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5-Azacytidine-Induced Conversion to Cadmium Resistance Correlates with Early S Phase Replication of Inactive Metallothionein Genes in Synchronized CHO Cells

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Abstract—*Previous studies have shown both hypermethylation and late replication of DNA sequences to be associated with gene inactivity. To determine whether there is a causal relationship between patterns of DNA methylation and replication timing during S phase, we have examined the timing of replication of the inactive, hypermethylated metallothionein (*MT*) I and II genes in synchronized, cadmium-sensitive (Cd^s) CHO cells. The time of S-phase replication of the* MT *genes was ascertained by (1) determining the period of S phase wherein cadmium-resistant (Cd^r) cells could be induced with highest frequency by pulse treatment of synchronized Cd^s cells with the hypomethylating drug 5-azacytidine (5-aza-CR), and (2) by analyzing Southern blots of density fractionated DNAs isolated from synchronized cells pulse-labeled with BrdU during different intervals after release from hydroxyurea blockade. Southern filter hybridization analyses demonstrated replication of both* MTI *and* II *gene sequences within the first half of S phase. Consistent with this result, phenotypic conversion of Cd^s to Cd^r was maximal immediately after hydroxyurea release and decreased abruptly within three hours. The replication of inactive hypermethylated* MT *genes in early S phase argues that transcriptional inactivity and gene-specific hypermethylation are not sufficient conditions for late DNA replication.*

INTRODUCTION

DNA methylation is one mechanism of gene regulation in mammalian cells; hypermethylated genes typically have reduced levels of mRNA transcription (see reference 1 for review). The effects of demethylation on gene expression can be studied with the hypomethylating agent, 5-azacytidine (5-aza-CR) (2). Substitution of 5-aza-CR for cytidine can enable the transcription of inactive genetic loci, and alter the phenotypes of certain cultured cell lines (1,2). Examples of transcriptional activation include metallothionein (*MT*) (3 and this report), thymidine kinase (4), glutamine synthetase (5), hypo-

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xanthine phosphoribosyl transferase (*HPRT*) (6), glucose-6-phosphate dehydrogenase (*G6PD*) (6), and phosphoglycerate kinase (*PGK*) (6), among others. Syntenic alleles for three of these loci (*HPRT, PGK,* and *G6PD*) were reactivated on the inactive human X chromosome, providing evidence that DNA methylation is involved with X chromosome inactivation during development (6).

Late replication is another property of the entire inactive X chromosome in eutherian mammals (see reference 7 for review). Late replication also has been correlated with transcriptionally inactive, heterochromatic DNA sequences on autosomes (8). The correlation between late replication and transcriptional inactivity has led to the hypothesis that the timing of a gene's replication during S phase is an important factor with respect to transcriptional control (9). There are no reported examples of transcriptionally active genes which are late-replicating. Goldman et al. (9) proposed that early replication during S phase is a necessary, but not sufficient, condition for gene transcription.

Although both hypermethylation and late S-phase replication of DNA sequences have been associated with transcriptional inactivity, it is unclear whether patterns of methylation govern the time of gene replication during S phase. In this study, we have examined this question by determining the time of S-phase replication of transcriptionally inactive, hypermethylated metallothionein (*MT*) gene sequences in a cadmium-sensitive (Cd^s) CHO cell line. In Chinese hamsters, two linked *MT* genes, *MTI* and *MTII*, have been identified on chromosome 3 (10, 11). The induction of *MT*s is a major factor in conferring cellular cadmium resistance (12); selection of Cd^s cells for the Cd^r phenotype then enables the identification of phenotypic variants which express *MT*s (10). The molecular events underlying such phenotypic conversion can then be characterized using appropriate DNA probes for the *MT* genes (13).

MATERIALS AND METHODS

Cell Growth and Synchronization. Chinese hamster cell line CHO was grown in suspension culture in F10 medium supplemented with 15% neonatal calf serum and antibiotics. Cultures were regularly verified to be free of *Mycoplasma* contamination. Cells were synchronized in G_1 arrest by resuspension in thymidine-free F10 medium supplemented with dialyzed serum (hereafter, Td-F10). To accumulate cells in very early S phase, cultures arrested in G_1 by isoleucine deprivation were resuspended in Td-F10 medium containing 10^{-3} M hydroxyurea (14). To release cells from this early S-phase blockade, the cells were washed once in drug-free Td-F10 medium, then resuspended in that medium. Cultures used for DNA isolation and hybridization studies were prelabeled for 1.5 generations during exponential growth and synchronization in G_1 in medium supplemented with 6 nCi/ml [^{14}C] thymidine (53.2 mCi/mmol, New England Nuclear Corp., Boston, Massachusetts); incorporated ^{14}C label was then used to quantitate DNA loaded on analytical gels.

Treatment with 5-Azacytidine and Detection of Cd-Resistant Cells. CHO cells were synchronized in isoleucine-deficient medium as described above. The early G_1 population of cells was resuspended in Td-F10 + 15% neonatal calf serum and 10^{-3} M hydroxyurea. This population was split into two cultures (1200 ml and 300 ml). At 2 h after release from G_1 block, 5-aza-CR (2 μM) was added to the 300-ml G_1 traversing culture, while the 1200-ml portion of the culture continued to traverse G_1 in the presence of hydroxyurea alone. At 10 h after release from G_1 arrest into medium containing hydroxyurea, cells from the culture treated with 5-aza-CR were resuspended and plated at densities of 5×10^5 cells/60-mm dish in 5 ml of Td-F10 containing 2 μM $CdCl_2$, to select for Cd^r variants. To determine plating efficiency in the absence of $CdCl_2$ selection, 200 cells

were plated in 60-mm dishes in medium without $CdCl_2$. After incubation in a 5% CO_2 atmosphere at 37°C for 1 week, plates were stained and scored for colonies of greater than 50 cells.

Also at 10 h after release from G_1 block into medium containing hydroxyurea, the 1200-ml portion was resuspended in Td-F10 and divided further into six 200 ml spinner cultures. At 6-hourly intervals after release from hydroxyurea blockade, 5-aza-CR (2 μM) was added to one 200-ml culture for a 1-h pulse period, after which time cells were plated to determine the frequency of Cd^r cells as described above.

BrdU Labeling and CsCl Density Gradients. After isoleucine deprivation, approximately 4×10^8 cells were pelleted and resuspended in 2100 ml of medium containing both isoleucine and 10^{-3} M hydroxyurea. This cell suspension was split into a 300-ml and 1800-ml spinner culture. After 4 h, BrdU (10 μM final concentration) was added to the smaller control culture for 7 h. The BrdU labeling period was stopped by extracting the DNA from the cells (15). The other pulse BrdU labeling periods were obtained by splitting the 1800-ml culture into six 300-ml spinner cultures after release from a 10-h hydroxyurea blockade. BrdU was then added in turn to each culture for 1-h intervals spanning the first 6 h following release from hydroxyurea blockade, and DNA was isolated immediately following each 1-h BrdU pulse label.

Two hundred micrograms of DNA from each population of BrdU-labeled cells were then digested to completion with BamHI by methods described by the supplier (New England Biolabs, Cambridge, Massachusetts), loaded onto CsCl density gradients (1.69 g/cc), and spun at 4°C, 38,000 rpm in a Beckman Ti 65 rotor for 72 h. BrdU (replicated heavy–light or HL DNA) and non-BrdU (nonreplicated light–light or LL DNA) labeled DNA were fractionated by a Buchler Auto Densi Flow II density gradient collector. Each fraction of HL and LL DNA was dialyzed 3 × with 1 × TE buffer, precipitated with 0.1 volumes 3 M Na acetate and 2.5 volumes EtOH, and resuspended in 1 × TE (buffer abbreviations are defined in reference 15).

Southern Blot Analyses. For Southern filter hybridizations, DNA samples were digested with restriction enzymes according to directions provided by the supplier (New England Biolabs), electrophoresed in 0.8% agarose gels (medium EEO grade; FMC Corp., Marine Colloids Div., Rockland, Maine), and transferred to Zetabind hybridization membranes (AMF Cuno, Meridan, Connecticut) by the method of Southern (16) as modified by Wahl et al (17). Prehybridization was performed for 8–16 h at 42°C in 5 × SSC, 1 × Denhardt's, 0.02 M $NaPO_4$ (pH 6.7), 100 μg/ml of sonicated denatured salmon sperm DNA, 10% dextran sulfate, and 40% formamide. Hybridization was performed at 42°C for 48 h in the prehybridization solution with 3.0×10^7 cpm of a 150-nucleotide hamster *MTII* coding sequence probe (13) labeled with [^{32}P]dCTP by the T_4 DNA polymerase 1 replacement synthesis method of O'Farrell (18). Washes were performed once for 15 min in 2 × SSC–0.1% sodium dodecyl sulfate (SDS) at 25°C; once for 15 min in 0.1 × SSC–0.1% SDS at 25°C; and twice for 30 min in 0.1 × SSC–0.1% SDS at 60°C.

RESULTS

Detection and Stability of Cd^{+2}-Resistant CHO Cells after 5-Azacytidine Treatment. We have described previously the isolation and characterization of Cd^{2+}-resistant (Cd^r) clonal variants derived from Cd^s CHO cells (10). Coordinate expression of both major isometallothioneins was observed in all Cd^r variants, whereas Cd^s CHO cells do not provide detectable MT (10). In general, the extent of Cd^{2+}-resistance can be correlated with *MT* expression, measured with respect to both induction kinetics and maximal rate of

MT synthesis. Variant cells resistant to 1–2 μM Cd^{2+} could be detected in single-step selection of CHO cell populations, at a frequency of approximately 10^{-5} or less; such clones produce demonstrable *MT*, and exhibit an *MT* gene copy number equivalent to that of wild-type, Cd^s CHO cells (10).

Given the observations of Compere and Palmiter (3) and Lieberman et al. (19) that DNA methylation involving the *MT* locus controls *MT* gene transcription in certain mouse cell lines, we investigated whether a similar mechanism could account for the Cd^s to Cd^r phenotypic switch in CHO cells. As a first test of this hypothesis, asynchronous cultures of CHO cells were grown in the presence of the nonmethylatable cytosine analog, 5-azacytidine (5-aza-CR, 1.0, 2.0, 5.0, and 10.0 μM), then sampled to determine the frequency of Cd^r variants. Treatment with 5-aza-CR increased the frequency of Cd^r variants resistant to 1 μM Cd^{2+} to as much as 2×10^{-2}, above a background frequency of less than 10^{-5} for CHO cells. (At 5-aza-CR concentrations 2.0 μM and below, phenotypic conversion was achieved without demonstrable cytotoxicity.) Using 2 μM 5-aza-CR as the drug concentration which achieved maximal phenotypic conversion without detectable cytotoxicity, the optimal duration of drug exposure for inducing Cd^r variants was determined for asynchronous cultures (Fig. 1). Maximal conversion to the Cd^r phenotype was achieved for cultures treated for 12–18 h with a pronounced increase in the Cd^r frequency observed in the interval 1–4 h after initiating treatment (Fig. 1, and data not shown). These results are consistent with the mechanism of 5-aza-CR action, which requires that cells engage in one cycle of DNA replication to establish hemimethylated DNA (1). Cultures of Cd^s CHO cells had a population doubling time of 16–18 h, with postreplication growth and mitotic phases of approximately 2 h. Thus, treatment coincided with the time expected for all cells in the exponentially growing population to complete one round of

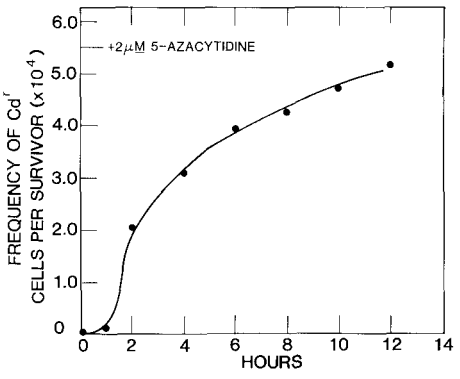

Fig. 1. Kinetics of appearance of Cd^r colonies during exposure of CHO cells to 2μM 5-Aza-CR. 5-Aza-CR was added to a suspension culture of exponentially growing CHO cells at time 0. Periodically thereafter, aliquots of the cell culture were removed and cells were replated in complete medium containing 2 μM $CdCl_2$. After incubation for 1 week, colonies were counted and results presented as the frequency of Cd^r colonies per surviving cell.

DNA replication. The initial lag in appearance of Cd^r cells after beginning the 5-aza-CR exposure can be attributed to time required for equilibration of the 5-aza-CR pool with the cytidine pool. The S-phase dependence of 5-aza-CR action is discussed in more detail below, in the context of experiments with synchronized cell cultures.

Five independent clonal isolates of Cd^r cells from 5-aza-CR-treated CHO populations were grown in cadmium-free medium for 30–35 population doublings, and then examined for their degree of cadmium resistance in comparison to wild-type Cd^s CHO cells. Each of the five clones examined retained its Cd^r phenotypes.

Appearance of Cd^r Variants without Phenotypic Lag. The use of cadmium selection to quantitate the conversion of Cd^s cells to an *MT*-expressing phenotype relies on the assumption that phenotypic lag prior to expression of the Cd^r phenotype is not a complication. When a hemimethylated state is produced either by inhibition of maintenance methylases by 5-aza-CR or by incorporation of the drug into newly replicated DNA strands (1), decreased methylation might be

expected to enable immediate transcription of *MT* genes in cells whose DNA is substituted at the *MT* locus. *MT* gene transcription, in turn, would be expected to confer the Cd^r phenotype.

A synchronous culture of Cd^s CHO cells was treated with 2 μM 5-aza-CR for 18 h, after which aliquots of 10^6 cells were diluted and plated in medium containing 1 μM Cd^{2+}, or maintained in nonselective medium, pending plating in Cd^{2+} at subsequent posttreatment expression times (data not shown) ranging from 0 h to 7 days. Immediately after 5-aza-CR treatment, the frequency of Cd^r cells was increased to 11×10^{-2}/survivor, an increase of more than 100-fold above the background Cd^r frequency of $<10^{-4}$. Subsequent expression times exhibited a plateau in the Cd^r frequency which did not exceed 3×10^{-2}, likely reflecting the limits of sampling error in these experiments. These experiments indicated that Cd^{2+} selection could be used in single-step selection assays to quantitate the conversion of Cd^s cells to a Cd^r phenotype, without serious complications of phenotypic lag.

Cd^r Variants Induced by 5-aza-Cytidine Treatment Exhibit Alterations in DNA Methylation at Metallothionein Locus. Using a Chinese hamster cDNA clone encoding *MTII* (13), Southern transfers of DNA cleaved with methylation-sensitive isoschizomers were probed to reveal alterations in the pattern of DNA methylation at the *MT* locus (Fig. 2). Hybridizations were performed under conditions which enabled detection of homologous *MTI* coding sequences (10). These analyses revealed a complex pattern of restriction fragments, as would be expected for cleavage by an endonuclease recognizing a four-base recognition sequence within the linked *MTI* and *II* genes which in turn comprise two alleles on Chinese hamster chromosome 3 (10, 11). While detailed interpretation of these restriction patterns must await sequencing of genomic clones encompassing the *MTI* and *II* loci, Fig. 2 demonstrates clear

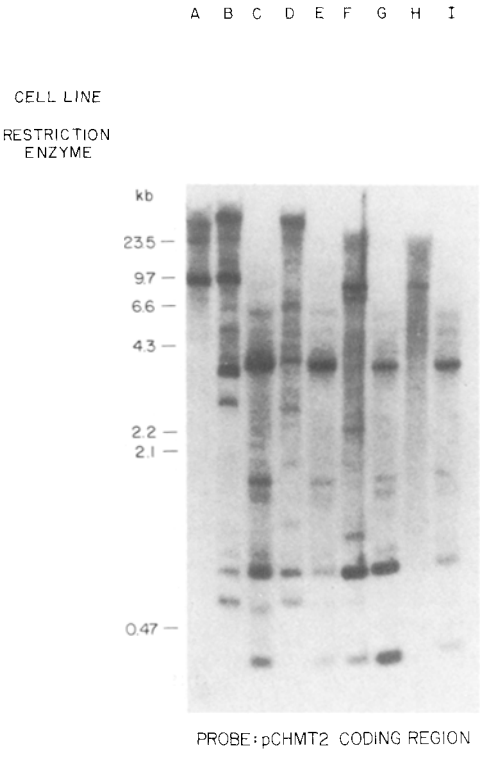

Fig. 2. Filter hybridization demonstrating hypermethylation of *MT* gene sequences in Cd^S CHO cells and hypomethylation in Cd^r CHO cells. DNAs were isolated from wild-type Cd^S CHO cells and their Cd^r derivatives, 5-AZCd^r 4-15, 5-AZCd^r 5-13, and Cd^r 30F9. Clonal Cd^r derivatives 5-AZCd^r 4-15 and Cd^r 5AZ 5-13 were obtained by selection in 2 μM $CdCl_2$ following exposure to 2 μM 5-Aza-CR. The origin of the Cd^r 30F9 CHO cell line was described previously (10). The Cd^s CHO DNA digested with HpaII (lanes A and H) revealed higher-molecular-weight *MT* gene sequences, distinct from those observed in the MspI-digested DNA (lane I), thus indicating that the majority of the HpaII/MspI sites are blocked from HpaII digestion due to methylation. However, in all Cd^r MT-inducible variants, HpaII digestion generated smaller fragments characteristic of MspI digestion, indicating hypomethylation of a subset of the HpaII/MspI sites.

differences in MspI and HpaII restriction patterns in Cd^s CHO cells, indicative of methylation of the *MT* loci in these cells. In contrast, Cd^r variants induced by 5-aza-CR harbor several new HpaII sites accessible to cleavage. The retention in Cd^r cells of some sites insensitive to HpaII cleavage suggests that perhaps not all methylcytosines were

converted to cytosine by 5-aza-CR. Indeed, it is likely that only one of the two *MTI/II* alleles need be activated to generate the Cd^r phenotype; the other allele thus would be expected to remain methylated. DNA from a previously described Cd^r variant, Cd^r30F9 (10), was used in these studies as a positive control and is best used for comparison to clones derived after 5-aza-CR treatment. This cell line expresses both *MTI* and *MTII* and has amplified both *MT* genes in response to continued Cd^{2+} selective pressure (10). Several predominant bands of hybridization detected in HpaII-cleaved DNA from these Cd^r cells were shared by 5-aza-CR-derived clones Cd^r 5-13 and Cd^r 4-15, yet were absent in Cd^s CHO cell DNA cleaved with this enzyme. These results confirm that conversion from the Cd^s to the Cd^r phenotype by 5-aza-CR can be attributed to changes in the pattern of DNA methylation at the *MT* locus.

Replication of Hypermethylated MT Sequences. Determining the time of inactive *MT* gene replication in Cd^s CHO cells involved arrest in G_1 by isoleucine deprivation, followed by arrest at the G_1/early S boundary by hydroxyurea (14, 20). Synchronized CHO cell cultures were pulse-labeled with BrdU during seven different periods of S phase, as summarized in Fig. 3. The first (control) period involved incorporation of BrdU during the hydroxyurea blockade, whereas the other six periods represent incorporation of BrdU at hourly intervals spanning the first 6 h after release from hydroxyurea. DNA was isolated from each cell culture immediately following each BrdU labeling period. The progression of cells from S phase through G_2 into mitosis was monitored by measuring the fraction of cells labeled with [^{3}H]TdR and the fraction of divided cells (Fig. 3).

DNA from each population of S-phase cells was digested with BamHI so that BrdU-labeled (replicated HL) and non-BrdU-labeled (nonreplicated LL) DNA fragments could be separated on CsCl density gradients by ultracentrifugation (Fig. 4). Southern fil-

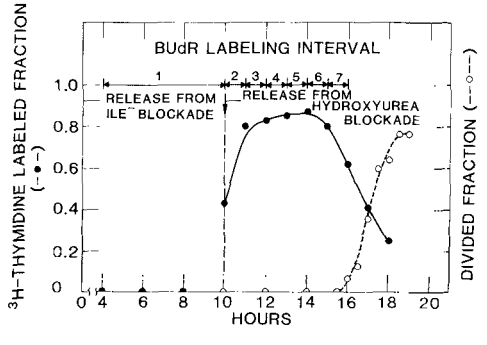

Fig. 3. CHO cells synchronized by isoleucine deprivation (36 h) followed by a 10-h hydroxyurea blockade. The lower abscissa shows the time elapsed after release from the Ile^- blockade. BrdU was added to the first cell population for 6 h during hydroxyurea arrest. Subsequent BrdU pulse-labeling periods involved adding BrdU to different aliquots of a synchronized cell population for hourly intervals after release from hydroxyurea. The percentage of cells incorporating [^{3}H]thymidine during S phase (—●—) and the divided fraction of cells following release from hydroxyurea (—O—) are shown.

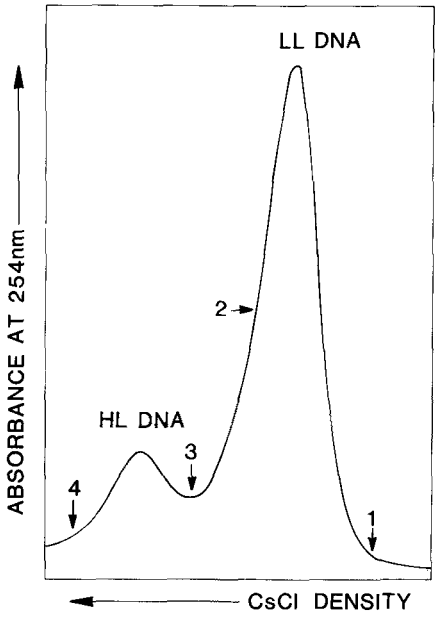

Fig. 4. Resolution of BrdU-labeled (HL) and non-BrdU-labeled (LL) DNA separated on CsCl gradients by ultracentrifugation. Fractions between arrows 1 and 2 were collected as LL DNA and the DNA between arrows 3 and 4 was collected as HL DNA. DNA in the interval between LL and HL (2–3) was discarded.

ter hybridization analyses were performed on HL and LL DNAs recovered from each period of S phase to determine when MT sequences were replicated (Fig. 5). A 150-nucleotide ^{32}P-labeled Chinese hamster MTII coding sequence (13) was used as a probe, under conditions which allowed cross-hybridization to MTI coding sequences. Figure 5 is a Southern blot showing the relative proportion of HL to LL (replicated–unreplicated) MT DNA sequences for each period of S phase. The intensities of the bands detected in each lane of the blot were quantitated for the *MTII* gene by densitometry so that accurate ratios of HL:LL DNA could be determined (Fig. 5). The HL:LL ratio for the first hour after release from hydroxyurea was >1.0, whereas ratios from all other periods of S phase were less than 1.0. We therefore conclude that the MTII coding sequence replicates during the first hour of S phase. Although not quantitated by densitometry, it was also clear that MTI coding sequences replicate coordinately with MTII. It should also be noted that HL:LL ratios for cells labeled with BrdU during the hydroxyurea blockade and for the second and third hour of S phase were also quite high, whereas MT replication declined substantially in the fourth, fifth, and sixth hours of S phase. A similar pattern also was observed for the frequency of Cd^r variants induced in synchronized cell populations treated with 5-azacytidine, as described below.

Cell-Cycle-Dependent Conversion of Cd^s CHO Cells to Cd^r Phenotype. The conversion of CHO cells from a Cd^s (MT noninducible) to a Cd^r MT-inducible phenotype by growth in 5-aza-CR, together with the observation that hypermethylated *MTI* and *II* genes are replicated in early S phase, suggest that interference with postreplication maintenance methylation in the region of the *MT* genes would lead to S-phase specific conversion of

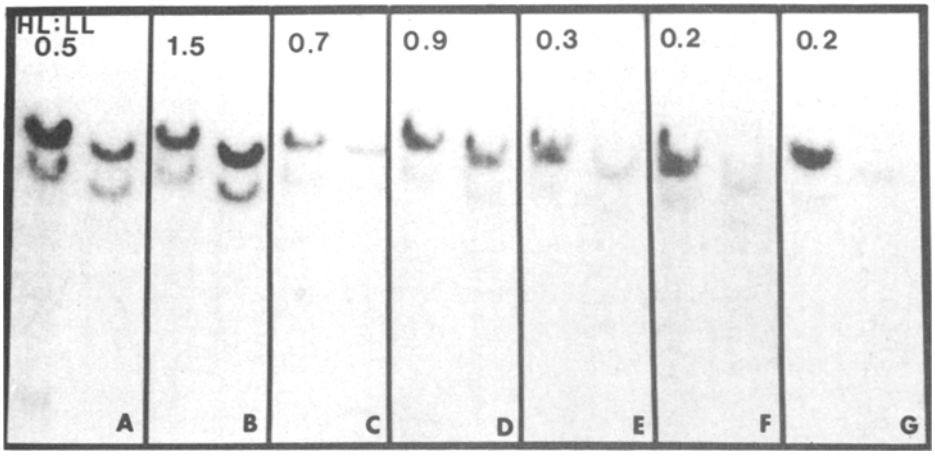

Fig. 5. The period of MT replication during different periods of S phase, determined by Southern blot analyses of BrdU-labeled DNA isolated from synchronized cells. DNAs were digested with BamH1 and centrifuged to equilibrium in CsCl to separate BrdU (HL) from non-BrdU (LL) -labeled DNA fragments. Paired lanes containing LL (left) and HL (right) DNAs from different labeling periods are shown in each panel. When electrophoresed in 0.8% agarose gels containing ethidium bromide, HL DNA consistently migrated faster than LL DNA. BrdU labeling periods include cells labeled with BrdU during the hydroxyurea blockade (A) and cells labeled with BrdU 1 (B), 2 (C), 3 (D), 4 (E), 5 (F) and 6 h (G) after release from hydroxyurea. Nine micrograms of DNA were loaded in lanes 1–4 and 7–14, whereas lanes 5–6 contain 4 μg of DNA. Both MTI and MTII sequences replicated during the first half of S phase. The larger BamH1 fragment (6.8 kb), which represents *MTII*, is more intense than the smaller 6.6 kb *MTI* fragment because a 150-bp Chinese hamster *MTII* coding sequence was used as a probe (see reference 10). The numbers at the top of each panel indicate the ratio of HL:LL *MTII* DNA as determined by densitometry.

CHO cells from a Cd^s to a Cd^r phenotype with high frequency. It was possible to test this prediction by pulse treatment with 5-aza-CR, which is incorporated rapidly into replicating DNA and, because it is blocked at the 5 position, prevents maintenance methylation of nascent daughter DNA strands (see reference 1 for review).

CHO cells were grown in isoleucine-deficient medium to accumulate cells in early G_1 and then released from G_1 blockade into complete medium containing hydroxyurea (HU) to synchronize the population in early S phase near the G_1/S boundary. Since we have shown previously (21) that a small amount of DNA is synthesized in CHO cells despite the HU block, 5-aza-CR was added to a control aliquot of the HU-treated culture during the 6 h of HU blockade. Following release from the G_1/S block into complete medium, synchronized cultures were treated with 2 μM 5-aza-CR for 1-h pulse intervals, then plated in Cd^{2+} selection medium as described in Materials and Methods. As expected, a small but detectable fraction of CHO cells underwent the Cd^s to Cd^r phenotypic switch during HU blockade. The Cd^r frequency increased significantly above this baseline immediately after release of cells from HU block and then declined rapidly as the population traversed further into S phase (where the bulk of DNA synthesis occurs) (Fig 6). Thus, the 5-aza-CR-mediated conversion of synchronized Cd^s CHO cells to the Cd^r phenotype was maximal in early S phase.

DISCUSSION

Since late replication and hypermethylation both have been correlated with transcriptional inactivity, we attempted to test the hypothesis that early replication requires hypomethylation of DNA sequences. Our results demonstrate, however, that unexpressed, hypermethylated MT DNA sequences in CHO cells replicate early during S phase. This was verified by Southern filter hybridization analyses of DNAs isolated from synchronized cells labeled with BrdU during different intervals of S phase as well as by demonstrating that 5-aza-CR can most effectively convert Cd^s cells to a Cd^r phenotype during early S phase. Our results support the conclusion that DNA sequences become late-replicating during S phase by mechanisms distinct from gene-specific methylation.

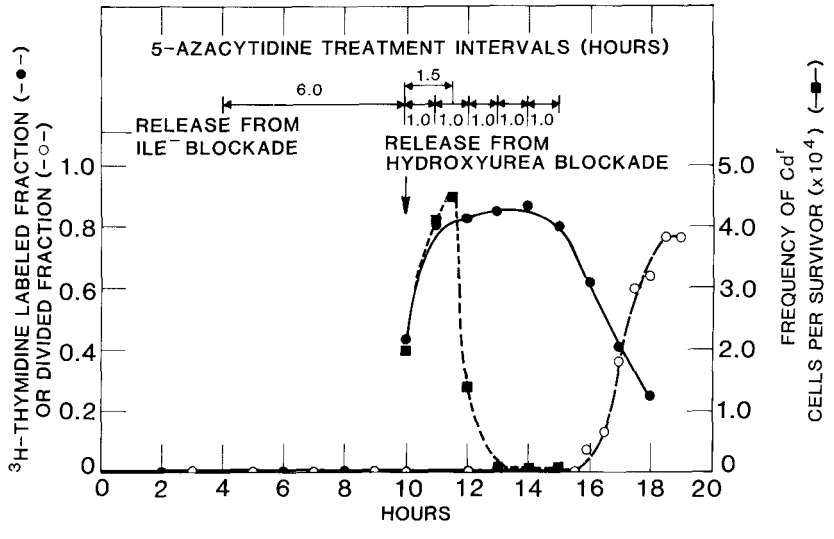

Fig. 6. Cell cycle dependence of Cd^r phenotypic conversion by 5-aza-CR. Data for the fraction of Cd^r colonies per 10^4 surviving cells are plotted at the end of the 5-aza-CR treatment interval.

Our conclusion that gene specific DNA methylation need not be directly involved with the temporal order of gene replication is supported by additional evidence. Although individual genes on the inactive X chromosome contains regions that are more heavily methylated than alleles on the active X (22–24), there are no profound differences in overall patterns of methylation between the active and inactive X chromosomes (25–27). In addition, Calza et al. (28) demonstrated that the chromosomal positions of genes appear to be important factors in influencing gene replication timing. Their work was consistent with the observation that late replication of the inactive X chromosome can spread by position effect to a translocated autosome (29, 30) and thereby inactivate autosomal genes (31, 32). This spreading inactivation event is difficult to explain by methylation alone. We would propose, therefore, that DNA methylation and late replication are two distinct and, perhaps, mutually exclusive mechanisms of silencing gene transcription.

In our experiments, the ratio of HL to LL DNA did not decline sharply until the last half of S phase. Likewise, the frequency of Cd^r revertants induced by treatment with 5-aza-CR declined gradually until the last half of S phase, whereupon the frequency declined sharply. These results might be attributable to a certain amount of asynchrony in our cell population. They might also indicate heterogeneity in the replication time of *MT* genes in CHO cells. A similar result was obtained by Jones et al. (33), who demonstrated that 5-aza-CR could convert the *HPRT* allele on the late-replicating, inactive X chromosome to a transcriptionally active state specifically during the last half of S phase. In their experiments, the frequency of $HPRT^-$ to $HPRT^+$ conversion did not significantly increase until late S phase (33). Such results reported here clearly indicate that 5-aza-CR must be incorporated into DNA to be effective.

Our study also indicated that both *MTI* and *MTII* genes replicate coordinately during the first hour of S phase. We previously reported the *MTI* and *MTII* genes to be located within 14 kb of each other on Chinese hamster chromosome 3 (10,11). Coordinate replication of linked gene families also has been reported for the histone genes, in studies which proposed a functional relationship between histone gene replication and expression (34). In this context it may be noteworthy that both *MTI* and *MTII* genes are coordinately expressed in Chinese hamster and mouse cells (10, 35) and that both loci are activated coordinately by either spontaneous or induced changes in DNA methylation (19, this report).

If late replication and DNA methylation are both important factors in regulating gene expression, we would speculate that late replication is the more stringent and permanent method of gene control, given the relative ease with which even linked loci can be activated by both spontaneous and 5-aza-CR-induced demethylation events. We would emphasize that our conclusions are based on the observation that DNA methylation sufficient to render *MT* genes transcriptionally inactive is insufficient to cause late replication of *MT* coding sequences. If *MT* loci are similar to those for HPRT and G6PD, then only a small region of the gene might need to be hypermethylated to result in transcriptional inactivity, whereas methylation of additional sites within or flanking the gene could still conceivably alter replication time. It is also possible that the methylation of very specific DNA sequences, such as origins of replication, could alter gene replication time. Recent studies showing that 5-aza-CR treatment can alter chromosome replication patterns are consistent with the concept that methylation may in some way be involved with DNA replication (36,37).

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