Regulation of cyclin E gene expression by the human papillomavirus type 16 E7 oncoprotein

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In this study, we characterized the 5′ regulatory region of the murine cyclin E gene and analysed activation of the gene by the E7 oncogene of human papillomavirus type 16 in transfection experiments. We found that the murine cyclin E promoter is composed of multiple regulatory elements, and we present evidence for at least two independent transcription units, designated P1 and P2. Overlapping binding sites for the cellular transcription factors Sp1 and E2F were identified in both promoters, and we found that E2F-mediated activation of transcription is inhibited by Sp1 in cotransfection experiments. The E2F/Sp1 binding sites contribute to transcriptional activation by E7, and the data suggest that the cyclin E gene is rendered E7-inducible through the combination of several cis-acting elements which display only weak intrinsic responsiveness to E7.

Introduction

Regulation of cell cycle progression in mammalian cells depends on the sequential activation of a series of cyclin-dependent kinases (cdk). Catalytic activity of a given cdk subunit depends on its correct posttranslational modification and on its association with the appropriate cyclin, which acts as a regulatory subunit (reviewed by Sherr, 1994). Thus, phasespecific fluctuations in the abundance of various cyclins constitute one mechanism by which timing of cdk activation is controlled. Cyclin E (Koff et al., 1991; Motokura et al., 1991) is expressed in the G1 phase of the mammalian cell cycle; it associates with and activates cdk2 kinase (Dulic et al., 1992; Koff et al., 1992). Biochemical and genetic evidence suggests that cyclin E controls S phase entry in eukaryotes from Drosophila to man (Knoblich et al., 1994; Ohtsubo et al., 1995; Resnitzky et al., 1994; Wimmel et al., 1994). These findings strongly suggest that cyclin E, and its associated kinase, are required for S phase entry in most if not all cells. In several mammalian cell types, cyclin E mRNA is absent from G0 cells and appears in mid G1 (Koff et al., 1991).

Regulation of cyclin E gene expression may be involved in transformation of mammalian cells by viral oncoproteins, since a rapid induction of cyclin E gene expression is observed when adenvirus E1A (Spitkovsky et al., 1994, 1996) or HPV-16 E7 (Zerfass et al., 1995) are expressed in resting fibroblasts. In both cases, the ability of the viral oncoproteins to activate cyclin E gene expression is genetically linked to their transforming potential (Spitkovsky et al., 1996; Zerfass et al., 1995). These results suggest that transcriptional regulation of the cyclin E gene contributes to the control of cell cycle progression in normal and virally transformed fibroblasts.

Here we report a structure/function analysis of the murine cyclin E promoter and identify promoter elements required for E7-dependent trans-activation of the cyclin E gene.

Methods

Reporter constructs and expression plasmids. Drosophila expression vectors pPAC, pPAC E2F1 and pPAC Sp1 (Hagen et al., 1994) (kindly provided by G. Suske, Marburg, Germany) and cyclin E promoter/reporter gene constructs pCE(−3565/+263), pCE(−543/+263), pCE(−94/+263) and pCE (+95/+263) (Botz et al., 1996) were described previously. Additional reporter gene constructs were produced by digestion of parental plasmids with suitable restriction enzymes and religation. Point mutations were introduced into constructs pCE(−94/+95) and pCE(+95/+263) by digestion with restriction enzymes NotI/KpnI and BamHI/XhoI, followed by insertion of synthetich oligonucleotides bearing the appropriate mutations. All constructs were sequenced before use.

Cell culture and transfection. NIH3T3 subclones, stably transfected with an expression vector for HPV-16 E7 (E7/2 cells) or the empty expression vector (pMo cells; Davies et al., 1993), were obtained from K. Vousden (Frederick, USA). Cells were grown in Dulbecco’s modified essential medium (DMEM) supplemented with 10% foetal calf serum.
(FCS). For transient transfections, cells were transfected by calcium phosphate precipitation as described elsewhere (Chen & Okayama, 1987). Besides the pCE luciferase reporter gene series, cells were cotransfected with a second reporter gene construct, pCMV-gal, as described (Botz et al., 1996), which was used to correct for transfection efficiency. At 16 h postincubation, cells were washed and placed in DMEM containing either 0.5% or 10% FCS. Luciferase and β-galactosidase assays were performed on cell extracts prepared 24 h after transfection as described previously (Zerfass et al., 1995).

Drosophila SL2 cells (kindly provided by G. Suske; Marburg, Germany) were transfected with the LipofectAMINE reagent (GibcoBRL). Cells were plated into six-well tissue culture plates and grown in Schneider’s Drosophila medium with 10% FCS at 25°C without CO₂. After 2 h transient transfection with solution A (2 μg DNA with serum-free medium) and solution B (LipofectAMINE reagent with serum-free medium) cells were washed and grown in Schneider’s Drosophila medium. At 24 h postincubation cell extracts were prepared and luciferase activity was measured.

### Preparation of protein extracts

Whole cell extracts were prepared as described (Schulze et al., 1996). For nuclear/cytoplasmic fractionation, cells were pelleted by centrifugation and incubated in hypotonic lysis buffer (10 mM HEPES pH 7.5, 10 mM KCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 mM NaF and 0.1 mM Na-vanadate) for 5 min on ice. After addition of NP-40 to a final concentration of 0.05% and incubation for 5 min on ice, nuclei were pelleted by centrifugation at 1250 g washed twice with hypotonic lysis buffer containing 0.05% NP-40 and extracted in high salt extraction buffer (10 mM HEPES pH 7.5, 500 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 35% glycerol, 1 mM DTT, 5 mM NaF, 0.1 mM Na-vanadate, 5 μg/ml aprotinin and 5 μg/ml leupeptin) by flash freezing. After rocking for 30 min at 4°C, cell debris was removed by centrifugation at 100,000 g. Cytoplasmic extracts were cleared by centrifugation at 100,000 g supplemented with glycerol to 35%.

### Western blotting

Cell extracts were separated by SDS–PAGE and probed with polyclonal antisera to Sp1 (Santa Cruz) and monoclonal antibodies to M2-PK (Schebo Tech). E2F-2 was detected by the monoclonal antibody WUF-11 (a gift from E. Harlow, Charlestown, USA), pRb by a polyclonal antiserum (C-20, Santa Cruz), DP-1 by monoclonal antibody TFD10 (Neomarkers), E2F-4 by monoclonal antibody WUF-11 (a gift from E. Harlow, Charlestown, USA) and Sp1 by polyclonal antibody PEP 2 (Santa Cruz). To define uncomplexed E2F2/DP heterodimers, 100 ng of a GST fusion protein containing the pocket domain of pRb (GST–Rb 1379–928; Spithkowsky et al., 1997) was added to the bandshift reaction.

### Electrophoretic mobility shift assay

Bandshift experiments were performed as described previously (Schulze et al., 1996). The following double-stranded oligonucleotide probes were derived from the murine cyclin E promoter:

- cyclElwt (5’ GATCGGGCGGCGGAGCGGGGACGGGGCAG-CTC 3’)
- cyclElmutA (5’ GATCGGGCGGCGGAGCGGGGACGGGGCAGGACGGGG-CTAC 3’)
- cyclElmutB (5’ GATCGGGCGGCGGAGCGGGGACGGGGCACTTCGGGC- GATC 3’)
- cyclElmutAB (5’ GATCGGGCGGAGCGGGGAAGATCTCGGGGC-GATC 3’)
- cyclElEIIw (5’ CCGGGCGAGGGGCGAGGGGAGGGGGCCCCTGCG-CGGGC 3’)
- cyclElEIImut2F (5’ CCGGGCGCAGGGGAGGGGAGGGGGCCCCTGCG-CGGGC 3’)
- cyclElEIImutSp1 (5’ CCGGGCGGAGGGGAGGGGACGGGGCCCTGCG-CGGGC 3’)
- cyclElEIImutCD (5’ GATCGGGCGGAGGATTCTCTCCTGTTCGCGCG-CTCCGGATC 3’)
- cyclElEIIImutC (5’ GATCGGGCGGAGGATTCTCTCCTGTTCGCGCGG-CTCCGGATC 3’)
- cyclElEIIImutD (5’ GATCGGGCGGAGGATTCTCTCCTGTTCGCGCGG-CTCCGGATC 3’)
- cyclElEIIImutCD (5’ GATCGGGCGGAGGATTCTCTCCTGTTCGCGCGG-CTCCGGATC 3’)

Oligonucleotides were chemically synthesized, 3’-end-labelled by Klenow DNA polymerase and incubated with cellular extracts, as described (Schulze et al., 1996, 1998). The oligonucleotides containing the E2F binding site of the adenovirus E2 promoter are described elsewhere (Schulze et al., 1995). Proteins in nucleoprotein complexes were analysed by the addition of specific antibodies to the bandshift reaction followed by incubation on ice for 50 min prior to electrophoresis. p107 was detected by monoclonal antibody BD15 (a gift from N. Dyson, Charlestown, USA), pRb by a polyclonal antiserum (C-20, Santa Cruz), DP-1 by monoclonal antibody TFD10 (Neomarkers), E2F-4 by monoclonal antibody WUF-11 (a gift from E. Harlow, Charlestown, USA) and Sp1 by polyclonal antibody PEP 2 (Santa Cruz). To define uncomplexed E2F2/DP heterodimers, 100 ng of a GST fusion protein containing the pocket domain of pRb (GST–Rb 1379–928; Spithkowsky et al., 1997) was added to the bandshift reaction.

### Results and Discussion

#### Two independent transcription units control cyclin E gene expression

To determine functional elements in the murine cyclin E promoter, we first performed a deletion analysis, starting with construct pCE(−3565/+263), which is known to contain all transcriptional start sites and sufficient regulatory sequences to confer G1-specific activation to a luciferase reporter gene (Botz et al., 1996). Activity of the resulting reporter gene constructs was analysed by transfection into asynchronously growing NIH3T3 cells. Deletion of 5’ flanking sequences between positions −3565 and −543 led to a slight increase in promoter activity, indicating that a weak negative regulatory element may be located in that region. Further deletion to position −94 led to a twofold reduction in promoter activity. It was shown before that in the murine cyclin E gene, besides the major transcriptional start site (referred to as +1), additional start sites are located at positions +12 and +191, respectively (Botz et al., 1996). To understand the role of the various transcription start sites for promoter function, additional reporter gene products were prepared, containing DNA sequences extending from −94 to +95 and from +95 to +263, respectively. As shown in Fig. 1, both constructs displayed similar promoter activity, indicating that at least two independent transcription units directly express the murine cyclin E gene; these promoter sequences are referred to as P1 and P2 in Fig. 1. A similar combination of several independent cis-acting elements was also reported to control activity of the human cyclin E promoter (Geng et al., 1996; Ohtani et al., 1995). However, a direct comparison of the genomic sequence obtained for the human (Ohtani et al., 1995) and mouse (Botz et al., 1996) cyclin E genes suggests that, while both promoters contain several functional E2F binding sites, the nucleotide
To determine the role of the A and B elements on the formation of the various complexes, increasing amounts of oligonucleotides with mutations in either the A or B element were added to bandshift reactions as competitors. The affinity of these oligonucleotides for individual complexes was then determined by the efficiency with which a given complex was abolished. As expected, the cycElwt oligonucleotide served as efficient competitor for all specific complexes, and simultaneous mutation of both the A and B elements (as in probe cycElmutAB; Fig. 2a) completely abolished the binding of all proteins involved in complexes I to IV, as shown by the inability of oligonucleotide cycElmutAB to compete for these nucleoprotein complexes (Fig. 2b). An oligonucleotide with a selective mutation in element B (cycElmutB, Fig. 2a) was unable to compete for complexes I and II, and was also significantly impaired in its ability to compete for the formation of complex III (Fig. 2b). This indicates that the proteins involved in complexes I and II interact with the probe through sequences contained in the B element; the protein in complex III also binds to the B element but may recognize a second, weaker binding site elsewhere. Since oligonucleotide cycElmutAB is unable to compete for complex III, the second low affinity binding site appears to localize to the A element. Finally, mutation of the A element, as in oligonucleotide cycElmutA (Fig. 2a), had no discernible effect on the ability of the oligonucleotide to bind proteins involved in any of the complexes (Fig. 2b). Taken together, these results indicate that element A may comprise a weak E2F binding site, while integrity of the B element appears crucial for the binding of at least two different DNA binding proteins, one of which shares DNA binding properties with E2F (see also below, Fig. 3b).

In the case of promoter P2, two GC-rich sequences were identified which bear identity to binding sites for the E2F and Sp1 transcription factors. These motifs are referred to as C and D in Fig. 2(c). To characterize cellular proteins that interact with these sequences, a double-stranded oligonucleotide (cycElIwt; Fig. 2a), spanning nucleotides −25 to +1 of the murine cyclin E gene, was synthesized, 3′-end-labelled and used as a probe in bandshift experiments. Four major complexes were formed with this probe in whole cell extracts from asynchronously growing NIH3T3 cells (Fig. 2b). Complexes designated I and II in Fig. 2(b) displayed quite similar mobility in the gel; in some experiments the double band is not easily visible, depending on the experimental conditions. Upon addition of an oligonucleotide (E2wt) comprising the high affinity E2F binding site derived from the adenovirus E2 promoter (Schulze et al., 1994), the abundance of complex III was strongly reduced, while this complex was not affected by mutated control oligonucleotides (E2mut) in which the E2F site was destroyed by point mutations (Schulze et al., 1994). These results indicate that complex III contains a DNA binding protein that specifically recognizes E2F binding sites. The abundance of complexes I, II and IV was not affected by the E2wt oligonucleotides, indicating that E2F is not present in these complexes (Fig. 2b).

To determine which of the individual binding sites are not conserved (Botz et al., 1996).

Close inspection of the nucleotide sequence in the P1 promoter region revealed several GC-rich sequences bearing identity to binding sites of the E2F and Sp1 transcription factors, and we had shown before that an oligonucleotide spanning positions −25 to −5 indeed binds E2F complexes in cellular extracts (Botz et al., 1996). Since there are at least two sequences with identity to E2F consensus sites (designated A and B in Fig. 2a) in this region of the cyclin E promoter, we carried out a detailed mutational analysis in order to identify the actual transcription factor binding sites. To characterize proteins interacting with the wild-type sequence, a double-stranded oligonucleotide (cycElwt; Fig. 2a), spanning nucleotides −25 to +1 of the murine cyclin E gene, was synthesized, 3′-end-labelled and used as a probe in bandshift experiments. Four major complexes were formed with this probe in whole cell extracts from asynchronously growing NIH3T3 cells (Fig. 2b). Complexes designated I and II in Fig. 2(b) displayed quite similar mobility in the gel; in some experiments the double band is not easily visible, depending on the experimental conditions. Upon addition of an oligonucleotide (E2wt) comprising the high affinity E2F binding site derived from the adenovirus E2 promoter (Schulze et al., 1994), the abundance of complex III was strongly reduced, while this complex was not affected by mutated control oligonucleotides (E2mut) in which the E2F site was destroyed by point mutations (Schulze et al., 1994). These results indicate that complex III contains a DNA binding protein that specifically recognizes E2F binding sites. The abundance of complexes I, II and IV was not affected by the E2wt oligonucleotides, indicating that E2F is not present in these complexes (Fig. 2b).

Fig. 1. Deletion analysis of the murine cyclin E promoter. The different reporter gene constructs derived from the murine cyclin E promoter are schematically shown; nucleotides are numbered relative to the major transcriptional start site, referred to as +1 (Botz et al., 1996). The major transcriptional start sites of promoters P1 and P2 are indicated by arrows. Asynchronously growing NIH3T3 cells were transfected with the reporter gene constructs. Luciferase assays were performed in cell extracts prepared 24 h after transfection. The activity of the longest construct was set as 100%. The relative promoter activities are shown for each construct.
Fig. 2. Analysis of cellular proteins binding to the cyclin E promoter. (a) Representation of the double-stranded synthetic oligonucleotides bearing mutations in the GC-rich elements A and B of the P1 promoter (see text). Nucleotides that are altered by mutation are included in shaded boxes. (b) Bandshift analysis of the P1 promoter. Oligonucleotide cycEIwt was used as labelled probe in bandshift experiments, applying whole cell extracts from asynchronously growing NIH3T3 cells. Four specific complexes were obtained, designated I to IV. Specificity of binding was assessed in a competition experiment by adding a 10-fold (++) or 100-fold (++++) excess of unlabelled oligonucleotides, as indicated. (c) Representation of the double-stranded
compete for complexes I and II and also had a weak influence on the formation of the other complexes. Together, these results suggest that element C is most critical for the binding of the proteins in complexes I and II, whereas proteins present in complexes III and IV apparently recognize both elements C and D, although with slightly different affinity.

**Antagonistic effects of E2F and Sp1 on cyclin E promoter activity**

To be able to discriminate between the binding of Sp1 and E2F to the overlapping binding sites in the P1 promoter, a new set of oligonucleotides was prepared, in which specific mutations were introduced in the B element that would selectively disrupt the consensus sequence for one DNA binding protein but leave the other consensus sequence intact. Oligonucleotide CycEIwt, extending from -20 to +14, was chosen as reference oligonucleotide, as it is centred on the potential E2F/Sp1 double binding site (Fig. 3a). It is known that many DNA binding proteins bind with reduced affinity to motifs which are located close to the ends of a DNA fragment. To produce a fragment deficient for E2F binding, oligonucleotide cycEIImutE2F was designed, in which the E2F consensus sequences in A and B were destroyed. In this case, the mutations were chosen such that the Sp1 consensus sequence (5’ G/A C/T T/C A/C C G C C C/T A/C 3’; Locker & Buzard, 1990) was retained. To create a fragment deficient in Sp1 binding (cycEIImutSp1; Fig. 3a), three nucleotides outside the E2F identity region (5’ T T T C C/C/G C G C; Lathangue, 1996) were mutated, thereby affecting the Sp1 consensus sequence. The mutated oligonucleotides were radioactively labelled and used as probes in bandshift experiments. Similar to oligonucleotide cycEIwt in Fig. 2(b), probe cycEIwt formed four different complexes with cellular DNA binding proteins (Fig. 3b). By competition experiments applying oligonucleotides E2wt and E2mut, we found that one specific complex, equivalent to complex III in Fig. 2(b), contains a protein with the sequence specificity of E2F transcription factors (Fig. 3b). Upon addition of antibodies to E2F-4 and DP-1, this complex was eliminated (Fig. 3b), indicating that it contains E2F-4/DP-1 heterodimers, most likely in the form of free E2F (see below, Fig. 5). Addition of antibodies to Sp1 revealed that the slower migrating complex, which is equivalent to complex I in Fig. 2(b), contains Sp1. Oligonucleotide cycEIImutE2F bound Sp1 with an affinity similar to wild-type but was unable to bind E2F, whereas cycEIImutSp1 was unable to interact with Sp1 but strongly interacted with E2F complexes (Fig. 3b). Addition of antibodies to p107 revealed weak supershifts with a subset of the labelled probes. Since p107 supershifts were visible with oligonucleotides cycEIwt and cycEIImutSp1 but not with cycEIImutE2F, these data suggest that a p107-containing E2F complex weakly interacts with the cyclin E promoter in NIH3T3 cells (see also below, Fig. 5).

Similar to the findings with the P1 promoter, E2F and Sp1 consensus motifs physically overlap in the C element of promoter P2 (Fig. 2c). The presence of E2F and Sp1 was also addressed for the complexes formed on this binding site, assayed in bandshift experiments using labelled oligonucleotide cycEIwt. By the addition of specific antibodies to E2F-4 and Sp1 we found that complex IV contains E2F-4, whereas complex I contains Sp1 (Fig. 3c). The faster migrating complex, which displays E2F-specific binding properties (Fig. 2d), was not affected by the E2F-4 antibodies, suggesting that E2F-4 is not part of this particular complex. Probably, this complex contains a different member of the E2F protein family, which is not recognized by the antibodies available to us. Antibodies to pRb, included as a control, did not react with any of the complexes. The results suggest that both Sp1 and E2F specifically interact with the C element of the P2 promoter.

Since Sp1 and E2F appear to interact with overlapping binding sites in both the P1 and P2 promoters, we analysed the influence of Sp1 and E2F on transcriptional activity of both promoters. For these experiments, Drosophila Schneider cells were used, which have been described as a suitable experimental system to test the trans-activation function of Sp1 in cotransfections (Hagen et al., 1994), as such cells are devoid of any endogenous Sp1. For cotransfection experiments, E2F-1 was used, as it is known that E2F-1, unlike other E2F family members, contains an autonomous nuclear localization signal, which enables it to reach the nucleus independent of any auxiliary proteins (Delaluna et al., 1996; Magae et al., 1996). Furthermore, it was shown previously that E2F-1 can act synergistically with Sp1 to induce transcription of the DHFR gene (Karlseder et al., 1996). Expression of E2F-1 in Schneider cells resulted in a 25-fold activation of transcription from the reporter gene construct pCE(-94/+95), whereas Sp1 induced only a weak trans-activation (about threefold). Interestingly, when E2F-1 and Sp1 were coexpressed, we did not observe any further trans-activation; in contrast, coexpression of Sp1 severely impaired E2F-1-mediated trans-activation (Fig. 3d). As in the case of the P1 promoter, E2F-1-induced transcription from the P2 reporter gene construct pCE(+95/+263) is downregulated by Sp1 (Fig. 3d), indicating that both transcription factors compete for the same site also in the P2 promoter. These experiments suggest that the presence of Sp1 and E2F compete for overlapping binding sites in the P1 and P2 promoters, thereby affecting the transcriptional activity of these promoters.

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**Activation of cyclin E gene by HPV-16 E7**

*Synthetic oligonucleotides bearing mutations in the GC-rich elements C and D of the P2 promoter (see text). cycEIwt extends from nucleotides +120 to +150. Nucleotides that are altered by mutation are included in shaded boxes. The consensus sequences for E2F and Sp1 are given for comparison (for details, see text). (d) Bandshift analysis of the P2 promoter.*

Oligonucleotide cycEIwt was used as labelled probe in bandshift experiments, applying whole cell extracts from asynchronously growing NIH3T3 cells. Four specific complexes were obtained, designated I to IV. Specificity of binding was assessed in a competition experiment by adding a 10-fold (+) or 100-fold (+++) excess of unlabelled oligonucleotides, as indicated.
Fig. 3. E2F and Sp1 interact with both transcription units of the cyclin E promoter. (a) Representation of the P1 oligonucleotides used in bandshift assays. cycEIIwt extends from nucleotides −21 to +14. The E2F and Sp1 binding sites are boxed. In the lower part of the panel, the two mutant oligonucleotides used in (b) are depicted; mutated bases are shown in bold. The consensus sequences for E2F and Sp1 are given for comparison (for details, see text). (b) E2F and Sp1 interact with the P1 promoter. Whole cell extracts from asynchronously growing NIH3T3 cells were incubated with labelled oligonucleotide cycEIIwt or with oligonucleotides bearing mutations in the E2F (cycEIImutE2F) and Sp1 (cycEIImutSp1) consensus sequences, as indicated. Specificity of binding was assessed by competition with excess unlabelled oligonucleotides carrying the wild-type (E2 wt) or mutated version (E2 mut) of the E2F binding site from the adenovirus E2 promoter, as indicated. Antibodies to p107, DP-1, E2F-4 and Sp1 were added as indicated. The positions of the Sp1-specific complex and E2F-4/DP-1 heterodimers are marked. (c) E2F and Sp1 interact with the P2 promoter. Oligonucleotide cycEIIIwt (see Fig. 2 c) was used as
Table 1. Activation of cyclin E reporter gene constructs by HPV-16 E7

Asynchronously growing NIH3T3 cells were transfected with the indicated reporter gene constructs and expression vectors for HPV-16 E7 wild-type or the GLY24 mutant, as indicated. Cells were maintained in DMEM with 0.5% FCS 24 h after transfection. Luciferase assays were performed in cell extracts prepared 24 h after transfection. Foldness of induction by E7 or the GLY24 mutant was then determined.

<table>
<thead>
<tr>
<th>Gene construct</th>
<th>Induction by HPV-16 E7</th>
<th>Induction by GLY24</th>
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<tbody>
<tr>
<td>pCE(-3565/+263)</td>
<td>5.6 (±1.1)</td>
<td>1.7 (±0.3)</td>
</tr>
<tr>
<td>pCE(-543/+263)</td>
<td>5.0 (±0.2)</td>
<td>1.4 (±0.4)</td>
</tr>
<tr>
<td>pCE(-94/+263)</td>
<td>2.7 (±0.2)</td>
<td>0.9 (±0.1)</td>
</tr>
<tr>
<td>pCE(+95/+263)</td>
<td>2.6 (±0.93)</td>
<td>0.9 (±0.22)</td>
</tr>
<tr>
<td>pCE(-94/+95)</td>
<td>3.4 (±0.55)</td>
<td>1.1 (±0.09)</td>
</tr>
</tbody>
</table>

of excess Sp1 limits the interaction of E2F with both transcription units of the cyclin E gene, resulting in a lack of *trans*-activation.

Previous studies from other laboratories suggest a functional interaction between Sp1 and E2F. It was shown that Sp1 and E2F-1 act synergistically to induce transcription of the DHFR gene, and synergistic activation may result from a physical interaction between both transcription factors (Karlseder et al., 1996; Lin et al., 1996). While these data provide evidence for cooperation between adjacent binding sites for E2F and Sp1, our finding that E2F and Sp1 apparently compete for overlapping binding sites in the cyclin E promoter suggests that Sp1 may modulate inducibility of the gene by E2F. These observations suggest that transcriptional activity of the cyclin E gene depends on a functional balance between transcription factors of the E2F and Sp1 families.

The E2F/Sp1 binding sites are involved in *trans*-activation of cyclin E gene expression by E7

Since expression of HPV-16 E7 prevents downregulation of cyclin E mRNA levels in serum-starved NIH3T3 cell clones (Zerfass et al., 1995), we asked if expression of E7 would lead to an activation of cyclin E transcription in NIH3T3 cells. To address this question, NIH3T3 cells were cotransfected with an expression vector for HPV-16 E7 and the cyclin E reporter gene constructs mentioned above. As could be expected from previous studies (Zerfass et al., 1995), expression of E7 did not significantly affect the activity of the cyclin E-derived reporter genes in asynchronously growing cells (data not shown). However, when expressed in serum-starved cells, E7 induced a five- to sixfold increase in the activity of constructs pCE(-3565/+263) and pCE(-543/+263) (Table 1). Further deletion of 5’ sequences reduced responsiveness to E7. Thus, construct pCE(-94/+263) responds by a threefold activation to E7 expression, indicating that a promoter element(s) located between -543 and -94 contributes to full inducibility of the cyclin E promoter by E7. Separate analysis of P1 and P2 revealed that P1, as in the reporter gene construct pCE(-94/+95), was induced about threefold by E7, while the activity of P2, as in construct pCE(+95/+263), was induced about twofold by E7 (Table 1). These results indicate that at least three different promoter elements, each of which is only weakly activated by E7, contribute to the observed significant *trans*-activation of the gene by E7.

Since both E2F and Sp1 are targeted by proteins encoded by the retinoblastoma gene family (for review, see Weinberg, 1995), we analysed the ability of an E7 mutant, GLY24, that is deficient for binding to pRb family members (Banks et al., 1990; Edmonds & Vousden, 1989), to activate cyclin E transcription. As shown in Table 1, cotransfection of an expression vector for GLY24 had a weak stimulatory effect on the activity of the full-length construct pCE(-3565/+263) but did not significantly affect the activity of the shorter reporter gene constructs. Together, the data indicate that the ability of E7 to interact with members of the pRb family may contribute to *trans*-activation.

To evaluate the role of the GC-rich elements for activation of cyclin E transcription by E7, specific point mutations were introduced into P1 and P2. Mutations that selectively disrupt either the Sp1 or the E2F binding site, as shown in Fig. 3, were introduced into the P1 reporter gene construct pCE(-94/+95) to yield pCE(-94/+95)mutE2F and pCE(-94/+95)mutSp1, respectively. Similarly, point mutations were introduced into elements C and D of the P2 reporter gene construct pCE(+95/+263) to yield construct pCE(+95/+263)mutCD. Mutation of the GC-rich elements affected induction of the promoter by E7. Thus, transcription from the

labelled probe in bandshift experiments as in (b). The composition of the individual complexes was analysed by the addition of antibodies to Sp1, E2F-4 and pRb as indicated. (d) Regulation of P1 and P2 promoter activity by Sp1 and E2F-1. Drosophila Schneider cells were transfected with the P1 reporter gene construct pCE(-94/+95)wt or the P2 reporter gene construct pCE(+95/+263)wt, as indicated. pPAC-based expression vectors for Sp1 and E2F-1 were cotransfected, as indicated. Relative luciferase activity is shown, determined from three independent experiments.
P1 promoter construct pCE(-94/+95) is induced three- to fourfold by HPV-16 E7, whereas simultaneous mutation of the E2F and Sp1 binding sites, as in pCE(-94/+95)mutAB, reduced inducibility to background levels (Fig. 4). The constructs in which either the E2F or Sp1 binding site was mutated, were both inducible about twofold by E7, indicating that both binding sites are required for maximal trans-activation. Transcription of the P2 promoter construct pCE(-95/+263) is induced twofold by E7, and this is reduced to background levels by simultaneous disruption of elements C and D, as in construct pCE(+95/+263)mutCD.

Although the foldness of E7-dependent trans-activation is quite weak for all constructs shown in Fig. 4, the observation that the GLY24 mutant has no significant effect in either case suggests that the effects observed for wild-type E7 are specific. Together, these results expand our previous conclusion that activation of cyclin E transcription by E7 results from the combination of several pathways which would only weakly affect promoter activity in isolation.

**E7-dependent modulation of transcription factor complexes**

To further investigate the mechanism underlying E7-dependent trans-activation, we prepared cellular extracts from E7-expressing and control NIH3T3 cells (pMo and E7/2; see Lam *et al.*, 1994; Zerfass *et al.*, 1995) and analysed the binding of cellular factors to the E7-responsive element of the P1 promoter, as defined by the experiments shown in Fig. 4. It was shown before that certain forms of free E2F/DP heterodimers localize to the cytoplasm (Delaluna *et al.*, 1996; Lindeman *et al.*, 1997; Magae *et al.*, 1996), where they cannot modulate transcription. To screen for relevant changes in the composition of transcription factor complexes, we therefore used nuclear extracts for the analysis. As shown by Western blotting (Fig. 5a), a clean separation of cytoplasmic proteins from the nuclear extracts has been achieved in these experiments; thus, M2 pyruvate kinase, a prototypical cytosolic protein (Tani *et al.*, 1988), was absent from the nuclear extracts, and the transcription factor Sp1 was exclusively present in the nuclear extract but absent from the cytoplasmic fraction. Under these conditions, p107 is strictly nuclear as expected; however, both E2F-4 and DP-1 are present in both nucleus and cytoplasm (Fig. 5a), in keeping with the current model of regulation of E2F activity (see Helin, 1998). When the nuclear extracts were analysed in a bandshift experiment using the wild-type sequence (cyclEIIwt) as labelled probe, we found that Sp1 was the major protein binding to the oligonucleotide in nuclear extracts (Fig. 5b). While we noted a slight increase in the abundance of Sp1 in extracts from E7-expressing cells, the amount of Sp1 binding activity in these

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**Fig. 4.** E7-dependent trans-activation of P1 and P2 reporter gene constructs. The reporter gene constructs derived by site-directed mutagenesis from constructs pCE(-94/+95) and pCE(+95/+263) are shown. Point mutations were introduced in the reporter genes, corresponding to the mutated oligonucleotides that were used in bandshift experiments (see text). Asynchronously growing NIH3T3 cells were transfected with the reporter gene constructs and the appropriate expression vectors, as indicated. Cells were maintained in DMEM with 0–5% FCS 24 h after transfection. Luciferase assays were performed in cell extracts prepared 24 h after transfection. Foldness of induction by E7 or the GLY24 mutant was then determined.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Induction by HPV-16E7</th>
<th>Induction by GLY 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclE (-94/+95) wt</td>
<td>3.4 (± 0.55)</td>
<td>1.1 (± 0.09)</td>
</tr>
<tr>
<td>cyclE (-94/+95) mut E2F</td>
<td>2.3 (± 0.23)</td>
<td>1.0 (± 0.13)</td>
</tr>
<tr>
<td>cyclE (-94/+95) mut Sp1</td>
<td>1.8 (± 0.37)</td>
<td>0.8 (± 0.25)</td>
</tr>
<tr>
<td>cyclE (-94/+95) mut AB</td>
<td>1.4 (± 0.21)</td>
<td>0.9 (± 0.71)</td>
</tr>
<tr>
<td>cyclE (+95/+263) wt</td>
<td>2.6 (± 0.93)</td>
<td>0.9 (± 0.22)</td>
</tr>
<tr>
<td>cyclE (+95/+263) mut CD</td>
<td>1.4 (± 0.99)</td>
<td>1.1 (± 0.69)</td>
</tr>
</tbody>
</table>
Activation of cyclin E gene by HPV-16 E7

Fig. 5. Effect of E7 on the binding of nuclear proteins to the P1 promoter. (a) Nuclear extracts and cytoplasmic fractions were prepared from E7-expressing (E7/2) and control cells (pMo). Equal aliquots of each fraction were separated by 10% PAGE, and proteins immunoblotted with antibodies to Sp1, M2PK, E2F-4, DP-1 and p107 as indicated. (b) Analysis of nuclear proteins interacting with a probe containing the wild-type cyclin E promoter sequence. Labelled oligonucleotide cycEIIwt was incubated with nuclear extracts from E7/2 and pMo cells, as indicated. The complexes were resolved in a bandshift experiment. Specificity of binding was assessed by competition with excess unlabelled oligonucleotides carrying the wild-type (E2 wt) or mutated version (E2 mut) of the E2F binding site from the adenovirus E2 promoter. Antibodies to p107, DP-1, E2F-4 and Sp1 were added as indicated. The presence of free E2F was assayed by addition of 100 ng of a GST–pocket fusion protein, which can associate with free E2F/DP heterodimers (Spitkovsky et al., 1997; see also c). The positions of Sp1-specific complexes are marked; n.s. refers to a nonspecific DNA binding protein that interacts with the probe (see text). (c) Analysis of nuclear proteins interacting with a probe deficient for Sp1 binding. Labelled oligonucleotide cycEIImutSp1 was incubated with nuclear extracts from E7/2 and pMo cells, as indicated. The complexes were resolved in a bandshift experiment. Specificity of binding was assessed by competition experiments as in (b). Antibodies to p107, DP-1, E2F-4 and were added as indicated. The presence of free E2F was revealed by addition of 100 ng of a GST–pocket fusion protein, which supershifts the free E2F-4/DP-1 heterodimers (Spitkovsky et al., 1997). The positions of free E2F and E2F/p107 complexes are indicated; n.s. marks a nonspecific DNA binding protein that interacts with the probe.

extracts was very similar. Under the conditions used here, an interaction of E2F with the probe was not observed. In control experiments, using a probe containing the E2F sites from either the cyclin A promoter (Schulze et al., 1995) or the adenovirus E2 promoter, strong E2F binding activity was observed in these extracts (data not shown), indicating that E2F is present in these extracts but fails to interact with its binding site in the cyclin E promoter. Nuclear extracts from both pMo and E7/2 cells yielded an additional nucleoprotein complex, which comigrates with free E2F (Fig. 5b, c). However, this band represents an unspecific complex, as its abundance and gel migration are not significantly affected by addition of any competitor oligonucleotide or antibody used in the experiments shown in Fig. 5.

Since the E2F binding site overlapped with the Sp1 consensus sequence, the failure to detect E2F complexes in nuclear
extracts raises the possibility that binding of Sp1 to its recognition site may prevent the binding of E2F species. To analyse this possibility, additional bandshift experiments were performed using a probe (cycEllmutSp1) in which the Sp1 binding site is destroyed, while the binding of E2F is not affected (Fig. 3 b). Under these conditions, E2F binding was readily detected (Fig. 5 c), indicating that also in nuclear extracts, E2F can interact with the P1 promoter when Sp1 binding is prevented. Different E2F complexes were revealed in extracts from control and E7-expressing cells. Thus, in nuclear extracts from pMo cells, complexes containing E2F-4/Dp-1 and p107 were the main DNA binding proteins, whereas in extracts from E7-expressing cells, p107-containing complexes were not observed; instead, these extracts contained predominantly free E2F, consisting of E2F-4 and DP-1, as revealed by supershift experiments. That the faster migrating complexes indeed represent free E2F is further suggested by the observation that this complex is supershifted upon addition of a recombinant GST–pocket fusion protein (Fig. 5 c) (Spitkovsky et al., 1997). These data indicate that the P1 promoter of the cyclin E gene can interact with distinct E2F complexes, and the composition of these complexes is modulated by E7.

The ability of E7 to sequester p107 from E2F (Fig. 5 c; see also Lam et al., 1994), which results in nuclear free E2F-4/DP-1 heterodimers (Fig. 5 c; see also Schulze et al., 1998) probably contributes to transcriptional activation, as in the case of the cyclin A gene (Schulze et al., 1998). In contrast, no evidence for stable association of Sp1 and pRb or p107 was found in our bandshift experiments, and no changes of Sp1 complexes were noticed when extracts of E7-expressing and control cells were compared (Fig. 5). While it appears possible that altered binding of E2F family members may affect the binding of Sp1 to the promoter, given the fact that both factors compete for overlapping binding sites, the mechanism by which E7 activates Sp1-driven transcription remains to be elucidated. There is precedent for the ability of E7 to activate transcription factors distinct from E2F. Thus, evidence was presented for functional interactions between HPV-16 E7 and the transcription factors AP-1 (Antinore et al., 1996), ATF, Oct-1 (Wong & Ziff, 1996), Oct-4 (Brehm et al., 1999), TBP (Massimi et al., 1996) and the TBP-associated factor TAFI10 (Mazzarelli et al., 1995). Our finding that multiple cis-acting elements in the cyclin E gene respond to expression of the E7 oncogene is reminiscent of the pleiotropic effects on gene expression exerted by the E1A gene of adenovirus 5, a well-known transactivator of several cellular genes (for review, see Nevins, 1993).

Recent work from other laboratories (Magnaghi-Jaulin et al., 1998; Brehm et al., 1998) implies a role for histone deacetylases, in particular HDAC-1, in the regulation of E2F-driven transcription, including transcription from the murine cyclin E promoter (Brehm et al., 1998). At present it is unclear if a functional interaction of E7 with histone deacetylases is relevant for transcriptional regulation of the cyclin E promoter. As a matter of fact, preliminary data suggest that E7 binds to an unknown cellular protein that displays histone deacetylase activity; however, this unknown cellular protein is apparently unrelated to either HDAC-1 or HDAC-2, as suggested by the results of coimmunoprecipitation experiments (W. Zwerschke, unpublished). Hence, the role, if any, of chromatin-modifying enzymes in transcriptional regulation by E7 remains to be clarified.

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References


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