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An Early S Phase Checkpoint Is Regulated by the E2F1 Transcription Factor

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The E2F1 transcription factor regulates transit of cells through the S phase checkpoint, dependent on its association with cyclin A/cdk2. Expression in cells of a mutant E2F1 lacking the cyclin A/cdk2 binding domain leads to partial arrest of cells at the S phase checkpoint. When subconfluent growing cells expressing this mutant E2F1 are analyzed in detail, it is shown here that they display a significantly reduced incorporation of ^{3}H-thymidine into the DNA of each S phase cell, compared to control cells or to cells overexpressing full-length E2F1. Further, when cells are blocked at the G1/S phase border and released, there is a clear reduction in the amount of ^{3}H-thymidine incorporated into the DNA of S phase cells by 1.5 hours post release. Considering a normal 6 hour S phase duration, the results show that the S phase checkpoint mediated by E2F1 is not a late S phase event but an early one.

The E2F transcription factor activates the expression of genes whose expression is needed for S phase, such as dihydrofolate reductase, DNA polymerase alpha, cyclin E and thymidine kinase (1-4). E2F is a multiprotein family (E2F1-5 and DP1 & 2) where the E2F's form heterodimers with the DP proteins (1,2,4). The amino-terminal domain of E2F1, 2 and 3 contains a site for cyclin A/cdk2 binding (5-7). Cyclin A is an S phase cyclin and cdk2 is its associated kinase. The cyclin A/cdk2 complex associates with residues 87-94 of E2F1 during S phase, resulting in a phosphorylation of both E2F1 and its heterodimer partner DP1 (8). This phosphorylation causes E2F1/DP1 to dissociate from DNA (5-7). When mutants of E2F1 that lack the cyclin A/cdk2 binding domain are expressed in NIH3T3 cells, profound effects are seen on cell cycle transit. The cells arrest in S phase and begin to undergo apoptosis (9). It has been proposed that the inability of the mutant E2F to dissociate from DNA at the end of S phase results in an S phase arrest and hence defines an S phase checkpoint (9). This is consistent with the requirement for cyclin A/cdk2 in the onset of DNA replication (10). However, it is not yet known what process this E2F1-mediated checkpoint senses. Possibilities include DNA damage, inefficient initiation of DNA at origins and elongation of the DNA polymerase complex.

Further insight into the S phase checkpoint has been gained by studies where a mutant E2F1 lacking the first 87 amino acids (termed E2F1d87) was constitutively expressed in NIH3T3 fibroblasts (11). This mutant E2F1 binds cyclin A/cdk2 with a 10-fold reduced affinity (12) and produces a number of effects on the phenotype of these fibroblasts. The cells have a lengthened S phase, a slow rate of proliferation and do not undergo apoptosis but are viable and can be carried as a cell line (11,13-15). Here we have used this mutant to better understand the nature of the S phase checkpoint. Assessment of ^{3}H-thymidine incorporation into the DNA of S phase cells indicates that E2F1d87 causes a significant decrease in the incorporation of nucleotide into DNA early in S phase.

MATERIALS AND METHODS

Cell culture and cell cycle analysis. NIH3T3 fibroblasts (ATCC) expressing the E2F1 and E2F1d87 transcription factors (11) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. To arrest cells in G0/G1 phase, the cells were cultured in reduced serum media (0.5% calf serum) for 48 hours. Cells to be processed for flow cytometry were rinsed twice in chilled PBS then trypsinized and resuspended in 10 mls DMEM plus 10% serum. The cells were then pelleted and resuspended in 70% ethanol. The cells were kept on ice for 10 minutes, pelleted and then treated with RNAase A (1.8 ug) for 30 minutes at room temperature. Propidium iodide (Sigma) was added to a final concentration of 2 ug/ml for an additional 15 minutes at room temperature. Cell cycle analysis was then performed on a Coulter Profile 2 Flow Cytometer.

Thymidine block and ^{3}H-thymidine incorporation. To block cells at the G1/S boundary, proliferating cells were treated with excess thymidine (2 mM) for 14 hours. The cells were washed in PBS and fresh media added containing 10% serum. After nine hours, excess thymidine (2 mM) was again added for an additional 14 hours. The cells were then washed in PBS, fresh media containing 10% serum was added and flow cytometry performed at the indicated times.

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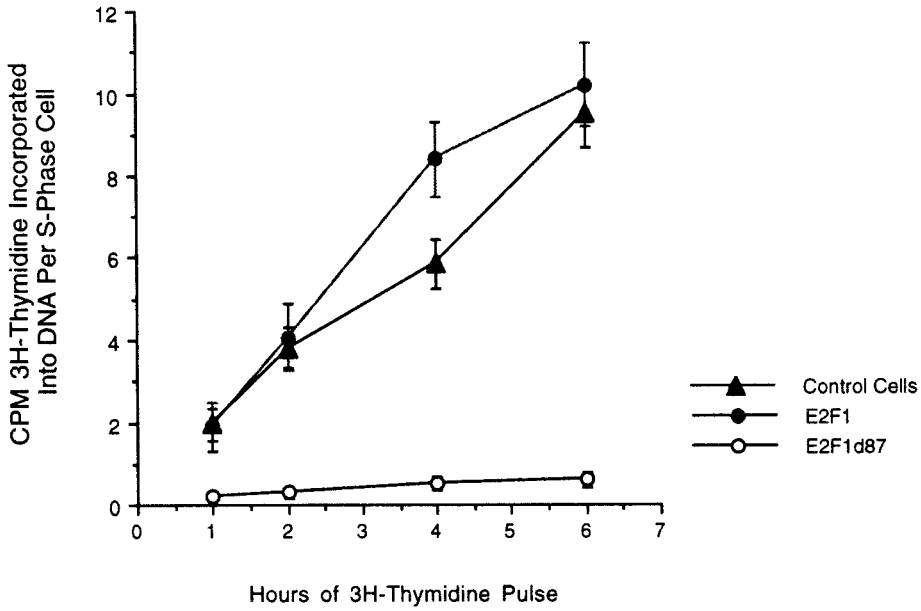

FIG. 1. Proliferating E2F1d87 expressing cells show a marked decrease in the level of ^{3}H-thymidine incorporated into the DNA of each S phase cell. Proliferating control cells (NIH3T3 fibroblasts) or NIH3T3 fibroblasts constitutively expressing the E2F1 transcription factor or the E2F1d87 mutant transcription factor were pulse labeled with ^{3}H-thymidine (5 uCi/ml) for varying lengths of times. Following the pulse, the cells were trypsinized and counted. An aliquot was processed for flow cytometry and another aliquot was processed for determination of incorporation of ^{3}H-thymidine into DNA. The incorporation of ^{3}H-thymidine into DNA is shown and given as CPM per S phase cell. The error bars indicate the standard deviation.

Cells were labeled for varying lengths of time with 5 uCi/ml of ^{3}H-thymidine at 20 Ci/mMole. The cells were washed once in PBS, trypsinized, resuspended in 10% serum containing DMEM and viable cells counted and aliquots were taken for flow cytometry analysis. Incorporation of ^{3}H-thymidine into DNA (acid insoluble material) was then assessed on aliquots of the cells (as in Ref. 16).

RESULTS AND DISCUSSION

It has been previously demonstrated that NIH3T3 fibroblasts constitutively expressing a mutant E2F1, lacking part of the cyclin A/cdk2 binding domain, have a longer S phase due to a reduced ability to transit the S phase checkpoint (9,13). As a result, these cells demonstrate a slow growth phenotype. To better understand the metabolic nature of this inefficient transit through S phase, labeling studies were undertaken. Control cells or cells constitutively expressing either E2F1 or the mutant E2F1d87 which lacks part of the cyclin A/cdk2 binding domain, were subcultured at low density (2 x 10^{5}/10 cm plate). At 24 hours post-plating, the cells were labeled with ^{3}H-thymidine for 1, 2, 4 and 6 hours. Aliquots of the cells were then taken for cell counts and assessment of cell cycle distribution by flow cytometry. Also, aliquots were taken for determination of the incorporation of labeled thymidine into DNA. In this way the counts per minute of ^{3}H-thymidine incorporated into DNA per the total number of S phase cells could be accurately measured. Shown in Figure 1 are the results of this determination. It is clear that the control cells and the cells constitutively expressing E2F1 show increasing levels of incorporation of ^{3}H-thymidine into the DNA of S phase cells with increasing duration of the pulse. Also, the levels of incorporation are nearly identical between the control cells and the E2F1 cell line. In contrast to the control cells and the E2F1 cell line, the E2F1d87 expressing cells show a markedly reduced level of incorporation of ^{3}H-thymidine into the DNA of each S phase cell. This is in keeping with a partial arrest of these cells at the S phase checkpoint. The level of reduction in incorporation at the longest pulse time (6 hours) is nearly a 20-fold. This indicates that the S phase arrest by E2F1d87 expression is mirrored by a significant decrease in the level of nucleotide incorporation into DNA. Note that the E2F1d87 cell line has a significantly increased proportion of cells in S phase during exponential growth (14).

It has been presumed (9) that the S phase arrest caused by the mutant E2F1 lacking the cyclin A/cdk2 domain, occurs near the end of S phase. This checkpoint regulates the completion of S phase, involving a dissociation of E2F1 from the DNA. An experiment was performed to determine if E2F1d87 causes an arrest near the beginning of S phase. Cells were arrested at the G1/S phase border with a double block of excess thymidine. When the cells were released and monitored at 1 hour intervals by flow cytometry (Figure 2A), it is clear that the time it takes to transit S phase is increased in the E2F1d87 cell line, as previously reported (13,14). If the S phase arrest by E2F1d87 is a

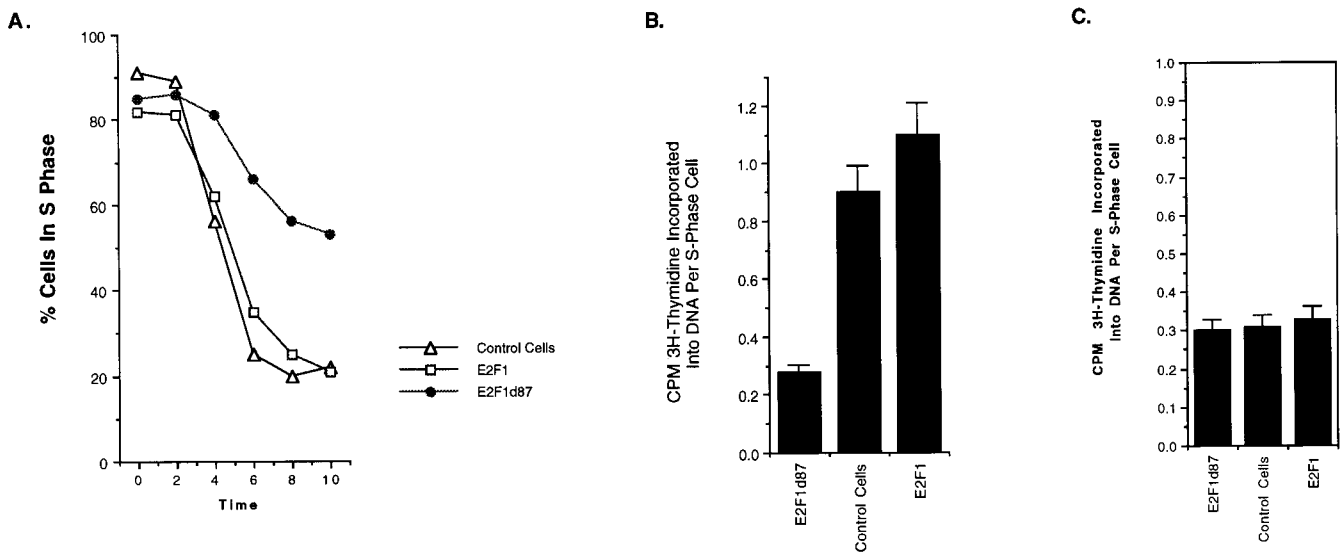

FIG. 2. E2F1d87 expressing cells show a decrease in the level of ^{3}H-thymidine incorporated into the DNA of cells early in S phase. (A) S phase duration is increased in the E2F1d87 cell line. Proliferating control cells (NIH3T3 fibroblasts) or NIH3T3 fibroblasts constitutively expressing the E2F1 or the E2F1d87 mutant transcription factors were double thymidine blocked (2 mM) to arrest cells at the G1/S phase border. The cells were released into thymidine free media and aliquots of the cells were processed for flow cytometry at 2 hour intervals. (B) Cells as in A were pulse labeled with ^{3}H-thymidine (5 uCi/ml) for 1.5 hours upon release of the thymidine block. The cells were trypsinized and counted. An aliquot was also processed for flow cytometry and another aliquot was processed for determination of incorporation of ^{3}H-thymidine into DNA. The incorporation of ^{3}H-thymidine into DNA is shown and given as CPM per S phase cell. The error bars indicate the standard deviation. (C) Cells were double thymidine blocked as in A but were not released from the thymidine block. They were pulse labeled with ^{3}H-thymidine (5 uCi/ml) for 1.5 hours. The cells were trypsinized and counted. An aliquot was also processed for flow cytometry and another aliquot was processed for determination of incorporation of ^{3}H-thymidine into DNA. The incorporation of ^{3}H-thymidine into DNA is shown and given as CPM per S phase cell. The error bars indicate the standard deviation.

late event, then there should be no decrease in the amount of ^{3}H-thymidine incorporated into the DNA of the E2F1d87 cells entering S phase just after release of the block. As seen in Figure 2B, this is not the case. When cells were released from the block and pulsed with ^{3}H-thymidine for 1.5 hours, there was a substantial decrease (>3 fold) in the incorporation of labeled nucleotide into the DNA of each S phase cell. This indicates that E2F1d87 may act earlier in S phase than previously thought, given that S phase duration is approximately 6 hours in control and E2F1 cell lines and 10-11 hours in the E2F1d87 cell line. As a control for these experiments, cells double thymidine blocked but not released from the block, were pulsed with ^{3}H-thymidine for 1.5 hours (the cells remain at the G1/S border). As seen in Figure 2C, there was no change in the incorporation of labeled nucleotide into the DNA of each S phase cell, between the three cell lines. This indicates that the E2F1d87 cell line is not deficient in the initiation of DNA synthesis. It should be noted that we have also used BrdU to determine the percent cells in S phase, as opposed to flow cytometry, and we obtained the same levels of CPM/S phase cell as described above (data not shown). This indicates that propidium iodide provides an accurate measurement of the numbers of cells in S phase.

It has been demonstrated that NIH3T3 cells constitutively expressing either E2F1 or E2F1d87 will enter S phase but not complete it, following culture in low serum (0.5%) containing media (11). This is shown in Figure 3A, where about 25-30% of the cells expressing either E2F1 or E2F1d87 enter S phase, compared to about 5% for the control cells. However there is no corresponding increase in the % cells in G2/M phase indicating that the cells do not complete S phase. When incorporation of ^{3}H-thymidine into DNA was assessed, it was found that the level of incorporation within the E2F1d87 cell population was significantly decreased relative to the control and E2F1 cells. While both the E2F1 and E2F1d87 lines initiate DNA synthesis during culture in low serum, the E2F1d87 line demonstrated a 3-fold reduced capacity to incorporate ^{3}H-thymidine into DNA. Further, while only 5% of the control cells have entered S phase, they nonetheless also have a 3-fold increase in the incorporation of 3H-thymidine into DNA of each S phase cell compared to the E2F1d87 cell line. This data indicate that even under conditions of enhanced entry into S phase, the cells expressing the mutant E2F1d87 have reduced incorporation of ^{3}H-thymidine into DNA, compared to the control cells or the E2F1 line. It should be noted that in the studies presented here we have used three independent E2F1d87 overexpressing cell lines, while we show only the data for one such line. Importantly,

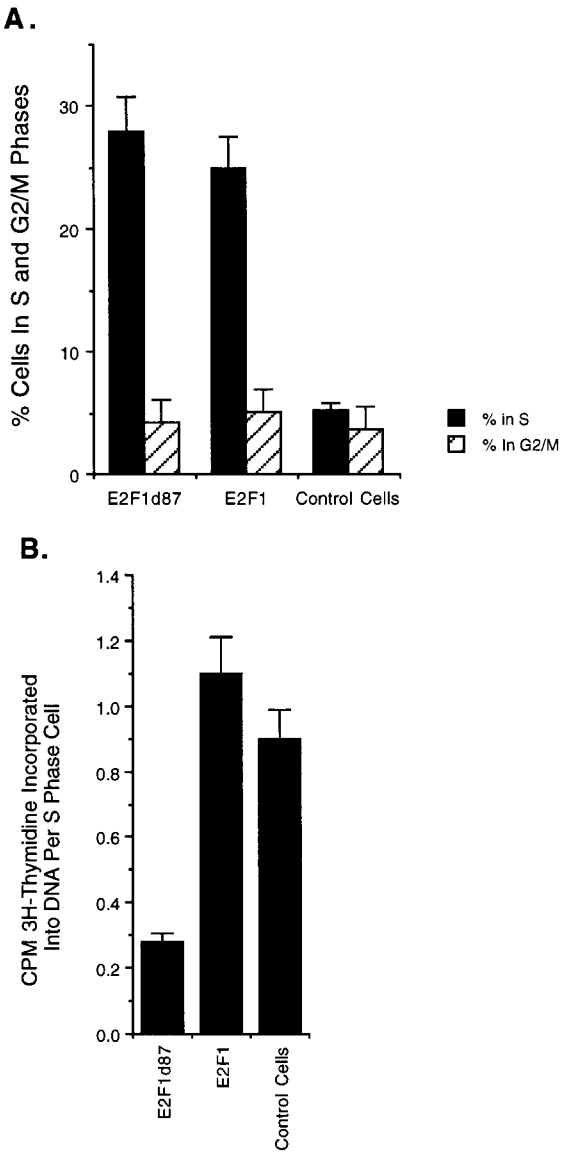

FIG. 3. E2F1d87 expressing cells show a decrease in the level of ^{3}H-thymidine incorporated into the DNA of cells following culture in low serum containing media. (A) The percentages of cells in S phase are increased in the E2F1d87 and E2F1 cell lines following culture in low serum media. Cells were cultured to confluence and then medium was changed to that containing 0.5% bovine calf serum. Cells were cultured in low serum containing media for an additional 48 hours. At the end of the 48 hour culture period, aliquots of the cells were processed for flow cytometry. The percentages cells in S and G2/M phases are shown. The error bars indicate the standard deviation. (B) Cells as in A were pulse labeled with ^{3}H-thymidine (5 uCi/ml) for 1.5 hours at the end of the 48 hour culture period in low serum. The cells were trypsinized and counted. An aliquot was also processed for flow cytometry and another aliquot was processed for determination of incorporation of ^{3}H-thymidine into DNA. The incorporation of ^{3}H-thymidine into DNA is shown and given as CPM per S phase cell. The error bars indicate the standard deviation.

all of the cell lines are nearly identical in their behavior with regard to the levels of ^{3}H-thymidine incorporated into the DNA per S phase cell.

Published studies indicate that the association of cyclin A/cdk2 with E2F1 is needed to establish an S phase checkpoint, yet the location of this checkpoint within S phase has not yet been identified (9). Mutant E2F1's lacking the cyclin A/cdk2 binding domain are unable to be phosphorylated by the kinase and hence remain bound to DNA, ultimately leading to a block to S phase completion (9,13). From the results presented here it appears that expression of one of these mutant E2F1's in fibroblasts inhibits the incorporation of thymidine into DNA, and that this inhibition occurs early in S phase. Evidence indicates that the cyclin A/cdk2 complex plays a role in the control of the early elongation phase of DNA synthesis (10,17-19). The results here are consistent with this notion and indicate that the association of cyclin A/cdk2 with E2F1 may be to regulate elongation of DNA replication early in S phase after initiation of replication has already occurred.

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