Functional Analysis of the Human Cyclin D2 and Cyclin D3 Promoters*

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The D-type cyclins promote progression through the G1 phase of the cell cycle and may provide a link between growth factors and the cell cycle machinery. We determined the nucleotide sequence of the 5′-flanking region of the human cyclin D2 and cyclin D3 genes and identified the transcription start sites. Analysis of the upstream sequences required for transcription of the cyclin D2 and cyclin D3 genes in continuously dividing cells revealed marked differences in their regulatory elements. In the cyclin D2 gene positive elements were localized between positions –306 and –114 relative to the ATG codon at +1. Additional positive elements were localized between –444 and –345, whereas sequences that reduced transcription were identified between nucleotides –1624 and –892. In the cyclin D3 gene all of the positive elements required for maximal transcription were localized between nucleotides –366 and –167, and no negative elements were found. The activities of a reporter gene linked to the upstream regulatory sequences of the cyclin D2 gene but not the cyclin D3 gene were induced when starved cells were serum stimulated. This suggests that although the abundance of both the cyclin D2 and cyclin D3 mRNAs is increased by serum stimulation, only the cyclin D2 gene is up-regulated at the transcriptional level. Sequences between nucleotides –306 and –1624 of the cyclin D2 gene were necessary for serum inducibility.

Progression of eukaryotic cells through the cell cycle is controlled by the activity of cyclin dependent kinases (cdks)1 (Morgan, 1995). Activation of cdks requires binding of a cyclin and phosphorylation by the Cdk-activating kinase (Morgan, 1995). In addition, the activity of the Cdk-cyclin complex is inhibited by binding to cdk inhibitory proteins (Morgan, 1995). Activation of cdks requires binding of a cyclin and phosphorylation by the Cdk-activating kinase (Morgan, 1995). Activation of cdks requires binding of a cyclin and phosphorylation by the Cdk-activating kinase (Morgan, 1995).

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1. The abbreviations used are cdk(s), cyclin dependent kinase(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); SEAP, secreted alkaline phosphatase; VSMC, vascular smooth muscle cell(s).

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Sac-BglII fragment from the plasmid pD3-G94 (Xiong et al., 1992) into pGL2Basic digested with Sac and BglII to generate a plasmid designated D3 Δ−1017/+112. To regenerate the entire 3.3 kb cyclin D3 upstream transfection was subcloned into the MluI site of D3 Δ−664 and pGL2Control (Promega) to study its effect on cyclin D3 and SV40 transcriptional activity, respectively. The same Sac-PstI fragment was also cloned into the BamHI site that lies farther upstream of the cyclin D3 and SV40 promoters in the plasmids D3 Δ−664 and pGL2Control.

In a similar fashion a Sac-Stul fragment and an Smal-PstI fragment from D2 −1624 were also cloned into the MluI site of D3 Δ−664.

Cell Culture—Primary cultures of rat aortic VSMC were prepared by enzymatic digestion as described (Owens and Thompson, 1986) and passaged in growth medium (Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum, 0.5 μg/ml insulin, 5 μg/ml transferrin, and 0.2 mM ascorbate). Cells were passages 2 and 7 were used for all experiments. MRC-5 cells, a normal human diploid lung fibroblast cell line, were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

Transient Transfections and Luciferase Assays—Primary rat VSMC were plated at 1–2 × 10⁶ cells/well in six-well plates, andould later 60% confluent duplicate wells were transfected with 16 μg of LipofectAMINE (Life Technologies, Inc.) complexed with 2 μg of the luciferase plasmid and 0.5 μg of the reference plasmid pCMV/SEAP (Promega) in which expression of secreted alkaline phosphatase (SEAP) is under the control of the cytomegalovirus promoter. Cells were exposed to lipid-DNA complexes for 5 h, washed once with Dulbecco’s modified Eagle’s medium, cultured in growth medium for a further 48 h, and the medium was assayed for SEAP using a chemiluminescent method (Promega Inc.). Cells transfected with the pCMV/SEAP plasmid had SEAP activities that were greater than 50-fold above non-transfected background activity, and SEAP activities were used to correct for differences in transfection efficiencies between different wells. Whole cell extracts were prepared and assayed for luciferase activity using a commercial luciferase assay kit (Promega) and read in a luminometer with automatic injection (monolight 1010; Analytical Luminescence Laboratories, San Diego). The luciferase activity of VSMC transfected with the luciferase vector lacking a promoter (pGL2Basic) was subtracted from all data before correction for SEAP activity. MRC-5 cells, a normal human diploid lung fibroblast cell line, were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

Preparation of RNA and Northern Blot Analysis—Primary rat VSMC were treated with equal aliquots taken from a single transfection mix. Cells were cultured in growth medium for 16 h, and the medium was assayed for SEAP activity to determine transfection efficiency. All four wells were washed twice with serum-free medium (Dulbecco’s modified Eagle’s medium, 5 μg/ml transferrin, 0.2 mM ascorbate) and maintained for 31 h in the same medium, changing the medium twice per day. Two of the wells were harvested for luciferase assays while the other two were transferred to growth medium supplemented with 5 ng/ml basic fibroblast growth factor and 1 ng/ml epidermal growth factor for 23 h and were then assayed for luciferase activity.

Preparation of RNA and Northern Blot Analysis—Primary rat VSMC were made quiescent by culturing them in medium containing 0.5% serum for 4 h, then stimulated to proliferate by the addition of 20% serum and 5 ng/ml basic fibroblast growth factor for 3, 6, 9, 12, 24, and 48 h after which RNA was extracted using RNA-STAT 60 (Tel-test “B”). For Northern blot analysis RNA was fractionated in formaldehyde:agarose gels, transferred to nylon membranes (Amersham Corp.), and fixed to the filter by UV irradiation. Radiolabeled probes were prepared by random primer labeling of the human cyclin D2 and cyclin D3 cDNA clones (kindly provided by D. Beach). Hybridization and washing of the filters were performed according to published methods (Church and Gilbert, 1984). Total RNA was prepared from normal human lung biopsy samples using RNA-STAT 60 (Tel-test “B”) or from MRC-5 cells by the method of Chirgwin et al. (1979).

Ribonuclease Protection Analysis—A Smal-SacI fragment from D2 Δ−306 spanning nucleotides −1 to −306 of the cyclin D2 gene was cloned into Smal/SacI-digested pSVSport-1 (Life Technologies, Inc.). The resulting plasmid was linearized with EcoRI and transcribed in vitro with T7 RNA polymerase in the presence of [α-32P]ATP to generate an antisense RNA probe. A SstI/Smal fragment spanning nucleotides 279 to 104 of the cyclin D3 gene was isolated from pD3G94 and cloned into the HindIII site of pET7.2B (Pharmacia Biotech Inc.). Orientation of the insert was determined by DNA sequencing, and after linearizing the plasmid with HindIII it was transcribed with T7 RNA polymerase as described above to generate an antisense RNA probe. Ribonuclease protection was performed as described (Gilman, 1987) using the probes described above and total RNA from MRC-5 cells or human lung. Yeast RNA was used as a negative control. The size of protected products was determined from a sequencing ladder run alongside the samples.

DNase I Footprint Analysis—DNA fragments were 5’ end labeled with [α-32P]ATP and poly(dIdC) kinase, then one end of the fragment was removed by digestion with an appropriate restriction enzyme. Footprinting with nuclear extract from HEK-293 cells (Santa Cruz Biotechnology) was performed as described (Briggs et al., 1986).

RESULTS

Induction of Cyclin D2 and Cyclin D3 mRNA by Serum in VSMC—Northern blot analysis of cyclin D2 and cyclin D3 mRNA in primary cultures of rat VSMC (Fig. 1) showed both were induced from low or undetectable basal levels by serum stimulation. A single cyclin D2 mRNA species with a size of approximately 3.4 kb (panel A) and two cyclin D3 mRNA species of 3.9 and 6.2 kb (panel B) were detected. Induction of the cyclin D2 mRNA was evident after 3 h of serum stimulation, reached a maximum at 9 h, and remained elevated at the 12 and 24 h time points. The cyclin D3 transcripts were both elevated after 6 h of serum stimulation and showed maximum induction by 12 h. The larger cyclin D3 mRNA species declined to near basal levels by 24 h, while the smaller mRNA species remained elevated at the 24 and 48 h time points. These data show that the mRNA for both cyclin D2 and cyclin D3 are induced rapidly when quiescent rat VSMC are stimulated to proliferate.
Sequencing of the Upstream Regions of the Cyclin D2 and Cyclin D3 Genes and Mapping of the 5'9 End of the mRNAs—We determined the nucleotide sequence of the 5' upstream regions of the human cyclin D2 and cyclin D3 genes (Figs. 2 and 3). The sequences are numbered relative to the ATG codon at +1. Ribonuclease protection was used to determine the 5' end of the cyclin D2 and cyclin D3 mRNAs using RNA from MRC-5 cells and human lung. Northern blot analysis demonstrated that the cyclin D2 and cyclin D3 mRNAs are both expressed in human lung and MRC-5 cells (data not shown). Using an RNA probe spanning nucleotides 2306 to 21 of the cyclin D2 gene, 11 protected fragments were evident after hybridization to both MRC-5 and human lung RNA, whereas no protected bands were detected with yeast RNA (Fig. 4, panel A). This indicates that multiple transcription initiation sites are utilized. The transcription start sites predicted from the sizes of these protected fragments are indicated by the # marks in Fig. 2. The major transcription start site corresponds to one of the start sites detected by ribonuclease protection (labeled 6 in Fig. 4, panel A). In addition one of the minor primer extension products equates to a start site at position −137, and this lies just 3 nt upstream of a cluster of three start sites detected by ribonuclease protection (7, 8, 9 in Fig. 4, panel A). This 3 nt difference lies within the margin of error expected for the calculation of the size of the protected bands in the ribonuclease protection assay. The more 5' start sites detected by ribonuclease protection may not have been detected by primer extension because of premature termination of the reverse transcriptase. The start sites detected by ribonuclease protection and primer extension span a region of 130 nt from position −243 to −113 of the cyclin D2 gene. The 5' end of the longest published cyclin D2 cDNA (Palmero et al., 1993) lies at position −155 on the sequence in Fig. 2 and is 9 nt 3' of a cluster of start sites detected by ribonuclease protection and primer extension. A probe spanning nucleotides −279 to +40 of the cyclin D3 gene was used in a ribonuclease protection assay with RNA from MRC-5 cells (Fig. 4, panel B). At least two major and four minor protected fragments were detected, and the locations of the start sites predicted by these bands are indicated by the stars above the sequence in Fig. 3. This indicates that the cyclin D3 gene also utilizes multiple transcription start sites. Attempts to map the 5' end of the cyclin D3 mRNA by primer extension analysis with several different primers gave inconsistent results that did not correspond to the ribonuclease
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Fig. 3. Nucleotide sequence of the upstream region of the human cyclin D3 gene. The symbols used are the same as in Fig. 2, except that ▼ indicates the location of a DNase I-hypersensitive site.

Protection data. This may have been caused by secondary structure in the mRNA due to its high G/C content in this region. The 5' end of the longest published cDNA sequence (Motokura et al., 1992) corresponds to position −162 on the sequence in Fig. 3 which is 15 nt upstream of the 5'-most start site detected by ribonuclease protection. This discrepancy could be a result of the different sources of RNA used in the synthesis of the cDNA library and the ribonuclease protection assay.

Putative transcription factor binding sites were identified in the cyclin D2 and cyclin D3 upstream sequences using the TFD data base (Ghosh, 1993) and the Macpattern Program (Fuchs, 1994) and are indicated below the sequence in Figs. 2 and 3. All of the transcription start sites identified in the cyclin D2 gene, with the exception of the two 3'-most ones, lie within, or a few nt away from a sequence matching the CAP site from other genes. The CAP site is the loosely conserved sequence within a maximum of 660% of the D2 gene. Further deletion to positions −1303, −1204, and −892 resulted in a stepwise increase in activity, reaching a maximum of 660% of the D2 −1624 construct (Fig. 5). This indicates that sequences between −1624 and −892 of the cyclin D2 gene contain a negative regulatory element. Further deletion to positions −444 had no significant effect upon promoter activity. Deletion of nt −444 to −345 resulted in a 10-fold fold in promoter activity, indicating that this region contains strong positive elements. Removal of sequences between −306 and −114 eliminated all remaining promoter activity. Thus, sequences between −306 and −114 which contain all of the transcription initiation sites are essential for basal promoter activity, whereas sequences between −444 and −345 appear to constitute a separate enhancer-like element that increases the activity of the basal promoter 10-fold.

Sequences between −3300 and −112 from the cyclin D2 gene (D3 −3300) also directed efficient luciferase activity in rat VSMC (Fig. 6). The activity of the D3 −3300 construct was on average 3−4×10^3 relative light units/9.6-cm^2 well. No activity was detected when the upstream sequence was inserted in the reverse orientation (D2 Rev), demonstrating the presence of orientation-dependent transcription. Progressive deletion to positions −1303, −1204, and −892 resulted in a stepwise increase in activity, reaching a maximum of 660% of the D2 −1624 construct (Fig. 5). This indicates that sequences between −1624 and −892 of the cyclin D2 gene contain a negative regulatory element. Further deletion to positions −444 had no significant effect upon promoter activity. Deletion of nt −444 to −345 resulted in a 10-fold fold in promoter activity, indicating that this region contains strong positive elements. Removal of sequences between −306 and −114 eliminated all remaining promoter activity. Thus, sequences between −306 and −114 which contain all of the transcription initiation sites are essential for basal promoter activity. Therefore, sequences between −444 and −345 appear to constitute a separate enhancer-like element that increases the activity of the basal promoter 10-fold.

Sequences between −3300 and −112 from the cyclin D3 gene (D3 −3300) also directed efficient luciferase activity in rat VSMC (Fig. 6). The activity of the D3 −3300 construct was on average 3−4×10^3 relative light units/9.6-cm^2 well, which was 10-fold higher than that of the D2 −1624 construct in the same cells. When the sequences between −3300 and −112 from the cyclin D3 gene were inserted in the reverse orientation (D3 Rev), activity was only 8% of that seen with the correct orientation. Deletion of sequences −1017 to −112 which included the transcription start sites (D3 Δ −1017/-112) completely abolished promoter activity. Deletion of the Bmal site at approximately −1090 resulted in a small increase in activity which was not statistically significant. Stepwise deletions down to position −366 had only a minimal effect (approximately 20% reduction) on promoter activity. However, further deletion to nt −264 reduced promoter activity to 22% of the
Deletion of sequences between the positive element in this region which had been seen in VSMC (Fig. 6, panel A), an antisense probe spanning nucleotides +40 to −279 from the cyclin D3 gene was hybridized to 20 μg of yeast RNA (lanes 1 and 3) or 20 μg of MRC-5 RNA (lanes 2 and 4) at 55 °C (lanes 1 and 2) or 62 °C (lanes 3 and 4). The arrows indicate specific protected fragments.

A subset of the same deletion constructs were assayed for promoter activity by transfection into the normal human lung fibroblast cell line MRC-5 (Table I). Deletion of the sequence between −1624 and −444 from the cyclin D2 promoter resulted in a nearly 2-fold increase in promoter activity. Although consistent with the finding of a negative element in this region in VSMC (Fig. 6, panel A) the magnitude of the effect was less. Deletion of sequences between −444 and −345 resulted in a 3.4-fold drop in promoter activity confirming the presence of the positive element in this region which had been seen in VSMC. Deletion to −306 did not alter promoter activity, whereas the further removal of sequences to −114 eliminated most of the activity, consistent with what had been seen in VSMC. The results with the cyclin D3 promoter in MRC-5 cells were also similar to those observed in VSMC. Deletion of sequences between −3300 and −366 had little effect on promoter activity, although a slight but inconsistent increase was observed. Further deletion to position −264 resulted in a 2-fold reduction in activity down to 68% of the full-length construct. This differs quantitatively from the results in VSMC where D3 Δ −264 had only 22% of the activity of the full-length construct. Deletion down to −167 eliminated most of the remaining promoter activity, consistent with what had been observed in VSMC.

Nuclear Factor Binding Sites in the Cyclin D2 and Cyclin D3 Promoters—To determine nuclear factor binding sites within the cyclin D2 and cyclin D3 promoters, we used nuclear extract from Hela cells, a cell type in which both promoters were functional (data not shown). DNase I footprinting of the region from −555 to +1 from the cyclin D2 promoter detected a single protected region (data not shown) designated footprint Ia (Fig. 2). This protected region contains potential binding sites for Sp1, AP2, and PuF. DNase I footprinting of a fragment from −664 to −121 of the cyclin D3 gene revealed two protected regions designated I and II in Fig. 7, panel A, which lie 76 and 19 nt upstream of the 5′-most transcription start site, respectively. Both of these footprints contain potential binding sites for Sp1 (see Fig. 3), and both lie within the region −264 to −167, which was shown to contain positive regulatory elements (Fig. 6 and Table I). A second footprint probe spanning nt −496 to −121 of the cyclin D3 gene revealed a site that was made hypersensitive to cutting by DNase I in the presence of Hela extract (Fig. 7, panel B). This may be indicative of binding of a nuclear protein, although no distinct footprint was observed in this region. This hypersensitive site lies within a region (−366 to −264) which, when deleted, reduced promoter activity from 78 to 22% of control in VSMC (Fig. 6) and from 144 to 68% in MRC-5 cells.

Fig. 4. Mapping the 5′ end of the human cyclin D2 and cyclin D3 mRNA by ribonuclease protection. Panel A, an antisense RNA probe spanning nt −1 to −306 of the cyclin D2 gene was hybridized to 40 μg of yeast RNA (lanes 1 and 4), 40 μg of human lung RNA (lanes 2 and 5), or 20 μg of MRC-5 RNA (lanes 3) at 58 °C (lanes 1−3) or 68 °C (lanes 4 and 5). After digestion with ribonucleases the protected fragments were analyzed on a sequencing gel. A sequencing ladder run alongside the samples was used to calculate the size of the protected fragments. Protected fragments are indicated by the arrows, and the star indicates some undigested probe. Panel B, an antisense probe spanning nucleotides +40 to −279 from the cyclin D3 gene was hybridized to 20 μg of yeast RNA (lanes 1 and 3) or 20 μg of MRC-5 RNA (lanes 2 and 4) at 55 °C (lanes 1 and 2) or 62 °C (lanes 3 and 4). The arrows indicate specific protected fragments.

full-length plasmid, indicating that strong positive elements are located between −366 and −264. Further deletion to nt −167 eliminated all remaining promoter activity. In conclusion, sequences between −366 and −167 contain all of the regulatory elements required for maximal expression of the cyclin D3 gene in dividing cells.

The Cyclin D2 Negative Element Functions in Heterologous Promoters and Is Position- and Orientation-independent—To confirm the presence of negative regulatory sequences between nt −1624 and −892 of the cyclin D2 gene we tested the ability of this region to affect transcription from two heterologous promoters. A 730-bp Sac-I-PstI fragment spanning nt −1622 to −892 from the cyclin D2 gene which includes all of the negative element was cloned upstream of either a cyclin D3 promoter/luciferase reporter construct containing 664 bp of cyclin D3 upstream sequence (D3 Δ −664) or upstream of the SV40 promoter. The fragment was placed in one of two locations: either immediately upstream of the promoter (proximal) or 2.9 kb upstream of the promoter (distal). These constructs are represented diagrammatically in Fig. 8 along with the results of transient transfections in rat VSMC. When placed proximal to the promoter, the forward and reverse orientations of the 730-bp Sac-I-PstI fragment reduced activity of the cyclin D3 promoter to 27 and 15% of D3 Δ −664, respectively. This shows that these sequences from the cyclin D2 gene contain negative regulatory elements that can affect a heterologous promoter and function in an orientation-independent manner. Furthermore, when placed distal to the cyclin D3 promoter the cyclin D2 negative element was still able to reduce markedly promoter activity, albeit less effectively than when placed closer to the promoter. Similar results were observed with the SV40 promoter; when placed proximal to this promoter, the negative element reduced its activity to 37% of control, whereas in the distal location promoter activity was reduced to 23%. Thus, the
cyclin D2 negative element affects both viral and cellular promoters. In MRC-5 cells the 730-bp SacI-PstI fragment containing the cyclin D2 negative element reduced the activity of the SV40 promoter to 48% (±8%), indicating that the negative element can also function in a human cell line (data not shown).

Interestingly, two smaller fragments from within the SacI-PstI region contain a negative regulatory sequence, and the stippled boxes are positive regulatory sequences. The cross-hatched box represents the luciferase gene.
The serum inducibility of the D2 gene was no greater than that seen with SV40. The difference in activity seen between starved and serum-stimulated cells in these experiments is not the result of some general effect of deletion of cyclin D2 upstream sequences eliminated serum induction that was significantly above that of SV40. The luciferase activity seen between starved and serum-stimulated cells in these experiments is not the result of some general effect of transcriptional activity of the activity of the construct containing the largest amount of 5'-upstream sequence and are the average of two independent transfections carried out in duplicate with standard deviations as shown.

| Cyclin D2 construct | Relative activity | Cyclin D3 construct | Relative activity |
|---------------------|-------------------|---------------------|-------------------|
| D2 1624             | 100 ± 5           | D3 -3300            | 100 ± 1           |
| D2 Rev              | −2 ± 0.4          | D3 Rev              | −0.2 ± 2          |
| D2 Δ - 892          | 140 ± 17          | D3 Δ Smal           | 136 ± 11          |
| D2 Δ - 444          | 185 ± 4           | D3 Δ -496           | 103 ± 30          |
| D2 Δ -306           | 55 ± 19           | D3 Δ -366           | 144 ± 25          |
| D2 Δ -114           | 44 ± 22           | D3 Δ -264           | 68 ± 11           |
| D2 Δ - 222          | 7 ± 7             | D3 Δ -167           | 9 ± 1             |

PstI fragment reduced transcription from the cyclin D3 promoter to 14 and 32% of control (Fig. 8). Thus the negative regulatory element appears to contain at least two redundant elements.

The Cyclin D2 and Cyclin D3 Promoters Are Up-regulated by Serum—Because the mRNAs for cyclin D2 and cyclin D3 were induced by serum we wanted to examine the response of the cyclin D2 and cyclin D3 promoters to serum. Cyclin D2 and cyclin D3 luciferase reporter constructs were transiently transfected into rat VSMC that were subsequently serum starved and then stimulated with serum and growth factors. The SV40 promoter linked to luciferase exhibited a 2.5-fold induction of luciferase activity by serum (Fig. 9) which we consider to be the baseline for a non-serum-inducible promoter in this system. The luciferase activity of the largest cyclin D2 construct (D2 –1624) was increased 6.8-fold by serum stimulation, and this induction was statistically significant compared with that of the SV40 promoter (p = 0.02). When the negative element was removed, as in D2 Δ –444, significant serum induction was also observed, but it was reduced to 4.3-fold. The difference in the serum inducibility of D2 –444 and SV40 was not quite statistically significant (p = 0.14). Despite having significant basal activity (see Fig. 5), construct D2 Δ –306 exhibited serum induction that was no greater than that seen with SV40. The difference in the serum inducibility of the D2 –1624 and D2 Δ –306 constructs was statistically significant (p = 0.02). The fact that deletion of cyclin D2 upstream sequences eliminated serum inducibility is further evidence that the difference in luciferase activity seen between starved and serum-stimulated cells in these experiments is not the result of some general effect of serum upon translation efficiency or stability of the luciferase mRNA. These data indicate that transcriptional activity of the cyclin D2 promoter can be induced by serum and that the sequences responsible for this induction lie between –306 and –1624. However, the sequences required for serum induction may not be localized to a single region within the 5' upstream sequence.

In contrast to cyclin D2, none of the cyclin D3 constructs exhibited serum induction that was significantly above that of the SV40 promoter (Fig. 9).

DISCUSSION

In this study we have sequenced the 5' upstream regions of the human cyclin D2 and cyclin D3 genes and mapped the start sites of transcription. More than 10 transcription start sites spread over a 130-nt region were identified for the cyclin D2 gene; in the cyclin D3 gene 6 start sites covering 70 nt were detected. Both the cyclin D2 and cyclin D3 genes are devoid of a TATA box, and this may explain the existence of multiple transcription start sites since this motif is thought to fix the site of transcription initiation. The existence of more than one start site has also been reported for the cyclin A (Henglein et al.

![FIG. 7. Footprint analysis of the cyclin D3 promoter. Panel A, DNase I footprinting of a PstI-HindIII fragment (see Fig. 3 for location of the PstI site) from the cyclin D3 gene promoter spanning nt −664 to −112 and labeled at the HindIII end (3' end of fragment). The four left lanes (GATC) are a sequencing ladder generated with a primer complementary to the labeled end of the probe. The fragment was incubated with 20 μg of HeLa nuclear extract (lanes 1 and 2) or no nuclear extract (lanes 3 and 4) and digested with 50 ng (lane 1), 150 ng (lane 2), 7.5 ng (lane 3), or 15 ng (lane 4) of DNase I. Panel B, a XmaI-HindIII fragment prepared from D3 Δ –496 and spanning nucleotides −496 to −112 from the cyclin D3 gene was end labeled at the XmaI site (5' end) and subjected to DNase I footprint analysis. The probe was incubated with 10 μg (lanes 1 and 2) or 30 μg (lanes 3 and 4) of HeLa nuclear extract or no nuclear extract (lanes 5–7) and digested with 25 ng (lane 1), 50 ng (lanes 2 and 3), 150 ng (lane 4), 5 ng (lane 5), 7.5 ng (lane 6), or 15 ng (lane 7) of DNase I. The arrow and the star indicate the location of a site that is made hypersensitive to cutting by DNase I in the presence of nuclear extract.](image-url)
al., 1994), cyclinD1 (Herber et al., 1994) and cdk2 genes, all of which are also devoid of a TATA box. The sequence at the site of transcription initiation, or CAP site, exhibits loose conservation, having the consensus sequence YYCAYYYYY, where Y is any pyrimidine (Azizkhan et al., 1993). Sequences matching the CAP sites from other genes and this consensus sequence were found to lie close to or overlap most of the transcription start sites identified in the cyclin D2 gene (see Fig. 2). In the absence of a TATA box, binding sites for transcription factors other than TATA binding protein are used to initiate transcription (Azizkhan et al., 1993). These include the factor YY1, as well as E2F and Sp1 (Azizkhan et al., 1993). In the cyclin D2 gene the sequence surrounding the two most 5' start sites, and a second cluster of start sites centered at nt −165, match the consensus binding site for YY1 at eight of nine positions (see Fig. 2), suggesting that this protein may be directing initiation at these sites. Two more start sites lie within a potential AP2 binding site, and yet another lies within a potential CTF/NFY binding site. Thus, multiple proteins could be involved in determining the start site of transcription. In the cyclin D3 gene one of the major start sites lies within a potential binding site for AP2. The presence of two potential Sp1 sites close to the other major start site, one of which was bound by nuclear proteins from HeLa cells (Figs. 3 and 7), suggests that Sp1 may play a role in transcription initiation from the cyclin D3 promoter.

By deletion analysis we have mapped the regulatory elements responsible for transcription of both the cyclin D2 and cyclin D3 genes. In the cyclin D2 gene deletion analysis identified a negative regulatory element localized between positions −2162 and −2892. This element also inhibited the activity of two heterologous promoters in a position- and orientation-independent manner. The stimulatory effect of the 5' deletions upon D2 promoter activity is unlikely to be caused by less efficient transfection of larger plasmids since a similar stimulation was not seen when even larger deletions of the cyclin D3 promoter were tested (Fig. 6). Furthermore, the fact that deletion of cyclin D3 upstream sequences did not result in a significant increase in expression makes it unlikely that the stimulatory effect seen in the cyclin D2 deletions was caused by bringing cryptic transcriptional enhancers within the cloning vector closer to the promoter since these would be expected to affect the cyclin D3 promoter in a similar manner. This explanation is also ruled out by the fact that the negative element was functional when transferred into the context of two other promoters and placed in two different locations (Fig. 8). In these constructs cryptic transcriptional enhancers within the vector would not have been brought closer to the promoter. Hypothetically, negative regulation of transcription, which has been documented for many genes, is a mechanism by which the rate of transcription can be exquisitely controlled. Specific re-

2 D. Shiffman, unpublished data.
expression of transcription can occur by several mechanisms. In the simplest situation the binding of a negative transcription factor prevents binding of a positive factor via steric hindrance. Alternatively, negative factors bound to a negative element may affect events occurring at a distance, either by interfering with transcriptional activators or by inhibiting the basal transcription machinery. Negative elements that interfere with specific activators bound to the promoter will act on some but not all promoters. In contrast, negative elements that interfere with the basal transcription machinery will affect all promoters. Our results show that the negative element from the cyclin D2 gene can inhibit the activity of both the cyclin D3 promoter and the SV40 promoter. Thus it is more likely that the cyclin D2 negative element functions by interfering with the basal transcription complex. However, we cannot rule out the possibility that the cyclin D2 negative element functions by inhibiting the activity of a transcriptional activator common to the cyclin D2, cyclin D3, and SV40 promoters. A negative element was also identified in the upstream region of the human cyclin D1 gene (Herber et al., 1994), although it reduced promoter activity by only 3-fold. Sequence comparison identified a 12-nt region from within the cyclin D2 negative element which matched a region of the cyclin D1 negative region at 10 positions, but the significance of this finding is unclear. The cyclin D2 negative element may contribute to the serum inducibility of this promoter since deletion of this element resulted in a reduction in serum inducibility from 6.8- to 4.3-fold. This reduction is the result of a greater increase in promoter activity after removal of the negative element under starved conditions than under conditions of serum stimulation. This suggests that one function of the negative element may be to keep transcription repressed in the absence of proliferative signals. Thus the negative element could potentially be activated by extracellular stimuli that inhibit cell growth and/or intracellular events in G_0 or late cell cycle which down-regulate G_1 events.

Two distinct positive regulatory elements were identified in the cyclin D2 upstream sequence. The first, which we consider to be the basal promoter, spans nucleotides −306 to −114 and has about 50% of the activity of the largest cyclin D2 construct. This region contains all of the transcription start sites as well as potential binding sites for CTF/NFY, AP2, and C/EBP. Sequences between −444 and −345 contain strong positive elements that increased the activity of the basal promoter by 10-fold in VSMC and by 3.4-fold in MRC-5 cells and thus seem to act like an enhancer. Putative binding sites for AP2 and PEA3 are present within this region. Interestingly, the addition of the cyclin D2 negative element almost neutralizes the effect of this enhancer upon promoter activity. Further experiments are needed to determine if the cyclin D2 negative element does in fact interfere with the function of the enhancer.

A single DNase I footprint was identified in the cyclin D2 promoter using HeLa cell nuclear extract. This footprint lies upstream of all of the positive elements identified by the deletion analysis, suggesting that it is not important for promoter activity in continuously dividing cells. However, we cannot rule out the possibility that it may be important for serum induction. The footprint contains sequences with similarity to the binding sites for PuF (a factor that binds to the c-myc promoter), AP2, and Sp1. Since HeLa nuclear extract contains Sp1 (Briggs et al., 1986) this may explain why this footprint was detected. Further work will be needed to identify the proteins interacting with the positive elements identified in the cyclin D2 promoter. In conclusion, cyclin D2 gene transcription is controlled by a combination of positive and negative elements and could potentially be modulated by changes in the activity of any of these elements.

The structure of the cyclin D3 promoter is very different from that of the cyclin D2 promoter; no negative element was identified, and all of the sequences required for full promoter activity were localized to a region between −167 and −366. Two DNase I footprints were detected in the cyclin D3 promoter. Both footprint I (−229 to −215) and footprint II (−171 to −161) lie within the positive regulatory element identified by deletion analysis, and both contain a sequence with similarity to the binding site for Sp1. The role of these potential Sp1 sites in the transcription of the cyclin D3 gene remains to be elucidated. Potential binding sites for Sp1 were also identified in the promoters of the cyclin A (Henglein et al., 1994) and cyclin D1 (Herber et al., 1994) genes as well as the cdk2 gene. The discovery of binding sites for Sp1, which is a ubiquitously expressed factor, is consistent with the requirement for expression of the cyclin genes in most cell types. Interestingly, a DNase I-hypersensitive site was detected at position −133 of the cyclin D3 gene. This is indicative of the binding of a nuclear protein which alters the conformation of the DNA such that it becomes more accessible to DNase I. This site lies within a region that, when deleted, reduced promoter activity from 78 to 22% in VSMC and from 144 to 68% in MRC-5 cells, suggesting that it may be important for transcription.

A similar analysis of the human cyclin D1 promoter in NIH3T3 cells identified positive elements between −848 and −742 and additional positive elements that were spread throughout the region between −742 and −29, rather than being localized to one particular region (Herber et al., 1994). Together these results show that there are marked differences in the arrangement of transcriptional regulatory elements between the three D-type cyclins. This suggests that transcription of these genes is independently regulated, supporting the idea that the three D-cyclins are not redundant but perform distinct functions. This was first suggested by the finding that the coding sequences of the human D-cyclins are more closely related to their mouse homologs than to each other (Inaba et al., 1992; Xiong et al., 1992). In addition, cyclins D2 and D3 form more stable complexes with the retinoblastoma protein (pRB) than does cyclin D1 (Ewen et al., 1993; Kato et al., 1993). Furthermore, cyclins D2 and D3 efficiently activated Cdk2 in insect Sf9 cells, whereas cyclin D1 could not (Ewen et al., 1993); and unlike cyclins D2 and D3, cyclin D1 was unable to induce retinoblastoma protein (pRB) phosphorylation in human diploid fibroblasts (Dowdy et al., 1993).

We examined serum inducibility of cyclin D2 and cyclin D3 reporter constructs in a transient transfection assay. The conditions used in this assay are similar to those in which we observed serum induction of the cyclin D2 and cyclin D3 mRNAs. However, two conditions that were necessary in these transient transfection assays would be expected to lead to an underestimate of the true level of serum inducibility of these promoters. First, to minimize the loss of the reporter gene plasmid that begins 4 days post-transfection the starvation period had to be limited to 30 h. Second, when starved rat VSMC were restimulated with serum less than 50% of the cells were found to have reentered the cell cycle (data not shown). Despite these limitations we observed that sequences between nt −1624 and −1 from the cyclin D2 gene conferred 6.8-fold induction by serum (Fig. 9), which is comparable to the induction of the endogenous rat cyclin D2 mRNA (Fig. 1, panel A). This demonstrates that increased transcription from the cyclin D2 promoter is at least partly responsible for the increase in cyclin D2 mRNA observed in response to serum. The sequences required for serum induction lie between −306 and −1624 but did not map to a single region within this sequence. Sequences between −1624 and −444, which contains the negative ele-
ment, appear to contribute to serum induction as do sequences between −444 and −306. A more comprehensive analysis is needed to localize further the DNA sequences required for serum induction. In the case of the cyclin D1 promoter (Herber et al., 1994), all of the deletions tested were inducible by serum, but a significant part of the serum inducibility could be attributed to a 100-bp region around position −900 (Herber et al., 1994). Within this region lie binding sites for a novel serum-inducible protein (Herber et al., 1994) and the AP1 family of transcription factors (Albanese et al., 1995; Herber et al., 1994). This AP1 site was shown to mediate induction of cyclin D1 transcription by transforming mutants of p21ras (Albanese et al., 1995). A second signal transduction pathway appears to function via a binding site for the ETS transcription factor within the proximal 22 bp of the cyclin D1 promoter, which is itself regulated by the activity of mitogen-activated protein kinase (Albanese et al., 1995). This minimal cyclin D1 promoter with just 22 bp of upstream sequence could be induced 3.5-fold by epidermal growth factor, and this was mediated by mitogen-activated protein kinase (Albanese et al., 1995). This contrasts with our results, which show that a cyclin D2 reporter construct that retains significant promoter activity (D2−2575 to −576) which matches the AP1 site from the cyclin D1 gene at six out of seven positions. These sequences lie within the region of the cyclin D2 promoter which we have shown is required for serum induction. Further experiments will be needed to determine the functional significance of these regions of homology.

None of the cyclin D3 promoter constructs tested exhibited induction by serum and growth factors which was above that of the SV40 promoter. One interpretation of these data is that the induction of the cyclin D3 mRNA observed by Northern blot (Fig. 1) is due primarily to post-transcriptional events such as stabilization of the mRNA. However, we cannot formally rule out the possibility that sequences outside of the largest construct tested are required for transcriptional induction of the cyclin D3 gene.

Induction of the cyclin D2 and D3 mRNA occurs after the induction of the immediate-early genes, suggesting that products of the immediate-early genes may be required for this induction. Therefore, products of the c-fos, c-jun, c-myc, and c-myb genes are good candidates for transcription factors that might be involved in the activation of cyclin D2 gene transcription in response to serum. Indeed, we found putative binding sites for AP1 (fos, jun) and myb in the upstream sequence of the cyclin D2 gene. Further experiments are needed to determine which if any of these transcription factors are involved.

Our findings are consistent with the concept that, like the cyclin D1 gene, the transcription of the cyclin D2 gene is a downstream target of mitogen-activated signal transduction pathways, and they suggest fine control of cell cycle progression by transcriptional as well as post-translational regulation. It seems likely that the D-type cyclins may play different functional roles in various cell types by responding to different signal transduction pathways. This work provides the basis for understanding the exact molecular mechanisms that link mitogens to the cell cycle.

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