NF-κB family members play a pivotal role in many cellular and organismal functions, including the cell cycle. As an activator of cyclin D1 and p21Waf1 genes, NF-κB has been regarded as a critical modulator of cell cycle. To study the involvement of NF-κB in G1/S phase regulation, the levels of selected transcriptional regulators were monitored following overexpression of NF-κB or its physiological induction by tumor necrosis factor-α. Cyclin E gene was identified as a major transcriptional target of NF-κB. Recruitment of NF-κB to the cyclin E promoter was correlated with the transrepression of cyclin E gene. Ligation-mediated PCR and micrococcal nuclease-Southern assays suggested the nucleosomal nature of this region while chromatin immunoprecipitation analysis confirmed the exchange of cofactors following tumor necrosis factor-α treatment or release from serum starvation. There was a progressive reduction in cyclin E transcription along with the accumulation of catalytically inactive cyclin E-cdk2 complexes and arrest of cells in G1/S-phase. Thus, our study clearly establishes NF-κB as a negative regulator of cell cycle through transcriptional repression of cyclin E.

The progress of the cell cycle is essential for normal development and homeostasis. It is controlled by sequential catalytic activation and inactivation of phase-specific cyclin-dependent kinases (cdks) combined with periodic synthesis and destruction of their cognate regulatory subunits or cyclins (1). There is a high degree of redundancy among the cyclins and cdks, and therefore both classes of molecules can be functionally replaced by corresponding homologues (reviewed in Refs. 2, 3). The real difference lies in their temporal expression during the cell cycle rather than in their effectors (4, 5). According to the prevailing hypothesis, D and E classes of cyclins are operative during the G1 phase of the cell cycle (1) where the D-family of cyclins (D1, D2, and D3) participates in the G1 phase while E type cyclins (E1 and E2) are involved in the G1/S phase transition (6–9). Assembly and activation of the cyclin E-cdk2 complex is essential to propel cells into the S-phase without further mitogenic cues (10, 11), while induction of cyclin E is considered as a rate-limiting step in the formation of cyclin E-cdk2 complexes (12). Therefore, the mechanisms that control the activation of cyclin E gene are crucial for the progression of cell cycle through G1-phase (13).

The expression of cyclin E gene is under the control of an autonomous mechanism that peaks around the G1/S phase boundary (10, 11). Transcription of the cyclin E gene is cell cycle-dependent and is known to be regulated positively by mitogens (14, 15). E2F binding sites present in the cyclin E promoter seem to control cell cycle-dependent expression of cyclin E by E2F (12, 16) and correlate with the phosphorylation status of retinoblastoma (Rb) protein (17). Apparently, multiple signaling pathways such as Hedgehog (18), integrin (17), and Akt (19) appear to control cyclin E expression and cellular growth. Myc activation of cyclin E/cdk2 kinase is associated with enhancement of the cyclin E gene transcription and accumulation of cyclin E protein (20, 21). Likewise, the Rho family GTPase, cdc42, is reported to increase cyclin E expression via p70S6 kinase (22). Among the factors that negatively regulate cyclin E gene expression include transforming growth factor–β (15). The Rb protein is known to cooperate with histone deacetylase 1 (HDAC1) to repress cyclin E expression (23). The Wilms’ tumor suppressor protein WT1 can also repress cyclin E promoter via Sp1 binding sites (24, 25). Interestingly, enforced expression of NF-κB/c-Rel family members also results in cell cycle arrest at G1/S-phase (26). Although NF-κB is known to directly regulate cyclin D1 transcription (27), the exact mechanism of NF-κB-mediated growth arrest is poorly understood. In the present study, we provide a detailed analysis of the regulation of cyclin E gene under the conditions of physiological stress. We report that multiple NF-κB binding sites present in the promoter region of cyclin E gene are associated with transcriptional repression without involving a chromatin remodeling.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors and Reporter DNA Constructs**—Description of different expression vectors can be found elsewhere: pCMV-E2F1 and pCMV-E2F1ΔC (1–374) (28); HA-p65 and HA-p50 (29); p65K218A, p65K222A, and p65K310A (30); IκBα (31); and human retinoblastoma, pRB (32). The human cyclin E promoter reporter construct pCycE-CAT (1–1195+79) was kindly provided by Prof. J. R. Nevins (Duke University Medical
Negative Regulation of Cyclin E by p65

Center) (16) while the interleukin 2 receptor-derived NFκB-CAT reporter (8/30) was from R. Sen (National Institutes of Health). pCycE-αNFκB-CAT, lacking putative NF-κB sites, was generated by deleting −575 to −452 region (between BstEII-EcoNI sites) in pCycE-CAT. pCycEαNFκB-CAT was constructed by cloning the 125-bp BstEII-EcoNI fragment at Smal site in front of the thymidine kinase minimal promoter (cloned at BglII site) in pCAT3-Basic vector (Promega, Madison, WI). The β-galactosidase expression plasmid pCH110 (Amersham Biosciences) was used as transfection control.

Chemicals, Radiochemicals, Enzymes, and Antibodies—The chemical inhibitors used and their working concentrations were: lipopolysaccharide (LPS, 100 ng/ml) was from Sigma-Aldrich. Tumor necrosis factor α (TNFα, 10 ng/ml) was from eBioscience, Inc., whereas histone H1 was from Roche Applied Bioscience. [α-32P]dCTP and [γ-32P]ATP were supplied by New England Nuclear.

All restriction enzymes (BstEII, EcoNI, Ndel, SacI, and SacII) and micrococcal nuclease (MNase) were from New England Biolabs. Antibodies against Ac-H3-K9, Ac-H4-K12, and HDAC1 were procured from Upstate, Temecula, CA. All other antibodies (p21Waf1, p50, p52, p65, p75, p300, cdk2, cyclin E, E2F1, 1κBα, c-Rel, and RelB) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Site-directed Mutagenesis—The p65 response element in cyclin E promoter of pCycE-CAT plasmid (−1195/+79) was mutagenized by PCR using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following set of primers were used: forward, CycEmut–F 5′-gggcccgtttgagctttgaagttgcccggcagg-3′ and reverse, CycEmut–R 5′-cctggcgggccccattataaggcttacgccc-3′. The mutated bases are underlined.

Cell Culture and Transfection—The human hepatoma HuH7, human embryonic kidney HEK293 (ATCC CRL-1573) and osteosarcoma U2OS (ATCC HTB-96) cell lines were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. The cells were transfected with different plasmids at 60% confluency with Lipofectin (Invitrogen) according to the manufacturer’s instructions. The cells were transfected in a 60-mm culture dish (5 × 105 cells). HuH7 cell lines, stably expressing the wild-type and lysine mutants of p65 were established by neomycin (G418, Sigma-Aldrich) selection. Cells were synchronized by serum starvation for 48 h and analyzed further for different parameters.

Flow Cytometry and Immunofluorescence—Flow cytometry and cell cycle analysis was done as described by Mukherji et al. (33). The nuclear-cytoplasmic trafficking of p65 was analyzed by indirect immunofluorescence using anti-mouse IgG-Alexa594 (Molecular Probes).

RNA Isolation and Real-time Reverse Transcription-PCR—Total RNA was isolated from cells using TRIzol reagent as per the supplier’s instructions (Invitrogen). The levels of cyclin E and p65 mRNAs were determined by real-time quantitative PCR (qPCR) (34) using specific primers (Table 1) in 1× PCR buffer (50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 7.6, containing SYBR Green at a final concentration of 0.5% and 5% DMSO). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. The PCR product size is given in Table 1. Results were analyzed by using the comparative Ct method (34).

Immunoprecipitation and Western Blot Analysis—Methods for immunoprecipitation and Western blotting have been described earlier (33).

In Vitro Kinase Assay and DNA Probes—In vitro phosphorylation was done as described by Hengst and Reed (35). A typical kinase reaction was performed using 10 μCi of [γ-32P]ATP and 1 μg of histone H1 for 20 min at 30 °C. The reaction was terminated by addition of 2× SDS-PAGE loading buffer, electrophoresed, and autoradiographed.

The radiolabeled DNA probes 1 and 2 were prepared using [α-32P]dCTP and a nick translation kit (Invitrogen) as per the manufacturer’s instructions and purified by using a Qiagen gel extraction kit. Probes for electrophoretic mobility shift assay (EMSA) were end-labeled according to Sambrook and Russell (36) using 10 μCi of [γ-32P]ATP and 5 pmol of each oligonucleotide.

CAT Assay—A chloramphenicol acetyl transferase (CAT) assay was performed as described earlier (37). Briefly, the β-galactosidase activity was determined using equal amounts of protein from each cell extract while CAT activity was measured using normalized amounts of protein in cell extracts. The autoradiographs were analyzed by densitometry, and the data were statistically evaluated by Student’s t test.

EMS—EMSA was carried out according to Donepudi et al. (38). Briefly, nuclear extracts (10 μg of protein) from HEK293 cells were incubated on ice for 60 min with 50 fmol of [32P]labeled EMSA probes (Table 2). Binding reactions contained 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 4% glycerol, 0.1% Nonidet P-40, and 0.25 μg of poly(dIdC). The free and protein-bound oligonucleotide probes were separated by electrophoresis on 5% polyacrylamide gel. Subsequently, the gels were dried, and the bands were visualized by autoradiography. The specificity of binding was examined by competition with a 100-fold molar excess of the unlabeled specific or mutant oligonucleotides. Super-shift analysis of nuclear extracts was done using NF-κB-specific antibodies.

ChIP Assay—A chromatin immunoprecipitation (ChIP) assay was performed according to Barre and Perkins (39). Briefly, cells were cross-linked with formaldehyde (1%), lysed, and sonicated on ice (5 pulses at 30% amplitude) and centrifuged at 13,000 rpm for 10 min to obtain the supernatant. Samples were pre-cleared for 2 h with protein A-Sepharose beads (Amersham Biosciences) and incubated overnight with 2 μg/ml specific antibodies (p50, p52, p65, p300, E2F1, HDAC1, c-Rel, RelB, Ac-H3-K9, and Ac-H4-K12). The immune complexes were pulled down using protein A-Sepharose beads. After a series of washing steps, the beads were extracted in 500 μl of elution buffer (0.1 M NaHCO3, 1% SDS) and analyzed by semi-quantitative PCR.

MNase-Southern Assay—An MNase-Southern assay was done according to Richard-Foy and Hager (40). Briefly, nuclei were isolated from HEK293 cells (107 cells) and incubated in 100-μl aliquots at 25 °C for 5 min with MNase (150 units in 10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM sperm
ine, 0.5 mM spermidine, and 1 mM CaCl₂). The reaction was stopped by adding equal volume of stop buffer (100 mM EDTA and 10 mM EGTA mix, pH 7.5) followed by overnight digestion at 37 °C with proteinase K as described. The samples were treated with RNase, extracted with phenol/chloroform, and ethanol-precipitated. DNA samples (20 μg) were electrophoresed in a 25-cm-long agarose gel (1%) in Tris acetate buffer and transferred to nylon membrane for hybridization with radiolabeled DNA probes.

**LM-PCR**—The nucleosomal boundaries were determined by ligation-mediated PCR (LM-PCR) according to Garrity and Wold (41). Briefly, nuclei were isolated from HEK293 cells and digested with MNase as described above. The 5’-end of DNA was phosphate-labeled with T4 polynucleotide kinase and first strand was synthesized separately with forward primers F1, F2, or F3 (0.3 pmol) (Table 3) and *Pfu* polymerase (1 unit). After overnight ligation of linker primers (L1 and L2) with T4 DNA ligase (3 units), the labeling reaction was performed as using 32P-labeled F1, F2, or F3 primers along with the reverse L2 primer. The amplicons were resolved in a denaturing gel (8% urea-polyacrylamide), followed by drying and autoradiography.

**Restriction Enzyme Accessibility Assay**—A modified protocol of chromatin accessibility by real-time PCR was adopted (42). Nuclei were prepared as described in MNase-Southern assay and incubated with the restriction enzymes (BstEII, EcoNI, and NdeI) at 37 °C for different time periods. DNA was extracted by phenol-chloroform method, and the cyclin E promoter region was amplified using cyclin E-NF-κB ChIP-PCR primer (Table 1). Percent protection from restriction digestion was determined by densitometry.

**Bioinformatic Analysis**—The TFSEARCH program owed to the TRANSFAC data base was used to predict transcription factor binding sites on human cyclin E promoter sequence (TFSEARCH is available on-line) (43).

**RESULTS**

The Human Cyclin E Gene Promoter Contains Putative NF-κB Binding Sites—To study the regulation of cyclin E gene, the promoter sequence of human cyclin E gene (GenBank™ accession no. L48996) was analyzed for transcription factor binding sites using the TFSEARCH program (43). The software predicted at least three potential NF-κB binding sites on cyclin E promoter in both orientations (−552 to −542 bp) (supplemental Fig. S1). Further, the putative sites were in close agreement with the canonical NF-κB-responsive elements (44).

**Cyclin E Gene Is a Transcriptional Target of NF-κB**—Because the bioinformatic search predicted cyclin E as a transcriptional target of NF-κB, the expression of cyclin E gene was measured by qPCR following transient expression of different combinations of p65, IκB, Rb, E2F1, and E2F1C in Huh7 cells. As shown in Fig. 1A, E2F1-mediated up-regulation of cyclin E transcript was specifically blocked in the presence of E2F1C, a dominant negative mutant lacking transactivation domain in E2F1, Rb, and p65. Further, p65 overexpression alone could inhibit the expression of cyclin E that was overexpressed in the presence of IκB. A marked decrease in cyclin E expression was also observed at protein level in the presence of p65 (Fig. 1B). Note that, under these conditions, the levels of cell cycle inhibitor p21Waf1, a well known target of p65, increased (45). The sequences are given in Table 1.

![Figure 1](https://example.com/figure1.png)

**TABLE 1**

| Oligonucleotide primers in sets for qPCR and ChIP-PCR | PCR product (bp) |
|------------------------------------------------------|-----------------|
| Cyclin E                                             | 205             |
| F: 5’ GTTATAAGGGAGACGGGAG 3’                         | 205             |
| R: 5’ TGCTCTGCTTACTCCGCTC 3’                         | 205             |
| NF-κB-p65                                           | 439             |
| F: 5’ AGCACCACATAACTATAGTATGCTTTC 3’                 | 439             |
| R: 5’ GAGTTATAGCTAAGGGTACTCCAT 3’                    | 439             |
| GAPDH                                               | 228             |
| F: 5’ CGACACCTTTTGTCAAGCCTCA 3’                      | 228             |
| R: 5’ AGGGGCTCACGCAGCAGT 3’                          | 228             |

**ChIP-PCR primers for Cyclin E promoter regions**

| Cyclin E-NFκB | PCR product (bp) |
|---------------|-----------------|
| F: 5’ AACTGGAAGGCTTAAAGTGAGAGAT 3’                    | 408             |
| R: 5’ AGCCCAAGGGGAGTTGCTGG 3’                         | 408             |
| Cyclin E-E2F1 |                 |
| F: 5’ CGACGCCCCTGTCACTTGG 3’                          | 416             |
| R: 5’ CAGTAGACTACACAGGGTCTG 3’                         | 416             |
Negative Regulation of Cyclin E by p65

FIGURE 2. Localization of the NF-κB response element in cyclin E promoter. A, schematic representation of the CAT reporter constructs having either wild-type sequence (pCycE-CAT), a deletion (pCycEΔNFκB-CAT), or a point mutation in the putative NF-κB site. The DNA sequence of the human cyclin E promoter with putative NF-κB-responsive element(s) can be found in supplemental Fig. S1. The NF-κB response elements are clustered between BstEII-Apal sites. B, HEK293 cells were transiently transfected with pCycE-CAT along with increasing concentrations (0.1 to 1.0 μg) of p65 expression plasmid, and the relative reporter gene activity was measured. C, cells were transfected separately with the above CAT reporter constructs and expression vectors for E2F1, p50, p65, or a combination of p65 and p50 (1:1 ratio) as indicated, and the relative reporter activity was measured. The bar graph shows mean of three independent experiments. The inset shows stimulation of interleukin 2 receptor-derived NF-κB element (κB) by p65. * and ** indicate statistically significant difference at p < 0.01 and p < 0.05, respectively.

NF-κB Binds to Putative Sites on Cyclin E Promoter—We employed EMSA to study the interaction between NF-κB and the putative responsive elements in cyclin E promoter. As shown in Fig. 3A, the HEK293 nuclear extracts showed typical macromolecular complexes of p65/p50 heterodimer and p50/p50 homodimer with the consensus NF-κB element (lane 2). The specificity of this interaction was evident from a competition experiment where the radiolabeled macromolecular complexes were displaced in the presence of an excess of unlabeled consensus or cyclin E-derived NF-κB elements (lanes 3 and 5) as also seen with p21-derived elements (lane 7). The corresponding mutated response elements did not compete with the NF-κB complexes (lanes 4, 6, and 8). The specificity of NF-κB binding was further confirmed by supershift analysis using a mixture of anti-NF-κB/p65 and anti-NF-κB/p50 antibodies, which showed formation of larger complexes (lane 9). As expected, none of the NF-κB elements used above interfered with Pax-5 binding in vitro (Fig. 3B). The DNA sequences of EMSA primers are given in Table 2.

We further checked the binding kinetics of NF-κB to the cyclin E promoter-derived element following induction by TNFα and LPS, which are known to activate NF-κB through a classic pathway (46). As shown in Fig. 3C, TNFα treatment resulted in a temporal increase in the binding to consensus NF-κB element as well as the element derived from cyclin E promoter. The recruitment of NF-κB to a particular κB site is controlled at multiple levels and might be achieved by...
NF-κB Recruitment to Cyclin E Promoter Initiates Transcriptional Repression—Because TNFα treatment is known to induce apoptotic cell death at the G1/S boundary (47) and we observed increased binding of NF-κB on cyclin E promoter under these conditions, it prompted us to monitor the transcription of cyclin E following TNFα-mediated NF-κB induction. Treatment of cells with TNFα resulted in a significant decline in the cyclin E reporter activity (Fig. 4A, lane 3) as in the case of p65-transfected cells (lane 2). Release from TNFα stress restored the reporter activity (lane 4). These observations were also confirmed at RNA levels by qPCR where TNFα treatment or enforced expression of p65 showed a marked decline in the endogenous levels of cyclin E transcripts (Fig. 4B). Because activation of NF-κB involves its nuclear translocation, the subcellular distribution of NF-κB was examined by indirect immunofluorescence following TNFα treatment. There was a rapid accumulation of p65 in the nucleus, which reversed upon withdrawal of TNFα stress (Fig. 4C). The qPCR analysis of TNFα-treated cells revealed a gradual decline in cyclin E expression with a concomitant surge in p65 mRNA levels (Fig. 4D). A specific but transient decline in cyclin E protein was also observed under these conditions (Fig. 4E). The level of 1κBα also declined slowly, but that of other key regulators such as cdk2 and p65 were maintained (Fig. 4E).

NF-κB-responsive Element on the Cyclin E Promoter Is Assembled into Nucleosome—The chromatin environment is one of the critical determinants that exercise control over transcription of genes (48). We therefore probed into the nucleosomal organization of the cyclin E gene promoter region spanning the NF-κB-responsive elements. Initially we performed a low resolution analysis of genomic DNA by MNase-Southern assay (40). Nuclei isolated from Huh7, HEK293, and U2OS cells were incubated with limiting amounts of MNase and we observed increased binding of endogenous NF-κB to the human cyclin E promoter in vivo and in vitro. As expected, anti-E2F1 antibody confirmed the occupancy of E2F1 on cyclin E promoter as opposed to a nonspecific antibody.

TNFα in the case of its recruitment to cyclin E promoter. Under similar conditions, LPS, a well known activator of TNFα, did not show any appreciable change in NF-κB binding. Therefore, TNFα-mediated NF-κB induction was followed up in subsequent studies. Next we analyzed the interaction of NF-κB with cyclin E promoter in a chromatin environment. Our ChIP results confirmed the binding of p65 to cyclin E promoter suggesting that endogenous NF-κB interacts with cyclin E promoter in vivo (Fig. 3D). As expected, anti-E2F1 antibody confirmed the occupancy of E2F1 to cyclin E promoter as opposed to a nonspecific antibody.

**TABLE 2**

| DNA sequence of NF-κB response elements used in EMSA |
|-------------------------------------------------------|
| **U**, upper strand; **L**, lower strand.              |
| **NF-κB consensus**                                  |
| U-5': AGTGGAGGCGGACCTTCCCAAGGC 3'                  |
| L-3': TCACTACCCTGAGAAAAGCCTCG 5'                   |
| **NF-κB-mut consensus**                              |
| U-5': AGTTGAGGCGGACCTTCCCAAGGC 3'                  |
| L-3': TCACTACCCTGAGAAAAGCCTCG 5'                   |
| **NF-κB Cyclin E**                                   |
| U-5': GCCGGTGGACCTTGGATGTCCCGCCCA 3'                |
| L-3': CCAGCGCATGAAACCCTCTACAGGCGGCGG 5'            |
| **NF-κB-mut Cyclin E**                               |
| U-5': GCCGGTGGACCTTGGATGTCCCGCCCA 3'                |
| L-3': CCAGCGCATGAAACCCTCTACAGGCGGCGG 5'            |
| **NF-κB p21 wt**                                     |
| U-5': CCAGCGGACCTGCGGCCGTC 3'                       |
| L-3': GNTAAACCCCGGGATCAC 5'                         |
| **NF-κB-mut p21 wt**                                 |
| U-5': CGTCTGGGAGCTTGGCCGCGTC 3'                     |
| L-3': GNTAAACCCCGGGATCAC 5'                         |
| **Pax1 response element**                            |
| U-5': GATAGGGGCTACGAGGCTGACACC 3'                   |
| L-3': GTTACCCCTGACTCCTCAGGTCGTGC 5'                 |

**FIGURE 3.** Binding of NF-κB to the human cyclin E promoter in vitro and in vivo. A, EMSA was performed to show the binding of a γ32P-labeled consensus NF-κB element to the nuclear extract from HEK293 cells (lane 2) and its competition by 100-fold molar excess of unlabeled consensus NF-κB elements (lane 3) and those derived from of cyclin E and p21wt promoters (lanes 5 and 7, respectively). The DNA sequences of EMSA primers are given in Table 2. Arrowheads indicate mobility of supershifted complexes incubated with anti-p65/p50 antibodies (lane 9). B, competition for Pax-5 binding site by 100-fold molar excess of unlabeled NF-κB response elements. C, binding kinetics of cyclin E promoter-derived and consensus NF-κB response elements to the nuclear extract from HEK293 cells treated with TNFα and LPS for indicated time periods. D, ChIP analysis showing recruitment of E2F1 and NF-κB on cyclin E promoter. IgG, antibody control; NE, nuclear extract.
fers requisite protection against the nuclease. Details of primer sequences can be found in Table 3.

**NF-κB-mediated Transcriptional Repression of the Cyclin E Gene Does Not Involve Chromatin Remodeling**—To determine whether NF-κB-mediated transcriptional repression of cyclin E is associated with chromatin remodeling, we performed dot blot hybridization on mono-nucleosomal DNA (by MNase digestion) before and after stress (TNFα and serum starvation). The soluble chromatin was immunoprecipitated with anti-H3 histone antibody or rabbit pre-immune serum (negative control) and subjected to dot blot hybridization with either 117-bp BstEII-EcoNI or 37-bp BstEII-ApaI fragments (Fig. 5B). Both probes bound to mono-nucleosomal DNA with equal efficiency suggesting that chromatin remodeling may not be critical for transcriptional regulation of cyclin E by NF-κB.

These results were further confirmed using restriction enzyme accessibility assay by real-time-PCR (49–51). The chromatin accessibility of BstEII, EcoNI, and NdeI restriction enzymes was determined in control and p65-stable Huh7 cell lines (supplemental Fig. S3). The chromatin from both cell lines was generally resistant to digestion with all three enzymes. Accessibility of these sites remained limited under asynchronous growth conditions in control cells and remained unchanged in p65 stable cell lines (supplemental Fig. S3B). The purified genomic DNA, on the other hand, did not lead to PCR amplification due to its accessibility to restriction enzymes (supplemental Fig. S3C). Thus, the restriction enzyme accessibility of the NF-κB binding region on cyclin E promoter is not altered due to p65 overexpression.

We next performed an LM-PCR assay on MNase-digested, mono-nucleosomal DNA preparations. Using nested primer sets spanning NF-κB-responsive elements shown in Fig. 5A, we obtained amplification in the 3′ direction, and a downstream nucleosomal boundary was mapped at position −501 relative to the translation start site (Fig. 5D, lane 3). No change in the position of downstream boundary of nucleosome was observed in cells grown in the presence of TNFα or in a serum-starved condition (lanes 4 and 5) substantiating our earlier observations that chromatin remodeling is not a prerequisite for NF-κB-mediated transcriptional repression of cyclin E.

**Cofactor Cross-talk Specifies Transcription of Cyclin E**—In view of earlier described role of cofactors p300 and HDAC1 in cyclin E gene expression (52), we monitored the recruitment of these factors on cyclin E promoter following serum starvation and release. Because cyclin E expression peaked around 15 h after serum stimulation (Fig. 6A), the kinetics of cofactor
We next examined the recruitment of cofactors on cyclin E promoter following induction of NF-κB by TNFα. At 15 min after TNFα stimulation, HDAC1 was efficiently recruited on the promoter with a concomitant loss of p300 binding (Fig. 6C) correlated perhaps with cyclin E expression (Fig. 4B). Interestingly, release from TNFα stress resulted in increased recruitment of p300 and simultaneous loss of HDAC1 binding (Fig. 6C). Exchange of dimers is a versatile regulatory mechanism to enhance the ability of different NF-κB dimers to redundantly bind several κB sites and generate different transcriptional effects (54, 55). Therefore, the recruitment of different subunits of NF-κB was monitored on the cyclin E promoter following TNFα treatment and under serum-starved conditions. We observed a decline in the promoter association of p65, p50, and c-Rel under the serum-starved conditions. No change in the status of RelB and p52 was observed (Fig. 6D). These results suggested that the cyclin E promoter regulation is specific to p65, p50, and c-Rel subunits of NF-κB and that the association between NF-κB and cofactors may be a key determinant for cyclin E transcription.

**NFκB Mediates Cell Cycle Arrest in G1/S-phase**—Because the transition from G1 to S phase of the cell cycle is controlled by the cyclin E-cdk2 complex (11), we wondered whether NF-κB-mediated transrepression of cyclin E would affect cell cycle progression. The qPCR analysis of cyclin E expression in stable cell lines for wild-type and acetylation mutants of p65 suggested that acetylation at Lys218 may be critical for repression of cyclin E expression (Fig. 7A). Because acetylation of RelA at lysine 221, alone or in combination with lysine 218, is associated with impaired interaction of ξB with acetylation of lysine 310 is essential for its full transcriptional activity, differential acetylation of p65 may be associated with regulation of cyclin E expression (30). As cyclin E-associated kinase activity is important for G1/S transition, we next examined the kinase activity in p65-stable cell lines. In agreement with qPCR results, we observed a marked reduction in the phosphorylation of histone H1 by cyclin E immunoprecipitates from these cell lines (Fig. 7B). Further, cell line expressing mutant p65K218A displayed better kinase activity than native p65 or K310A mutant. Cell cycle analysis of these cell lines showed increased accumulation of cells in G1 phase as compared with control cells or cells expressing p65K218A mutant (Fig. 7C). Similar results (G1 arrest or delayed onset of S phase) were also obtained when p65 was

---

**TABLE 3**

| Sequence of oligonucleotide primers for LM-PCR |
|-----------------------------------------------|
| **Forward**: | **F1** | 5\' GTTCTAGGCAAGGCAAGGCAAGG 3\' |
| | **F2** | 5\' CAGGACGATTGCCCCCGGAGCAGGACTC 3\' |
| **Reverse**: | **L1** | 5\' GCCGATGACCGGAGGAGATCTGAATT 3\' |
| | **L2** | 5\' GAATCAGATC 3\' |

recruitment was investigated within a window of 24 h. Using ChIP, we observed that p65 subunit of NF-κB was constitutively associated with cyclin E promoter, which persisted until 24 h of observation (Fig. 6B). Interestingly, p300 association was detectable around 6 h, which peaked at 12 h and declined thereafter. Dissociation of p300 was accompanied by concomitant recruitment of HDAC1. As expected, acetylation of the lysine 9 residue of histone H3 and lysine 12 of histone H4, which are considered as hallmarks of active transcription (53), were associated with the cyclin E promoter within 3–6 h post-stimulation. In contrast, HDAC1 was associated with the promoter region during starvation, but it dissociated soon after serum stimulation (3 h) and reappeared after 24 h. As expected, the recruitment of E2F1 was exclusively in a cell cycle-dependent manner. Thus, the expression of cyclin E gene seems to involve interplay of specific cofactors.

**FIGURE 5.** Nucleosomal localization of NF-κB-responsive element on the cyclin E promoter. A, nucleotide sequence of the 595 to 448 region of the cyclin E promoter showing an NF-κB response element (boxed), key restriction enzyme sites (underlined), and the position of LM-PCR primers (forward F1 and F2). Details of primer sequences can be found in Table 3. The core nucleosomal DNA is shown by a dotted line. B, ethidium bromide-stained gel of MNase-digested genomic DNA from HEK293 cells. The arrowhead shows the position of positive probes, probe 1 and probe 2 (117-bp BstEII-EcoNI fragment). * and ** indicate statistically significant difference at p < 0.001 and p < 0.01, respectively. **C, Lanes 2 and 3 show MNase Southern assay of the mono-nucleosomal DNA from asynchronous, serum-starved, serum-stimulated, or TNFα-treated cultures of HEK293 cells. The dot blots were hybridized to two different 32P-labeled probes, probe 1 and probe 2 (117-bp BstEII-EcoNI fragment) of cyclin E promoter (117 bp). M, Nase Southern assay of the mono-nucleosomal DNA from asynchronous, serum-starved, serum-stimulated, or TNFα-treated cultures of HEK293 cells. The dot blots were hybridized to two different 32P-labeled probes, probe 1 and probe 2 (117-bp BstEII-EcoNI fragment) of cyclin E promoter (117 bp). M, Nase Southern assay of the mono-nucleosomal DNA from asynchronous, serum-starved, serum-stimulated, or TNFα-treated cultures of HEK293 cells. The dot blots were hybridized to two different 32P-labeled probes, probe 1 and probe 2 (117-bp BstEII-EcoNI fragment) of cyclin E promoter (117 bp).
transiently expressed in other cells, like HEK293 and U2OS, which was most probably due to transrepression of cyclin E or induction of p21Waf1 expression (supplemental Fig. S4).

DISCUSSION

NFκB is a well described key regulatory transcription factor regulating numerous processes such as inflammation, development, oncogenesis, or cellular stress (56). However, the biological role of NFκB in stress response is a subject worthy of intense investigation. In the case of genotoxic stress, it is generally believed that activation of NFκB generates an anti-apoptotic signal to provide a window of opportunity for cells to repair the damage (56, 57). NFκB is shown to be activated during the G1/S transition (58) and is involved in the regulation of cyclin D1 expression and G1 to S-phase transition (59). Although some reports suggest that p65 or c-Rel could be involved in cell cycle arrest, others have shown their role in cell proliferation (26, 60). It seems that, depending on the cell type and signaling pathway(s) involved, NFκB would support both apoptosis as well as cell proliferation. Although a vast majority of published work relates to the inflammatory response and transactivation functions of NFκB, the functional significance of association between cell cycle regulation and NFκB needs to be better defined. Using two kinds of physiological stresses, namely, TNFα treatment and serum starvation known to be associated with NFκB activation (46, 61), we now establish p65/RelA as a negative regulator of cyclin E gene expression.

The bioinformatic analysis of the human cyclin E promoter led to the identification of a cluster of potential NFκB binding sites around positions −562 to −551 bearing close homology to the NFκB consensus binding sequence, namely, GGG(G/A)NNYYCC (N = any nucleotide and Y = pyrimidine) (supplemental Fig. S1). Transcriptional regulation of the cyclin E gene by NFκB and analysis of the putative NFκB binding sites on the cyclin E promoter were verified in cell culture using reporter gene and EMSA. We observed a dra-
matic reduction in the reporter gene activity in the presence of exogenous p65 compared with p50. Further, co-expression of p65 and p50 also exhibited a similar inhibition suggesting the involvement of the p65/p50 heterodimer rather than p50/p50 homodimer. The specificity of NF-κB interaction with the cyclin E promoter was also evident from the EMSA results. The cyclin E-derived NF-κB elements competed with the consensus NF-κB sites, and antibodies specific to p65 and p50 subunits supershifted the retarded bands. Further, mutational analysis of the putative NF-κB binding sites on cyclin E promoter confirmed its involvement in the regulation of cyclin E gene.

Cyclin E gene is reported to be independently regulated by the Rb-HDAC1-Brg complex and p300, E2F1, and Sp1 transcription factors. Many of these factors are known to associate with NF-κB (29, 62, 63). Our qPCR results clearly indicated a transcriptional repression in the presence of p65, although it did not rule out the possibility of involvement of other transcription factors or cofactors in the process. Further, our ChIP studies confirmed that p65 is stably associated with cyclin E promoter in a nucleoprotein environment. We believe that constitutively bound NF-κB may regulate target genes by recruiting co-repressors or co-activators (64). Exchange of dimers is a versatile regulatory mechanism reflecting the ability of different NF-κB dimers to redundantly bind to several κB sites and to generate different transcriptional effects (55, 62). Our result suggested the involvement of p65, p50, and c-Rel in the regulation of cyclin E gene expression among other NF-κB family members (Fig. 6D). We observed that p65 and p300 were recruited to the cyclin E promoter after mitogenic stimulation with simultaneous loss of HDAC1 (Fig. 5B) suggesting that p300 and HDAC1 may substitute each other in the NF-κB-mediated regulation of cyclin E expression (29, 65). Overall, there is a switch among the different members of co-activators in the course of cyclin E gene transcription.

Nucleosomes are known to regulate gene expression either through regulating the access to the transcription initiation site (66) and proximal promoter elements (67) or by acting as a barrier to control the escape of RNA polymerase II from the promoter region (68). Irrespective of the initiation mechanisms, the regulatory nucleosomes are positioned near the transcription start site. To investigate whether NF-κB-respon-
Negative Regulation of Cyclin E by p65

... elements were assembled into nucleosomes or located within the linker region, an MNase-Southern assay was performed as a first step toward the analysis of nucleosome positioning and/or chromatin remodeling. Southern hybridization of complete and partial MNase-digested genomic DNA from three different cell lines, namely, HEK293, Huh7, and U2OS, showed a typical array of positioned nucleosomes (supplemental Fig. S2). A nucleosome harboring NF-κB binding sites was positioned at ~500–600 nucleotides upstream of the transcription start site.

Nucleosome repositioning by the ATP-dependent nucleosome-remodeling complexes and histone chaperones is considered to play an important role in the regulation of most, if not all, genes (69–71). To monitor the remodeling events on the cyclin E promoter, we performed a restriction enzyme accessibility assay for three different enzymes (BstEII, EcoNl, and NdeI) in the presence of p65. These studies (supplemental Fig. S3) indicated that the p65-responsive element on the cyclin E promoter was assembled as phased nucleosomes that adopted “open” chromatin architecture both in the presence or absence of p65. Therefore, it appears that p65 may be required for the recruitment of other cofactors on the promoter. However, our LM-PCR results ruled out the possibility of a nucleosome repositioning during the transcriptional regulation of cyclin E gene by p65. It appears that the nucleosome on cyclin E promoter may occupy multiple frames and was thus easily remodeled without nucleosome repositioning. Therefore, nucleosomal positioning is not the key determinant of chromatin accessibility at this locus.

Because the nucleosome positioning of NF-κB elements in cyclin E promoter did not appear to be critical for transrepression, we analyzed the recruitment of other transcriptional cofactors in this region. The co-activators of NF-κB include p300/CREB, p/CAF, and p160 proteins (SRC-1, SRC-2, and SRC-3) (63, 72), whereas the co-repressor molecules include WT1, SMRT, NCoR, HDAC1, HDAC2, and HDAC3 (24, 73, 74). HDAC-1 is a major isoform of HDAC in mammalian cells, which catalyzes the removal of acetyl moieties from core histones. Its role in regulating cyclin E promoter activity has been already reported (75, 76). The present study confirmed the association of HDAC1 with NF-κB-responsive elements under serum-starved conditions, which were abrogated after mitogenic stimulation.

We also observed that mitogenic stimulation led to a marked increase in the recruitment of p300/CBP histone acetyl transferase at the NF-κB-responsive elements on cyclin E promoter suggesting its involvement in the opening of chromatin structure and increased accessibility of transcription factors. This is corroborated by the fact that the SWI/SNF complex binds to acetylated histones, and histone loss is dependent on SWI/SNF, suggesting a direct connection between histone acetylation and nucleosome displacement (77–79). Accordingly, we observed a rapid recruitment of Brq-1 in this region following serum stimulation (data not shown). Interestingly, according to a more dynamic model, rapid turnover of acetylation can also be observed at non-transcribing “poised” genes and, thus, could be an important determinant of transcriptional efficiency upon gene induction (80, 81).

Binding of regulatory proteins to DNA is well known to alter the conformation of the latter, making specific bends and changing spatial orientation (82, 83). The cis-acting regulatory elements such as enhancers, silencers, or insulators may act by causing conformational and structural changes in the chromatin. Thus, it is reasonable to expect that the spatial configuration of chromatin at the cyclin E promoter could have a considerable effect on the interaction between transcription factors and the promoter. Cyclin E is essential as well as rate-limiting for entry of cells into the S-phase of the cell cycle. The periodic appearance of cyclin E is also indicative of transient activation of its promoter during G1/S transition, which decays in S-phase. New lines of evidences suggest that E2F-mediated activation and repression could be involved in the control of the cyclin E promoter (16, 84). However, the molecular mechanism that determines whether E2F would function as a positive or negative regulator is still poorly understood. In addition, HDAC-Rb-Brg1 complex may have a role in the inhibition of cyclin E gene leading to a blockade at the S-phase entry of cells (75). Rb has been shown to repress the cyclin E promoter by modulating the levels of histone acetylation, whereas pRb and HDAC1 remain bound to the nucleosome during the period of transcriptional repression (85). Because NF-κB associates with these regulatory proteins, it will be interesting to see how such interactions are translated into transrepression of a key cell cycle regulator like cyclin E.

Lastly, the activation of NF-κB is known to be helpful to cells in surviving damage induced under stress conditions by allowing them an extended window of repair. This is quite evident from the fact that the TNFα-treated cells show a dramatic decrease in the levels of cyclin E transcripts, which is mediated by NF-κB. This action of TNFα may be critical for its proposed application in cancer therapy (86). Thus, NF-κB also functions as a negative regulator of the cell cycle under unfavorable conditions. This is further substantiated by the fact that treatment of cells with LPS or hydroxyurea also results in repression of cyclin E mRNA (data not shown). Although these agents induce growth arrest by different mechanism(s), they share a common intermediate like NF-κB that imposes anti-proliferative effects by regulating the levels of cyclin E.

Acknowledgments—We are grateful to the following scientists for the generous gift of the following recombinant constructs: Dr. X. Lu (Ludwig Institute for Cancer Research, Cambridge, UK) for pCMV-E2F1 and pCMV-E2F1(1–374), Dr. S. Ghosh (Yale University) for HA-p65 and HA-p50, Dr. Warner C. Greene (Gladstone Institute of Virology and Immunology) for p65 mutants (K218A, K222A, and K310A), and Dr. P. D. Robbins (University of Pittsburgh) for human retinoblas-toma gene. The pCycECAT reporter was provided by Dr. J. R. Nevins (Duke University Medical Center), whereas NF-κB-CAT reporter (8/30) was from Dr. R. Sen (National Institutes of Health). R. Kumar helped in cell culture work.

REFERENCES
1. Vermeulen, K., Van Bockstaele, D. R., and Berneman, Z. N. (2003) Cell Prolif. 36, 131–149
2. Sherr, C. J., and Roberts, J. M. (2004) Genes Dev. 18, 2699–2711
3. Malumbres, M., and Barbacid, M. (2005) Trends Biochem. Sci. 30,
Negative Regulation of Cyclin E by p65

81. Shahbazian, M. D., and Grunstein, M. (2007) *Annu. Rev. Biochem.* **76**, 75–100
82. Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002) *Science* **295**, 1306–1311
83. Tolhuis, B., Palstra, R. J., Splinter, E., Grosveld, F., and de Laat, W. (2002) *Mol. Cell* **10**, 1453–1465
84. Polanowska, J., Fabbrizio, E., Le Cam, L., Trouche, D., Emiliani, S., Herrera, R., and Sardet, C. (2001) *Oncogene* **20**, 4115–4127
85. Morrison, A. J., Sardet, C., and Herrera, R. E. (2002) *Mol. Cell. Biol.* **22**, 856–865
86. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) *Science* **274**, 784–787