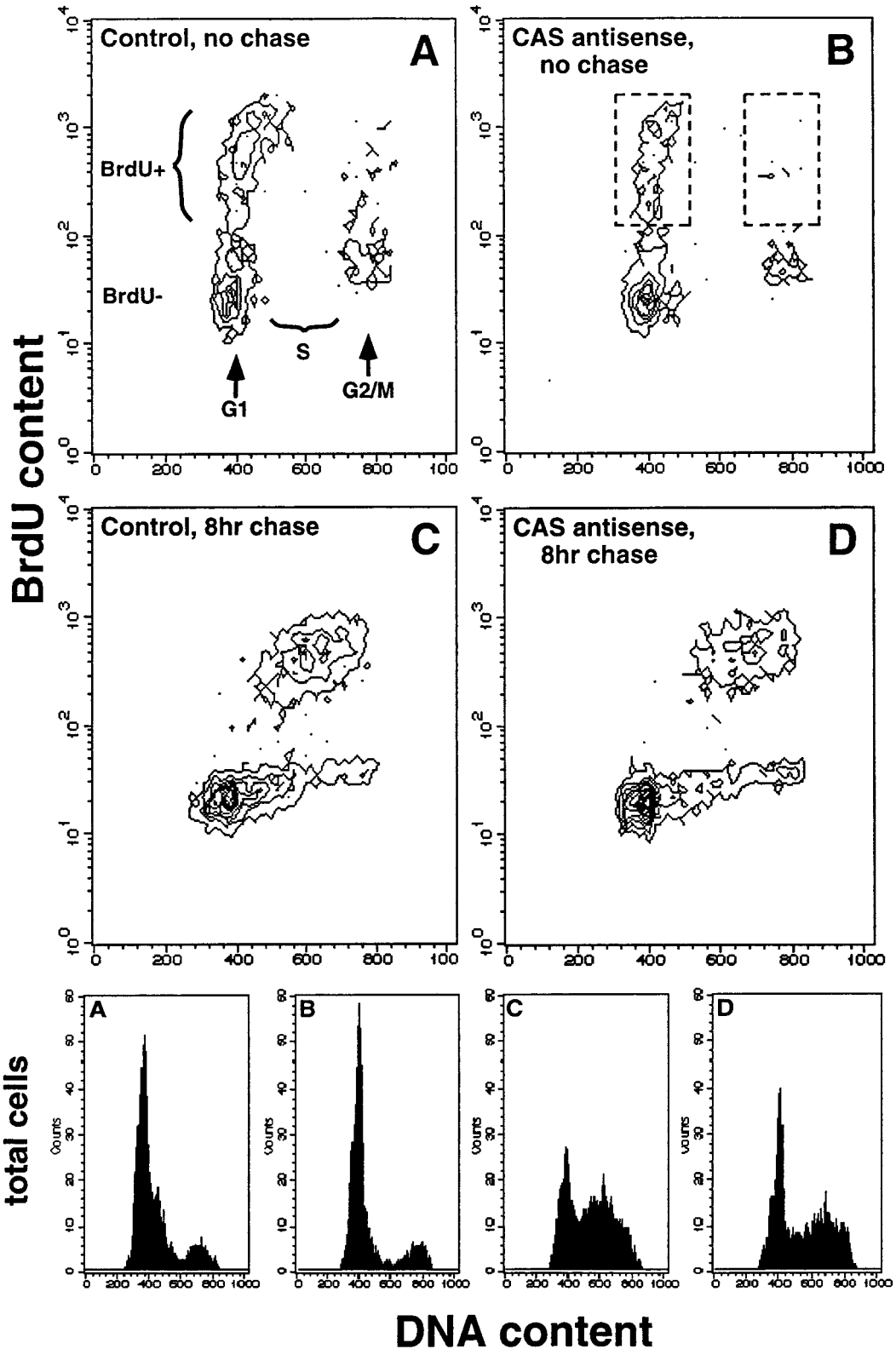

FIGURE 3: Progression of transfected HeLa cells after BrdU pulse-labeling and chase. Double parameter flow cytometric analysis of HeLa cells subjected to BrdU pulse after transfection with control plasmid pCDNA3 (A, C) or pCDNA3/CASR5 (B, D). Cells were sorted immediately after the BrdU pulse (A, B) or washed out of BrdU and allowed to replicate in conditions of chase for 8 h before sorting (C, D). BrdU labeling of isolated nuclei was detected with anti-BrdU antibodies and DNA content by propidium iodide (PI) staining. The horizontal axis (DNA) denotes DNA content (PI staining), and the vertical axis (BrdU) represents staining with fluorescein-labeled anti-BrdU antibody. The percentages of G1/S vs G2 cells or BrdU− vs BrdU+ cells (see Table 1) were obtained by gating (indicated in panels A and B) and then counting the appropriate cell populations. The bottom panel shows the same data (A−D) as one-dimension histograms (cell number vs DNA content). HeLa cells are refractory to transfection in S phase, and electroporation therefore produces a "synchronized" population of transfectants that are enriched in G1 and G2.

equal amounts of total cell extracts from CAS antisense and control cells showed that antisense reduction of CAS expression increases the cyclin B signal approximately 2-fold which coincides with an increase of G2 cells in the CAS antisense population (13% vs 6%). The CAS antisense cell population contains 2-fold more cyclin B than the controls,

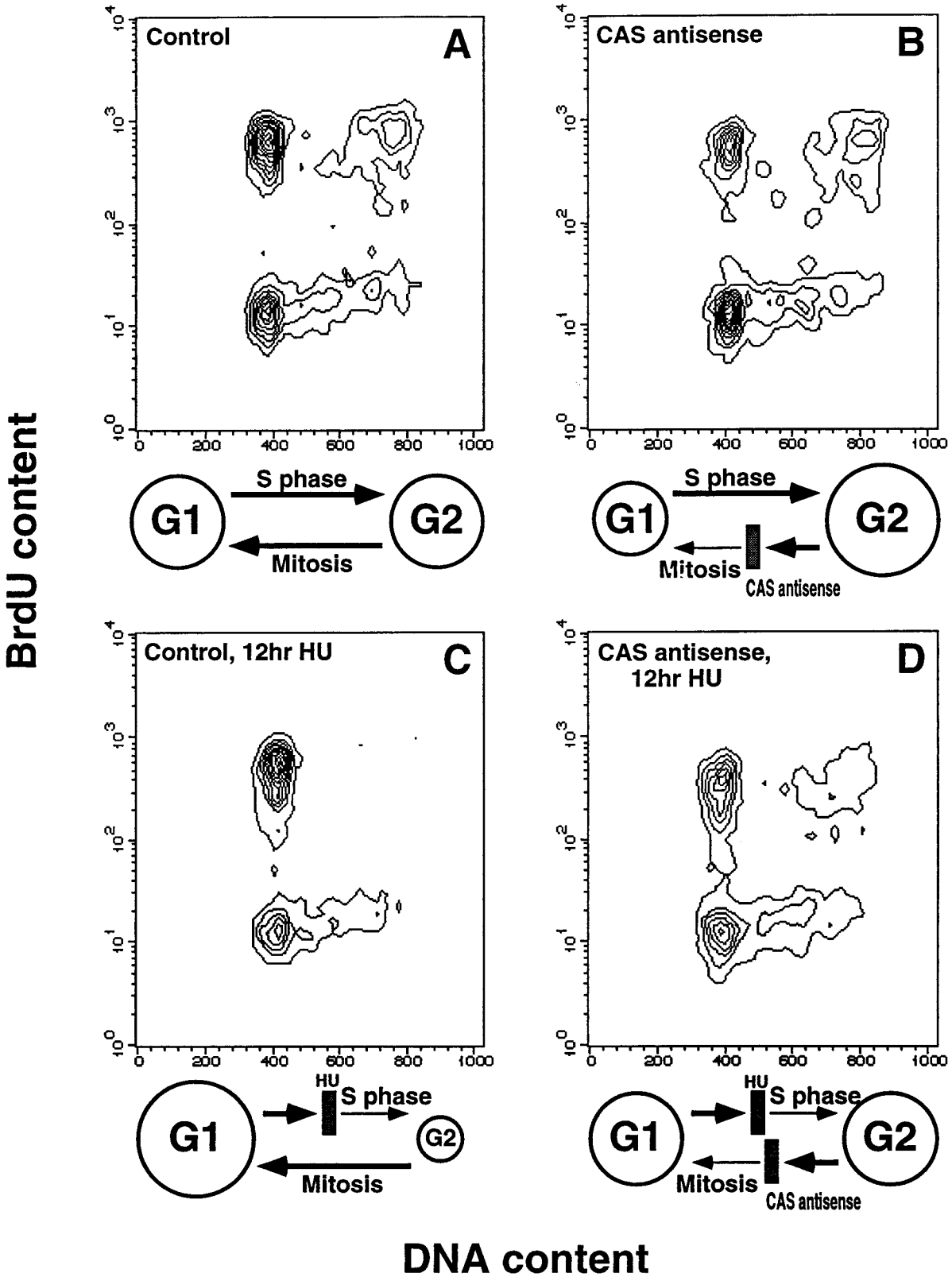

FIGURE 4: Delay in G2 to G1 transit in CAS antisense transfected HeLa cells. (A, B) Cells were treated and analyzed as in Figure 3, except the chase time before the sorting was 12 h. (C, D) after 12 h of chase, 2 mM HU was added to the cell, and they were sorted 12 h later. (A, C) Control transfection with pCDNA3; (B, D) transfection with pCDNA3/CASR5.

possibly because reduction in the level of CAS protein and a subsequent cell cycle arrest prevent the degradation of cyclin B.

DISCUSSION

CAS is the human homologue to the yeast chromosome segregation gene CSE1. To analyze the role of CAS in the cell cycle, we reduced the intracellular levels of CAS protein in HeLa cells by expression of antisense cDNA. We found that CAS reduction causes accumulation of G2-arrested cells which coincided with elevated cyclin B levels.

Analysis of Effects of CAS Antisense on G2 to G1 (Mitosis) and G1−S Transition. We have used a combination of affinity sorting of transiently transfected cells and double-

Table 1: Effects of CAS Antisense on the Cell Cycle[a]

	control	CAS antisense	*x*-fold change	effect
CAS protein	100%	~40%	2.5	CAS ablation
cyclin B1	100%	~200%	2	cyclin accumulation
G2:G1/S ratio (BrdU, 12 h chase)	0.66 (40/60)	0.92 (48/52)	1.4	G1/S + mitosis
G2:G1/S ratio (BrdU, 12 h HU)	0.11 (10/90)	0.42 (31/69)	3.8	only mitosis
BrdU −/+ ratio (no chase)	0.78 (44/56)	0.47 (30/70)	1.7	G1/S + mitosis
BrdU/PCNA negative cells (%) (8 h chase)	23%	46%	2	resting/blocked cells

[a] G2:G1/S represents the percent ratio of BrdU-labeled cells that are either in G1/S phase or in G2 phase as determined by PI stain (see Figure 4A,B for 12 h chase and Figure 4C,D for 12 h HU blocked cells). The actual percent data of G1/S and G2 are cited in parentheses next to the ratios. Those ratios reflect the effect of CAS protein reduction on mitosis. The data set "12 h HU" reflects G2 to G1 transition under experimental conditions that eliminate any effect of CAS antisense on G1 to S transition from the analysis of mitosis (see also Table 2). BrdU +/− is the ratio of cells that can be labeled by BrdU vs unlabeled cells (see Figure 3). Since BrdU labeling measures the rate of DNA synthesis, these ratios reflect the effect of CAS antisense on G1−S transition.

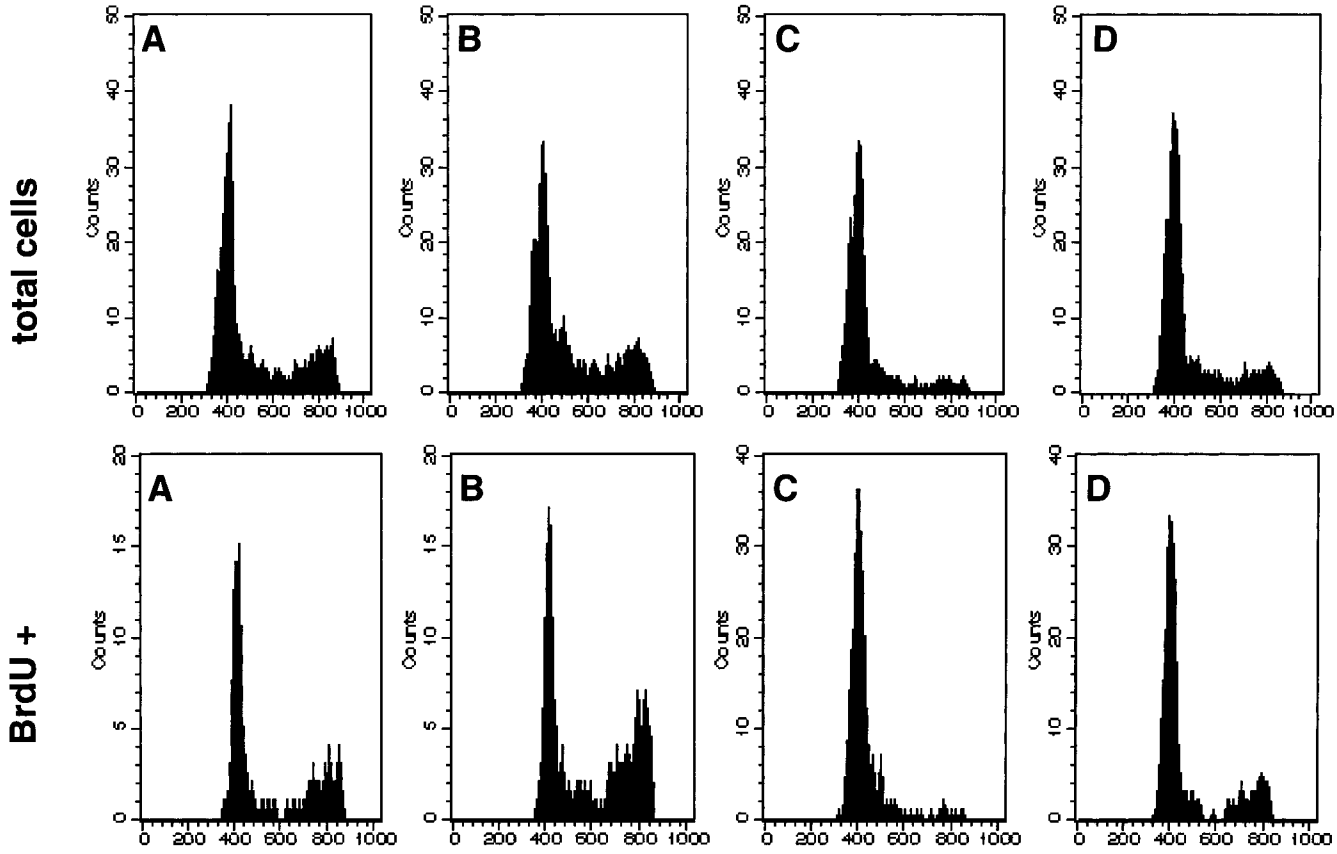

FIGURE 5: Delay in G2 to G1 transit in CAS antisense transfected HeLa cells. One-dimensional presentation (cell number vs DNA content) of the experiment shown in Figure 4 (HU block and progression through mitosis). Panels A−D are the same as in Figure 4. Upper panel, total cells; lower panel, BrdU positive cells. The G2 gate (shown in Figure 3) may contain in addition to G2 cells a small fraction of late S phase cells. Because S phase progression is identical in control of CAS antisense cells (Figure 3), the presence of a minor fraction of late S phase cells in our G2 gate is neglectable, and a CAS antisense block in late S (instead of G2) is therefore very unlikely. Hydroxyurea treatment does not affect the viability of the majority of the cells (neither control nor CAS antisense). We cannot exclude that some S phase arrested cells die, but this would not influence the G2 differences between control and CAS antisense populations because the numbers of potentially HU-sensitive late S phase cells are the same in control and CAS antisense populations.

parameter flow cytometry of isolated nuclei to analyze the effects of CAS protein reduction on the cell cycle. The reason that this technique was chosen over a simple analysis of stably transfected cells was that analysis of stable transfectants is questionable for any gene or situation that negatively affects cell growth (selective pressure during cell propagation favors compensatory mutations or loss of expression). The only other possible way to analyze the effects of CAS depletion without encountering the lethal effect of constitutive antisense expression would be to use tightly regulated inducible antisense expression constructs. The CAS homologue yeast CSE1 gene is essential, and homozygous CSE1 mutations are lethal (*2*). Since the human homologue CAS might have the same or a similar function as CSE1, we reasoned that CAS attenuation by expression of antisense DNA might be toxic to cells. Transfection of CAS antisense plasmids into mammalian cells results in unusually low numbers of transfectants, and stable "survivors" show only modest reduction in CAS protein levels (*7*), which is a clear indicator of the toxicity of CAS antisense. The results described below confirm and provide a plausible explanation for this toxicity, i.e., inhibition of G1−S transition and mitosis.

One disadvantage in analyzing a population of transiently transfected cells is that the expression levels in the population may vary widely. Overall, we measured a 60% decrease in

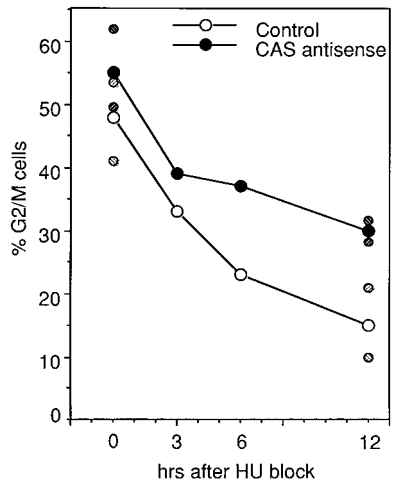

FIGURE 6: Time course of G2 to G1 transition in CAS antisense transfected HeLa cells. The cells were treated and analyzed as in Panels C and D of Figures 4 and 5, except the times of sorting after the HU block varied from 3 to 12 h. Horizontal axis denotes time after HU block; vertical axis represents percent of BrdU-labeled cells still in G2 phase. Repeated data points at 0 and 12 h show the results of completely independent additional experiments. Closed circles and dark background are control cells; open circles and white background are CAS antisense cells.

the amount of CAS protein in the sorted CAS antisense cell population. We consider this to be a "good" and clear antisense effect considering the high expression level of CAS in growing cells (~1 000 000 molecules/cell, *11*). It is also reasonable to assume that this reflects a mixture of cells, some with greater reduction in CAS protein and some with little or no CAS ablation. Possibly, the incomplete effects of CAS antisense that we observed (only partial block of mitosis and G1 to S transition) are due to this variability. The time course of elimination of CAS antisense transfected G2 cells by mitosis (Figure 6 could be a biphasic curve) could support this assumption; cells with low antisense expression would undergo mitosis at a similar rate as the control cells (first slope of the curve), and the remaining cells would undergo mitosis much more slowly (second slope). Since our results represent a mean of the whole transfected population (including cells with low antisense expression), these observations provide a conservative and probably understated assessment of the effects of CAS depletion.

One other difficulty that we faced during the analysis of CAS antisense cells was that not only the G2 to G1 transition but also the G1 to S transition appeared to be affected by CAS antisense. This made simple PI analysis of the transfected cells difficult because both effects can "neutralize" each other. A mitotic block normally leads to G2 accumulation, but a simultaneous G1/S block can prevent the production and thus the accumulation of G2 cells. This leads to a situation in which two major phases of the cell cycle, G1 and G2, appear "frozen". To overcome this problem, we used a combination of BrdU pulse-chase and hydroxyurea block to eliminate any influence of CAS on G1−S transition while analyzing its effect on mitosis.

CAS and CSE1 in Mitosis. The yeast CSE1 gene was originally isolated as a chromosome segregation gene (*2*), i.e., a gene with a function in mitosis. Recently it was shown that CSE1 mutations are deficient in cyclin B degradation (*3*). The human CSE1 homologue CAS was isolated because a CAS antisense containing plasmid rendered breast cancer cells resistant to apoptosis induced by certain bacterial toxins, immunotoxins, and tumor necrosis factor (hence the name CAS for Cellular Apoptosis Susceptibility gene; *1, 7*). We do not yet know the molecular basis of the function of CAS in apoptosis; it may be a specific component in the TNF and toxin pathway, or a secondary consequence of a proliferation function of CAS (see below; *7*).

However, we found that CAS has a function not only in apoptosis but, consistent with its homology to yeast CSE1, also in cell proliferation (*1, 9*). The observation presented here, that antisense inhibition of CAS expression retards G2 to G1 transition (mitosis) which also coincides with increased cyclin B levels, is in full agreement with observations that yeast with mutations in the homologous CSE1 gene are arrested in mitosis and defective in cyclin B degradation (*2, 3*). Thus, our study indicates that the human homologue CAS has a function in cell cycle progression of higher eukaryotes similar to that of the corresponding CSE1 gene in yeast.

So far, little is known about the molecular function of CSE1 or CAS. The chromosome segregation defect associated with CSE1 mutations is one phenotype, and a genetic screen turned out CSE1 as one of three genes that are necessary for cyclin B degradation (*3*). But while the other two genes isolated in that screen, CDC16 and CDC23, could be assigned a function (participation in the cyclin B ubiquitinylation/degradation reaction), the function of CSE1 remained elusive. The two main possibilities for CAS/CSE1 function are as follows: (i) CSE1 and CAS participate directly in cyclin B degradation, e.g., as a component of the degradation complex. (ii) CAS/CSE1 has another function before the "cyclin B checkpoint" which leads to mitotic arrest and cyclin accumulation as a secondary event. The localization of CAS on microtubules and the mitotic spindle which coincides with the location of the anaphase promoting complex that ubiquitinylates cyclin B for degradation might support the hypothesis of a direct involvement of CAS in cyclin B degradation. However, direct biochemical analysis of *Xenopus* egg extracts indicates that CSE1/CAS is not a subunit of the anaphase promoting complex that ubiquitinylates cyclin B in mitosis (J.-M. Peters, A. Georgi, and M. Kirschner, personal communication and ref *17*). This finding argues in favor of the hypothesis that CAS/CSE1 have a function close to but before the cyclin B checkpoint (e.g., in a chromosome alignment checkpoint; *20*). The "CAS/CSE1 step" would have to be completed to initiate or permit cyclin B degradation. We would then observe the CAS/CSE1 phenotype because CAS reduction prevents initiation of cyclin B degradation, which then leads to a G2 (mitotic) arrest (or vice versa).

So far, the molecular function of CAS/hCSE1 in cell cycle progression of mammalian cells as well as in yeast is unknown, and unfortunately our analysis method (trypsization of cells, sorting of transfectants, and isolation of nuclei) does not permit the microscopic observation of the transfectants to obtain clues of the exact stage of mitosis that becomes blocked. The absence of CAS/hCSE1 in the APC cyclin ubiquitinylation complex (the most thoroughly analyzed mitosis regulator) and the fact that no other protein shows a significant homology to CAS and CSE1 make the identification of its molecular function even more difficult (there is no obvious target of CAS). Since CAS/hCSE1 protein is quite abundant (10^6 molecules in proliferating cells), it is possible that it is not a cell cycle regulator but an

Table 2: Kinetics of Mitosis-Block by CAS Antisense[a]

h after HU block	G2:G1/S ratio (BrdU, 12 h HU)			% undivided cells	
	control	CAS antisense	*x*-fold change	control	CAS antisense
0	0.66 (40/60 ± 4.5)	0.92 (48/52 ± 4.6)	1.4	57 ± 5	65 ± 5
3	0.49 (33/67 ± 5.2)	0.65 (40/60 ± 6)	1.3	49 ± 6	57 ± 6
6	0.28 (22/78 ± 5.5)	0.61 (38/62 ± 3.5)	2.2	36 ± 8	55 ± 4
12	0.11 (10/90 ± 9.5)	0.42 (31/69 ± 6.7)	3.8	18 ± 16	47 ± 8

[a] The G2:G1/S ratios were determined as described in the legend to Table 1 at different time points after blockage of mitosis with HU (see Figures 4−6). The actual percent data of G1/S and G2 are cited in parentheses. HU block eliminates any effect of CAS antisense on G1−S transition. Therefore, the changes in G2:G1/S population are attributable solely to an effect of CAS reduction on mitosis. Since two G1 cells arise from one G2 cell, these data can also indicate how many cells have not divided at the end of the 12 h BrdU chase/HU period which is indicated in the column "% undivided cells". The percentage was calculated as % undivided cells = 1 - {(G1/S:2)/[G2 + (G1/S:2)]}.

effector; e.g., it could have a function in the process of aligning or moving chromosomes along the mitotic spindle. Such a function would not only be consistent with its localization on microtubules and the mitotic spindle but also could explain the chromosome segregation defective phenotype of CSE1 mutations in yeast.

A Possible Role of CAS/CSE1 Also in G1−S Transition? One additional interesting observation of this study is that in CAS antisense cells not only G2 to G1 transition (mitosis) but possibly also G1 to S transition appears to be affected. We noted in the course of our experiments that the ratio of BrdU-labeled cells versus unlabeled cells (without chase) was reproducibly lower in CAS antisense transfected cells than in controls (44% BrdU positive control cells entering or in S phase 12 h after transfection vs 32% BrdU positive CAS antisense cells). To further address this phenomenon, we used three-color staining of BrdU pulse-chased cells to simultaneously analyze the content of PCNA bound to the nucleus as well as BrdU and PI staining of DNA. BrdU incorporation shows cells that were in S phase at the moment of labeling and might have exited S phase during the chase; PCNA shows cells in S phase at the moment of harvest. BrdU and PCNA negative cells were not replicating any time during the labeling and chase periods. In this analysis, the contrast between control and CAS antisense was even more dramatic. The control cell population contained only 23% cells that were negative with BrdU as well as PCNA, but the CAS antisense population contained twice as many (46%) cells that did not synthesize DNA or replicate during that time period (BrdU and PCNA negative cells, see Table 1).

One likely interpretation of these differences between control and CAS antisense expressing cells is that the CAS antisense induced delay of G2 to G1 transit is responsible for reduction of BrdU-labeled cells: If a cell is transfected in G2, its appearance in the following S phase could be delayed by of suppression of transition from G2 to G1. Therefore, less cells are available for labeling, and the changes in the G1 to S transition would be merely an artifact caused by the "mitotic effect" of CAS antisense. It is likely that the observed effect of CAS antisense on the G1 to S transition is an assay artifact due to a "delayed mitotic effect".

Another possibility (which we cannot rule out completely, but which is unlikely) is that CAS may play a role not only in mitosis but also in G1 to S transition. However, such a dual role of a cell cycle protein in different checkpoints is unusual for mammalian cells. In yeast, cyclin B is involved not only in mitosis but also in G1 to S transition (*16*). Thus, it may be not too surprising to find that the human homologue to a yeast gene that is necessary for degradation of cyclin B (directly or indirectly) may also affect mitosis and G1 to S transition. An alternative explanation for the effects of CAS antisense on mitosis as well as possibly an G1 to S transition could be a so far unknown cell cycle "feedback" mechanism which prevents DNA synthesis in cells that do not possess the molecular "setup" for successful completion of the following mitosis.

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