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Exit from G_0 and entry into the cell cycle of cells expressing $p21^{Sdi1}$ antisense RNA

(Cip1 protein/Waf1 protein)

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ABSTRACT **$p21^{Sdi1}$ (also known as Cip1 and Waf1), an inhibitor of DNA synthesis cloned from senescent human fibroblasts, is an inhibitor of G_1 cyclin-dependent kinases (Cdks) *in vitro* and is transcriptionally regulated by wild-type p53. In addition, $p21^{Sdi1}$ has been found to inhibit DNA replication by direct interaction with proliferating cell nuclear antigen. In this study we analyzed normal human fibroblast cells arrested in G_0 and determined that an excess of $p21^{Sdi1}$ was present after immunodepletion of various cyclins and Cdks, in contrast to mitogen-stimulated cells in early S phase. Expression of antisense $p21^{Sdi1}$ RNA in G_0-arrested cells resulted in induction of DNA synthesis as well as entry into mitosis. These results suggest that $p21^{Sdi1}$ functions in G_0 and early G_1 and that decreased expression of the gene is necessary for cell cycle progression.**

The terminal loss of division potential observed in normal human cells in culture, cellular senescence, is well documented and utilized as a model for aging at the cellular level (1, 2). Although cell fusion studies have demonstrated that there is a genetic basis for cell senescence (3–6), the causal molecular mechanisms have not been determined. However, a series of genetic (7, 8), biochemical (9, 10), and microinjection (11) studies have provided evidence for the expression of an inhibitor(s) of DNA synthesis in senescent but not young, actively proliferating cells. To clone this inhibitor(s), we used an expression screen and identified three cDNAs (Sdi1, -2, and -3) in a senescent cell cDNA library that could inhibit DNA synthesis when transfected into proliferating cells (12, 13). The Sdi1 gene was subsequently cloned as the gene encoding a cyclin-dependent kinase (Cdk) inhibitor (named Cip1 or p21), which is present in various cyclin–Cdk complexes during the cell cycle (14–16). It has also been found to directly inhibit DNA replication by binding to proliferating cell nuclear antigen (PCNA) (17). In addition, it was cloned as a gene that was transactivated by p53 (*WAF1*) (18). However, we have evidence for a p53-independent pathway(s) for up-regulation of the gene (19) activated by DNA-damaging agents, and more recently $p21^{Sdi1}$ was cloned as a melanocyte differentiation-associated factor also activated independent of p53 (20). These observations have led to the convergence of the fields of cellular senescence, cell cycle regulation, cell differentiation, and tumor suppression.

Cell cycle regulation in eukaryotes involves the regulated synthesis, activation, and degradation of a family of cyclins that act as the regulatory subunits of Cdks (21). Since the Cdks are known to be necessary for initiation and progression through S phase (22–25), and also for mitosis (26), we explored in more detail the role of this kinase inhibitory protein, $p21^{Sdi1}$, during the cell cycle. We analyzed levels of the protein and its interaction with cyclin–Cdk complexes in G_0-arrested and serum-stimulated cells. The results demonstrate that high levels of $p21^{Sdi1}$ protein were present in G_0-arrested cells after immunodepletion of various cyclins and Cdks, in contrast to cells entering the S phase, 16 hr after serum stimulation. More importantly, induction of antisense $p21^{Sdi1}$ RNA sequences caused G_0-arrested cells to reenter the cell cycle and synthesize DNA. The cells also completed G_2 and entered mitosis. These results suggest that $p21^{Sdi1}$ is a key regulatory molecule in cell cycle entry and progression.

MATERIALS AND METHODS

Cell Culture. HCA2 cells, human diploid fibroblasts isolated in this laboratory from neonatal foreskin, were grown in minimal essential medium with either Earle's or Hanks' balanced salt solution, supplemented with 10% fetal bovine serum (FBS) (27). G_0-arrested cells were obtained by plating young cells (<30 population doublings), into standard cell culture medium, replacing this 6 hr later with culture medium containing 0.5% FBS, and incubating the cells for at least 1 week. DNA synthesis was determined by tritiated thymidine autoradiography (13).

RNA and Protein Analysis. Northern analysis was performed according to standard procedures (13). ^{32}P-labeled DNA probes for human $p21^{Sdi1}$ and glyceraldehyde-3-phosphate dehydrogenase RNAs were prepared by random oligonucleotide primer synthesis. $p21^{Sdi1}$ protein was analyzed by immunoprecipitation, immunoblotting, and immunostaining. Total cell extracts were prepared by lysing cells in ice-cold lysis buffer containing 0.5% Nonidet P-40, 20 mM Tris·HCl (pH 7.40), 0.25 M NaCl, 1 mM EDTA, soybean trypsin inhibitor at 20 μg/ml, aprotinin at 2 μg/ml, phenylmethylsulfonyl fluoride at 100 μg/ml, leupeptin at 2 μg/ml, 100 μM sodium orthovanadate, 50 mM sodium fluoride, and *p*-nitrophenyl phosphate at 5 mg/ml. Cell lysates were immunoprecipitated with normal mouse serum, mouse or rabbit IgG coupled to agarose beads (Sigma) (as controls), or a monoclonal antibody to a glutathione *S*-transferase (GST)-$p21^{Sdi1}$ fusion protein (PharMingen). Immunoprecipitates were separated by SDS/PAGE, transferred to Immobilon membranes (Millipore), and probed with a monoclonal antibody to $p21^{Sdi1}$ (1:1250; PharMingen). Protein was detected by enhanced chemiluminescence (ECL, Amersham) and Kodak XAR-5 film.

For immunostaining cells were fixed with 4% paraformaldehyde and treated with 1:500 dilution of the anti-$p21^{Sdi1}$ monoclonal antibody. Staining was visualized by an anti-mouse IgG avidin–biotin complex with horseradish peroxidase enhanced with nickel (Vector Laboratories).

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For metabolic labeling with [^{35}S]methionine, cells were washed with warmed labeling medium [methionine- and cysteine-free Dulbecco's modified Eagle's medium (ICN) supplemented with dialyzed fetal bovine serum]. After a 30-min incubation with the labeling medium, [^{35}S]methionine was added at 100 μCi/ml (1 μCi = 37 kBq) and incubation was continued for 2 hr. The cells were lysed as described above and cell lysates were immunoprecipitated with rabbit IgG coupled to agarose beads or polyclonal rabbit antiserum raised against human cyclin A, cyclin D1, cyclin E, Cdk2, Cdk4 (Upstate Biotechnology, Lake Placid, NY), Cdk5, Cdk6 (Santa Cruz Biotechnology, Santa Cruz, CA), or p21^{Sdi1} (PharMingen). Proteins were separated on polyacrylamide gels, which were fixed with 25% isopropyl alcohol/10% acetic acid/65% water (vol/vol) for 30 min, enhanced with Amplify (Amersham) for 30 min, and exposed to x-ray film at −70°C.

Construction of the Antisense Vector. The antisense expression vector was constructed by inserting p21^{Sdi1} antisense sequences into pMET, an inducible expression vector containing an altered human metallothionein promoter. The metallothionein promoter in pMET was derived from pM26 (28) (provided by M. J. Sleigh, Commonwealth Scientific and Industrial Research Organization Division of Biomolecular Engineering, New South Wales, Australia), which has undergone a deletion in the basal promoter and the addition of synthetic metal-response elements in triplicate. For construction of pMET, adenovirus sequences containing the *E1A* gene 12S and 13S introns (nucleotides 917–1673) were first inserted into the mammalian expression vector pRc/CMV (Invitrogen) at the *Not* I and *Apa* I sites of the multiple cloning region of this plasmid to create pRc/CMV-Ad. This plasmid also contained the neomycin-resistance gene under the control of the simian virus 40 promoter, to allow for selection of transfected cells. To ensure that no translation of *E1A* sequences occurred and to create an *Spe* I cloning site in this vector, an *Spe* I linker containing a stop codon was inserted between the cytomegalovirus (CMV) promoter and the *E1A* splice sequence in frame with the *E1A* sequence. The CMV promoter of plasmid Rc/CMV-Ad was then replaced with the pM26 promoter to create pMET. To accomplish this, the metallothionein promoter was excised from pM26 by *Bgl* II digestion and inserted into the *Bam*HI site of pBluescript KS (+) (Stratagene). An *Eco*RV and *Not* I fragment containing the promoter was then subcloned from pBluescript into a filled-in *Bgl* II site and *Not* I site of Rc/CMV-Ad. Antisense sequences from the p21^{Sdi1} cDNA were derived from the full-length cDNA inserted into the *Bam*HI site of pBluescript. An *Spe* I linker was inserted at the *Apa* I site at nucleotide 322 to create pSdiSpe322. This construct was digested with *Spe* I and inserted in the antisense orientation into the *Spe* I cloning site of pMET, thus creating pMETAS322.

Isolation of Stable Cell Lines Containing Either p21^{Sdi1} Antisense or Control Vector Sequences. We obtained several G418-resistant clones of normal human fibroblast cells after transfection with the pMETAS322 plasmid, which contains p21^{Sdi1} antisense sequences under the control of an inducible metallothionein promoter and the neomycin-resistance gene (28). We observed various degrees of induction of antisense p21^{Sdi1} RNA in the individual clones and chose to work with AS322 clone 6 (AS322), which was highly induced after addition of metal. Control clones transfected with vector alone were also isolated.

HCA2 cells were transfected with either the control vector or the metal-inducible antisense construct by calcium phosphate precipitation (29). Twenty-four hours later, the cells were subcultured into medium containing G418.

To determine the effect of induction of p21^{Sdi1} antisense sequences, cells were grown to confluence in medium containing fetal bovine serum (10%). Fresh medium was then added to the cells, and 5 days later they were treated with 117 μM $ZnCl_2$ and 2.3 μM $CdCl_2$ for 48 hr. Cells were labeled with [^{3}H]thymidine at 1 μCi/ml for 24 hr, beginning 24 hr after addition of metal. Autoradiographic analysis identified cells that synthesized DNA as having heavy silver deposits over the nuclei.

Determination of Mitotic Index. To determine the mitotic index, Colcemid was added to the cultures at 0.1 μg/ml for 6-hr periods beginning at 32 to 54 hr after the induction of p21^{Sdi1} antisense. Cells were fixed with formaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI) to allow for analysis with a fluorescence microscope. Cells with condensed chromosomes were scored as in mitosis.

RNase Protection Assay. RNase protection analysis was performed with an antisense RNA probe derived from the pMET vector (30). After *Spe* I digestion, transcription from the SP6 promoter in the pMET vector yields an antisense labeled probe that hybridizes to RNA from the expression vector. As a control, β-actin mRNA was measured in all assays. The actin probe contains nucleotides 2124–2189 inserted between the *Eco*RI and the filled-in *Bam*HI sites of pBluescript.

RESULTS

p21^{Sdi1} RNA and Protein Levels During Cell Cycle Progression. To determine the role of p21^{Sdi1} in regulating cell cycle progression, we measured the amount of p21^{Sdi1} RNA and protein during the cell cycle and found that these were increased in G_0-arrested cells compared with actively proliferating cells. Serum stimulation of arrested cells resulted in a further increase in the amount of RNA and protein at 4 hr, followed by a rapid decline to low levels 24 hr after stimulation, at which time the majority of the cells were synthesizing DNA (Fig. 1 *A* and *B*). Similar results were obtained from immunostaining of cells with a monoclonal antibody specific for p21^{Sdi1} (Fig. 1*C*). These results suggest that p21^{Sdi1} may function in control of early G_1 progression by preventing activation of cyclin–Cdk complexes. This possibility is supported by the observations that cyclin D1–Cdk4, D1–Cdk2, and E–Cdk2 kinase activities are not detected in fibroblasts before mid-G_1, even though these protein complexes are present earlier in the cell cycle (31, 32). In this regard, it is of interest that although nuclear staining of p21^{Sdi1} is undetectable in the majority of cells stained 14 hr after serum stimulation as compared with G_0-arrested cells (Fig. 1*C*), Western analysis demonstrates that protein is present in the cell population 16 hr after stimulation (Fig. 1*B*). It is possible that protein accumulation in the nucleus or cytoplasm is different in G_0 versus late G_1 cells.

Expression of p21^{Sdi1} and Maintenance of Cells in G_0. To determine the role of p21^{Sdi1} in the maintenance of cells in G_0 and whether this involved association with cyclin–Cdk complexes, we prepared ^{35}S-labeled extracts from G_0-arrested cells. Sequential immunoprecipitation of the extracts with antisera to cyclins A, D1, and E and to the cyclin-dependent kinases Cdk2, Cdk4, Cdk5, and Cdk6 demonstrated that p21^{Sdi1} was present in all the complexes in quiescent cells except for those with cyclin A, which could not be detected (Fig. 2*A*). The identity of the 21-kDa band was determined by specific binding to antibodies against p21^{Sdi1} (data not shown). Interestingly, a large amount of p21^{Sdi1} protein was present after the immunodepletion with the various Cdk and cyclin antibodies, suggesting involvement of this gene in maintaining cells in G_0, because it is expressed in excess of that needed to complex with several of the known G_1 cyclins and cyclin-dependent kinases. A similar immunodepletion analysis of cells 16 hr after stimulation with serum revealed no such excess of the protein (Fig. 2*B*). In G_0-arrested cells there is an excess of the protein which probably results in inhibition of cyclin-dependent kinase activity, but in late G_1 the amount of p21^{Sdi1} has decreased to levels that would permit activation of kinase activity.

Effect of Expression of p21^{Sdi1} Antisense Sequences in G_0-Arrested Cells. To more directly examine the role of p21^{Sdi1} in maintaining the G_0 state we used antisense RNA expression to reduce the amount of p21^{Sdi1} in growth-arrested cells. After arrest in G_0 by either serum deprivation or growth to high density in 10% serum and then induction with metal ions, a high percentage of AS322 cells (transfected with the antisense plasmid) synthesized DNA, whereas a control cell line (transfected with vector alone) failed to do so (Fig. 3*A*). The maximum number of cells synthesizing DNA was observed when the cells were labeled 24–48 hr after induction (Fig. 3*B*), consistent with the result of RNase protection assays, which demonstrated a peak in the amount of antisense RNA 12 hr after induction (Fig. 4*A*). Induction of antisense RNA also resulted in a decrease in the amount of p21^{Sdi1} protein synthesis (Fig. 4*B*), though some synthesis continued to occur. This is consistent with the observation that a small amount of p21^{Sdi1} in cyclin–Cdk complexes promotes activity of the kinase and also appears to be involved in stabilizing the complexes (33). The antisense results were confirmed by microinjection of p21^{Sdi1} antisense synthetic phosphorothioate oligonucleotides into the cytoplasm of G_0-arrested cells. Thirty percent of the cells injected with the antisense oligonucleotides entered the cell cycle and synthesized DNA, whereas 7% of the cells injected with oligonucleotides of the same base composition in random order synthesized DNA.

To determine whether antisense-expressing cells could progress to mitosis we induced p21^{Sdi1} antisense RNA in contact-inhibited cultures and added Colcemid at 0.1 μg/ml at

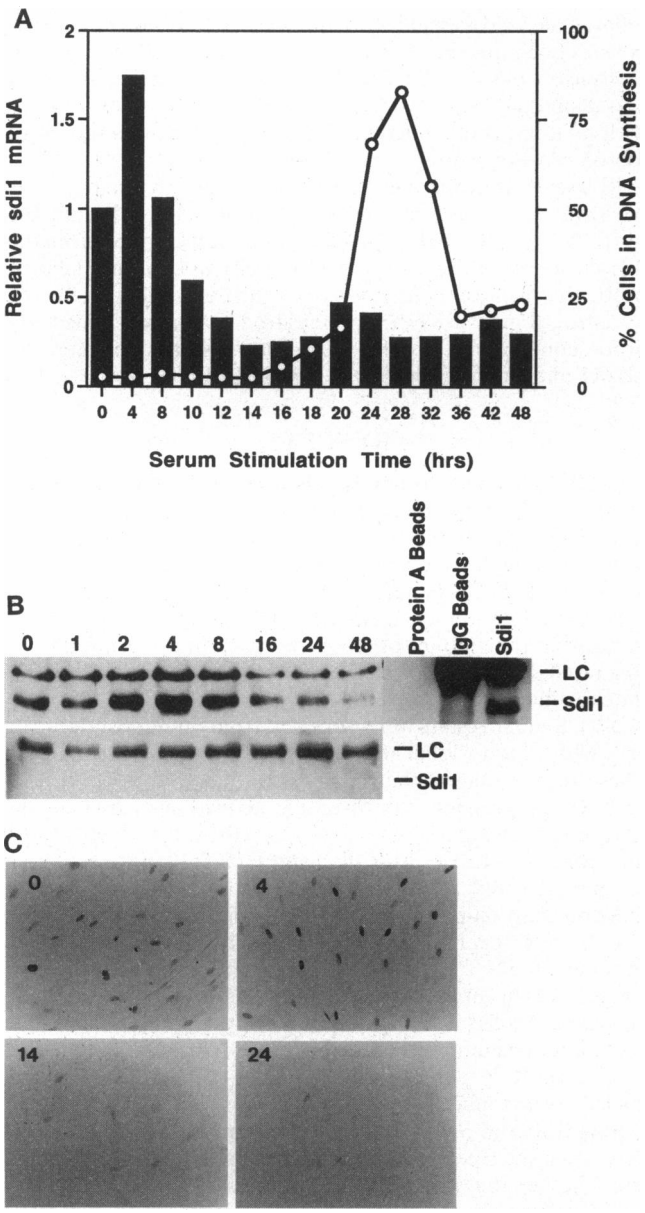

FIG. 1. p21^{Sdi1} RNA and protein levels in G_0-arrested and serum-stimulated human diploid fibroblasts. (*A*) Comparison of the amount of p21^{Sdi1} RNA and the percentage of cells synthesizing DNA after stimulation of G_0 cells with serum. Total RNA was harvested from quiescent, serum-deprived cells (0 time) and at various times after serum stimulation. The percentage of cells synthesizing DNA was determined by incubating cells for 1 hr prior to RNA harvest with tritiated thymidine (5 μCi/ml) (○). p21^{Sdi1} RNA was assayed by Northern analysis. p21^{Sdi1} and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA bands were quantitated with a β-scanner and the ratio of p21^{Sdi1} RNA to GAPDH RNA at each time was normalized to that ratio in quiescent cells (black bars). (*B*) (*Upper*) Immunoblot of p21^{Sdi1} protein after immunoprecipitation with a monoclonal antibody of total cell lysate (500 μg) from G_0-arrested cells at the indicated times (hr) after serum stimulation. Control lanes are protein A beads, mouse IgG beads, and p21^{Sdi1} immunoprecipitated from quiescent cell extract. (*Lower*) Same blot stripped and reprobed with normal mouse serum. LC, immunoglobulin light chain. (*C*) Immunostaining of p21^{Sdi1} in G_0-arrested cells at 0, 4, 14, and 24 hr after serum stimulation as indicated.

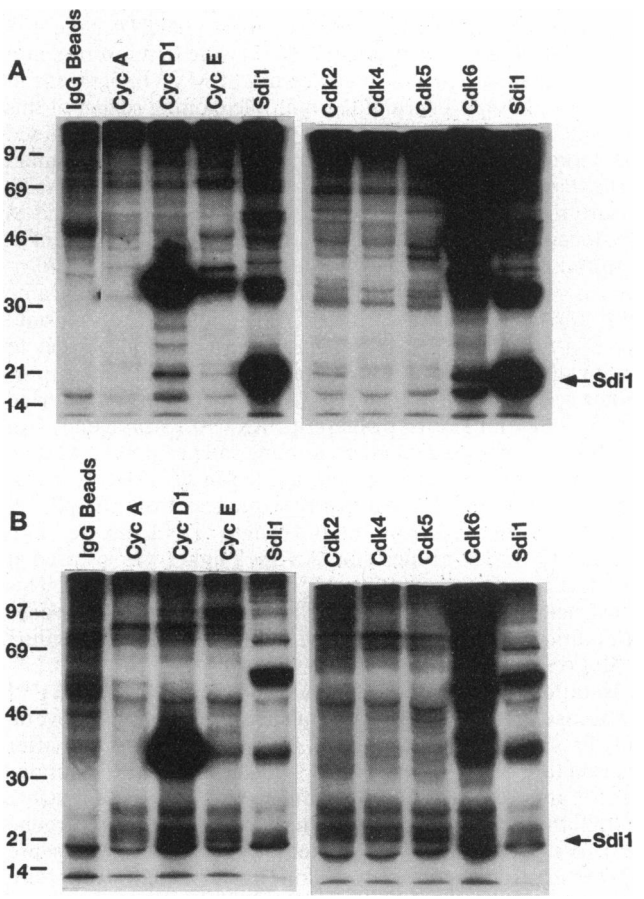

FIG. 2. Association of p21^{Sdi1} with cyclin–Cdk complexes in G_0-arrested and serum-stimulated human diploid fibroblasts. (*A*) ^{35}S-labeled extracts from G_0-arrested total cell lysates (2 mg) were sequentially immunoprecipitated with antisera against various cyclins or Cdks and each lysate was finally immunoprecipitated with p21^{Sdi1} antiserum to determine the amount of p21^{Sdi1} not complexed with these cyclins and Cdks. Control lane is cell extract immunoprecipitated with rabbit IgG beads. Positions of molecular mass markers (kDa) are indicated on the left. (*B*) ^{35}S-labeled extracts from G_0 cells 16 hr after serum stimulation sequentially immunoprecipitated as described for Fig. 1*B*.

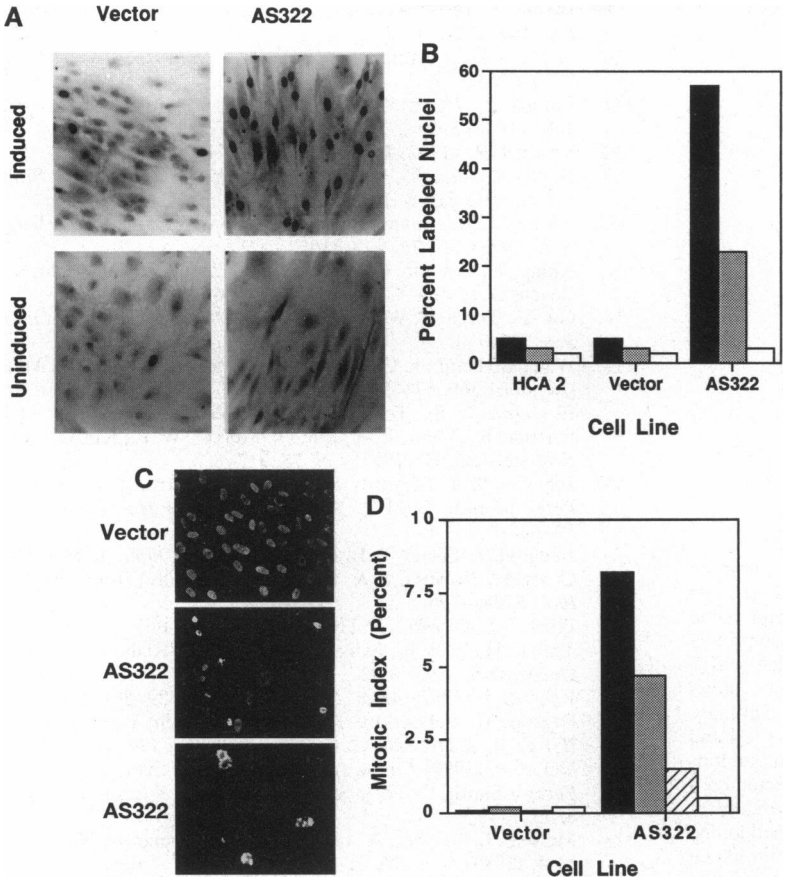

FIG. 3. Expression of antisense p21^{Sdi1} sequences causes G_0-arrested cells to synthesize DNA and enter mitosis. (*A*) Autoradiographic analysis of cells undergoing DNA synthesis with and without treatment with metals. (*Upper*) Control (Vector) and antisense cell line (AS322) induced by addition of metal ions. (*Lower*) Same cell lines, untreated. (Original magnification ×200.) (*B*) Quantitation of percentage of cells undergoing DNA synthesis after treatment with metal. Control (Vector) and antisense (AS322) transfected cell lines were treated as described for Fig. 2*A*. Cells were labeled with tritiated thymidine for 24–48 (■), 48–72 (▨), or 72–96 (□) hr after induction by metal ions. Percentage of cells that had synthesized DNA was determined as in *A*. (*C*) Analysis of mitotic cells in cultures of cell lines expressing control vector (*Top*) or p21^{Sdi1} antisense RNA (*Middle* and *Bottom*). (*Top* and *Middle* ×200, *Bottom* ×500.) To visualize mitotic cells in the AS322 line the photographs were taken in a different plane from adherent cells. (*D*) Mitotic index with time after induction of p21^{Sdi1} antisense RNA. Control (Vector) and antisense (AS322) cell lines were treated with metal ions as described for *A*. Cells were exposed to Colcemid at 0.1 μg/ml for 32–36 (■), 36–42 (▨), 42–48 (▨), or 48–54 (□) hr after induction by metal ions. A minimum of 300 cells were analyzed to determine the percentage in mitosis during each exposure period.

various times to arrest cells in mitosis. The cells were fixed and stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence microscopy confirmed that cells with condensed chromosomes, typical of cells arrested in mitosis by Colcemid, were present in antisense cultures and not in vector controls (Fig. 3*C*). Less than 0.5% mitotic cells was observed in the control cell line, whereas the mitotic index in the antisense cell line was 8% at 32–36 hr (Fig. 3*D*). These results suggest that removal of the G_0-to-G_1 block by p21^{Sdi1} antisense RNA results in not only entry into S phase but also progression through G_2 to the M phase of the cell cycle.

Fifteen percent of the cells entered mitosis within 48 hr after induction of p21^{Sdi1} antisense RNA (Fig. 3*C*), whereas 50% had synthesized DNA in that time (Fig. 3*B*). This apparent discrepancy can be explained by a number of possibilities: (*i*) A large percentage of the cells might have synthesized DNA in the last hours of the labeling period and not completed G_2 at the time of analysis, since many events must occur for a cell to complete S and progress from G_2 to mitosis. (*ii*) Some other factor(s) might be needed to enhance the effect of p21^{Sdi1} antisense in cell cycle progression. (*iii*) Technical aspects could also explain the result, since Colcemid does not arrest cells in mitosis with 100% efficiency and mitotic cells are loosely attached and easily lost during fixation and staining.

DISCUSSION

The results presented here implicate p21^{Sdi1} as a negative regulatory gene that maintains cells in G_0. The mechanism of action appears to involve binding of p21^{Sdi1} to cyclin–Cdk complexes, resulting in inactive complexes in G_0 cells. These complexes most likely remain inactive until an increase in the amount of cyclin–Cdk expression occurs, concomitant with a decrease in p21^{Sdi1} protein levels. This allows for activation of the kinases and cell cycle progression (34, 35).

Recently other cyclin–Cdk inhibitors, p16 (36), p15 (37), and p27 (38–40), have been identified. In particular p27, which shares a region of sequence similarity with p21^{Sdi1}, has been found in cyclin E–Cdk2 complexes in cells that have been growth arrested by either treatment with transforming growth factor β (TGF-β) or contact inhibition (38). The complex of p27 with cyclin E–Cdk2 is not present in proliferating cells, indicating that this protein also functions at the transition from G_0 to G_1. Therefore, p21^{Sdi1} and p27 may act together to cause a more stringent block of cells in G_0. Alternatively, the activity of these genes may be specific to different cell types and tissues.

The fact that p21^{Sdi1} expression increases during the first 4 hr after stimulation of cells with serum (13, 41) suggests that the normal function of this protein is to prevent premature entry into the mid and late G_1 phase of the cell cycle. The protein is localized in the nucleus (42), and the nuclear content increases immediately after mitogenic stimulation (Fig. 1*C*). This may act to block cyclin-dependent kinase activity until sufficient G_1 cyclin–Cdk complexes have accumulated to overcome the inhibitory activity of p21^{Sdi1} (34, 35). Thus, p21^{Sdi1} might be the first hurdle cells must overcome to successfully traverse the cell cycle. Additionally, since antisense RNA expression of this gene results in cell cycle progression to mitosis, progression through S phase could be the result of removal of the block to DNA replication caused by the interaction of p21^{Sdi1} with proliferating cell nuclear antigen (17). A recent report demonstrates that CAK, a Cdk-activating kinase, can activate other Cdk2–, cdc2–cyclin complexes (43). Therefore, another possibility is that once the initial hurdle of G_0 to G_1 is bypassed (44), a series of additional pathways, independent of the negative regulatory effects of p21^{Sdi1}, are triggered, and this allows for progression through the entire cell cycle.

We thank W. Brinkley for help in staining and identifying mitotic

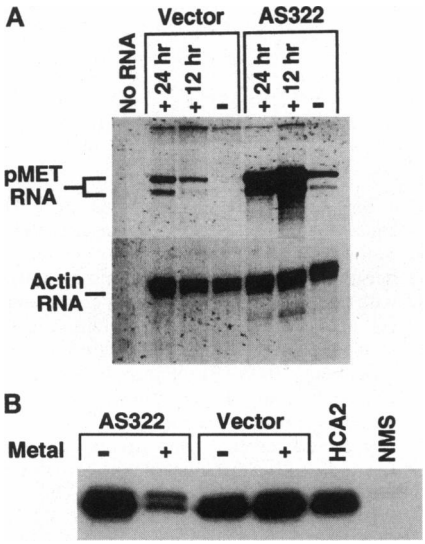

FIG. 4. Cells induced to express $p21^{Sdi1}$ antisense exhibit an increase in antisense RNA and a reduction in protein levels. (*A*) Analysis of total cell RNA from cell lines containing vector alone (Vector) and $p21^{Sdi1}$ antisense (AS322), performed by RNase protection analysis, using an antisense RNA probe derived from the pMET vector sequence, internally labeled with [^{32}P]UTP. This probe allows detection of RNA induction in both control and antisense cell lines. The probe detects two splice variants of the vector. Lanes: +, 24 and 12 hr after addition of metal ions (same as Fig. 3*A*); −, no metal ions added. (*B*) Control (Vector) and antisense (AS322) transfected cells were treated as described for Fig. 3*A*. Twenty-four hours after treatment of the cells with $ZnCl_2$ and $CdCl_2$ they were labeled with [^{35}S]methionine at 100 μCi/ml for 2 hr. $p21^{Sdi1}$ was then immunoprecipitated from 500 μg of total cell lysate as described for Fig. 1*B*. AS322, antisense-transfected cells, uninduced (−) and metal-induced (+). Vector, control cells uninduced (−) and metal-induced (+). Control lanes: HCA2 (^{35}S-labeled quiescent-cell extract isolated from untransfected cells) immunoprecipitated with $p21^{Sdi1}$ monoclonal antibody and NMS (normal mouse serum) as in Fig. 1*B*.

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