Identification of a Set of Protein Species Approximately 40 kDa as High-Affinity DNA Binding Factor(s) to the Cell Cycle Regulatory Region of the Human Thymidine Kinase Promoter

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Abstract
Promoter elements that are important for the G1-S induction of the human thymidine kinase (htk) promoter reside within the core of the cell cycle regulatory unit, positioned between −110 and −84 upstream of the TATA element. Within this 27-bp region are three GC-rich motifs, which resemble the E2F binding site. By site-directed mutagenesis, we identified a 14-bp region, between −97 and −84, critical for the htk promoter transcriptional activity. Methylation interference studies indicate that the sequences between −97 and −84 are major protein contact points, correlating with the functional significance of this sequence in vivo. Although the core of the cell cycle regulatory unit contains three E2F-like sites and can form minor S-phase-specific complexes containing p107, cyclin A, and cdk2, the major complex that binds to this region is not competed by E2F binding sites. Through DNA affinity chromatography, we identified a set of protein species of approximately 40 kDa that copurified with the htk DNA binding activity. From gel shift assays and Western blot analysis, this protein species is antigenically distinct from E2F-1, E2F-2, E2F-3, and E2F-4. Our studies raise the possibility that other members of the E2F protein family or a novel protein(s) with preferred binding affinity for the htk promoter exert(s) control on the G1 to S regulation of the htk promoter through their interactions with cyclins and kinases.

Introduction
The cell cycle is a highly regulated progression of events that directs cell growth and division. Mammalian cells can either enter the cell cycle after a period of quiescence, as in the case of terminally differentiated fibroblasts stimulated to divide in response to growth factors, or they can be continuously cycling through the cell cycle, as in the case of stem cells and transformed cells. Each of these systems is important for understanding the selective control mechanisms adapted by different cell populations in preparing a cell for commitment into S-phase.

One of the best studied replication-dependent enzyme systems is tk.2 tk expression is controlled at multiple levels; the relative contribution of each level of control is highly dependent on the growth state of cells and the type of cells (1, 2). At least two signal transduction systems have been postulated to direct cell progression through the cell cycle. The first system regulates the transition of cells from a non-growing, quiescent state to G1 phase. For example, it has recently been reported that the immediate early transcription factor, Egr-1, the expression of which is regulated by serum or growth factors during the G0 to G1 transition, could stimulate the mouse tk promoter activity (3). Interestingly, the mouse tk promoter is different from that of the human, hamster, and chicken, in that it is devoid of the canonical TATA and CCAAT elements but contains GC-rich motifs found in all other tk promoters (3, 4).

After the G0 to G1 transition, the next crucial step concerns the G1 to S transcriptional activation. Previously, using deletion and site-directed mutagenesis, it was established that within the htk promoter, a GC-rich region, located between −133 and −64, was required for S-phase stimulation (5, 6). Furthermore, this 70-bp promoter subfragment, referred to as the CCRU, is able to confer G1-S regulation on a heterologous promoter (5). Within the CCRU, a 27-bp region spanning −110 and −84 is critical for G1-S activation (7) and is a cellular target for cyclin A, p107, and p33GSK2 complexes in serum-stimulated cells (8). To understand the mechanism for G1-S activation mediated by this cell cycle control element, we seek to determine the identity of the nuclear factors that bind specifically to this regulatory domain. Of the candidate proteins, the E2F family of proteins is known to bind to GC-rich DNA motifs and form complexes with cyclins and cdks (9–11). In cotransfection studies, E2F-1 is capable of activating promoters containing E2F binding sites (12). Another candidate is E2F-4, a new member of the E2F transcription factor family, which interacts with p107 (13). Re-

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2 To whom requests for reprints should be addressed. Phone: (213) 764-0507; Fax: (213) 764-0094.
3 The abbreviations used are: tk, thymidine kinase; htk, human tk; CCRU, cell cycle regulatory unit; EMSA, electrophoretic mobility shift assay; wt, wild type; CHO, Chinese hamster ovary; GST, glutathione S-transferase; NE, nuclear extract; poly(dI-dC), poly(dI-dC); DHFR, dihydrofolate reductase; LS, Linker scanner.
cently, E2F-4 has been shown to account for the vast majority of the endogenous E2F DNA-binding activity (14).

In this report, through site-directed mutagenesis, we further reduced the transcriptional control element to a 14-bp region within the CCRU. Using EMSAs, we discovered that the nuclear protein complex that binds with high affinity and specificity to the htk site shows little affinity for the canonical E2F sites found in two other G1–S-regulated promoters. Furthermore, the methylation interference pattern for the htk complex is distinct from that formed with the DHFR E2F site. Through DNA affinity chromatography, we identify a set of protein species of approximately M, 40,000 that copurifies with the htk binding activity. The purified protein complex does not cross-react immunologically with E2F-1, -2, -3, or -4. Our results lend support to the recent finding that factors other than E2F-1 are needed for the G1 to S activation of the endogenous tk gene (15) and to the hypothesis that there may be novel, as yet unidentified, E2F-like proteins targeting specific G1–S-regulated promoters (11, 14).

Results

Transcriptional Regulatory Element within the CCRU of the htk Promoter. A schematic drawing of the known regulatory motifs of the htk promoter located upstream of the TATA element is shown in Fig. 1A. They include the two inverted CCAAT elements at −70 and −38, the 70-bp CCRU, two GC-rich motifs, and a Sp1 binding site. Also within the CCRU, there is a nest of three GC-rich motifs. Site 1 (TTTGGCCGC) closely resembles the consensus E2F site, whereas site 2 (TCTCCCGGCC) and site 3 (CTTGGCCGGG), which are overlapping, diverge more from the standard E2F binding site. To define further the contribution of these sites to the transcriptional activity of the CCRU, site-directed mutagenesis was performed to destroy either site 1 or the overlapping sites 2 and 3 (Fig. 1B). In exponentially growing cells transiently transfected with the htk/CAT fusion genes, mutation of site 1 and part of site 2 [phtk(−112/+98)CAT] had no effect on the overall promoter activity. In contrast, mutation of the overlapping sites 2 and 3 [phtk(−97/−84)CAT] almost completely eliminated the promoter activity (Fig. 1B). These results, coupled with the previous finding that mutation of sites 2 and 3 resulted in the loss of transcriptional induction at the G1–S border in stable transfectants (8), indicate that the 14-bp region spanning −97 and −84 within the CCRU is critical for the htk promoter activity, as well as for cell cycle transcription induction.

The CCRU Binding Complex Exhibits High Affinity for the htk Sequence. To determine the specificity of the transcriptional factor(s) that binds to the CCRU, EMSAs were performed using the radiolabeled oligomer wt(−110/−84) as a probe (Fig. 2). Previously, this promoter subfragment was found to form a minor S-phase-specific complex (III) involving p107 and cyclin-A/cdk2, and, in addition, formed a major complex (I) with a lower electrophoretic mobility throughout the cell cycle (8). We speculate that this binding activity initiates the higher-order S-phase-specific complex. Because this region has three elements that are similar to the consensus E2F binding site, a member of the E2F family would be a likely candidate for this activity. First, we examined the pattern of the complex formed with both human and hamster NEs with this site. A major complex (complex I), with a doublet band pattern, was observed consistently with independent extract preparations from exponentially growing cells (Fig. 3A). Thus, a similar activity resides in both human and hamster cells. To test the specificity of this complex to the htk sequence and its relationship to E2F, competition experiments were performed. Synthetic oligomers containing site 1 mutation [LS(−109/−98)] or site 2 and 3 mutations [LS(−97/−84); Fig. 2] competed less efficiently for the formation of complex I than the wt sequence containing all 3 E2F sites [wt(−110/−84)]. The oligomers containing E2F sites from the dhtr or cdc2 promoters were unable to compete for complex I. They were as ineffective as the heterologous lacZ competitor (Fig. 3B).

Methylation Interference Pattern of htk Complex I. To determine the contact points of the factor(s) binding to the htk E2F-like motifs, we performed a methylation interference analysis. For comparison, we also used the DHFR E2F site
known to interact with E2F. Methylation probes of both wt(-110/-84) and DHFR E2F sites were prepared and mixed with NEs. The probe recovered from the major complex I and the free, unbound probe following base cleavage were resolved on sequencing gels. The methylation interference patterns for the two sites were different. Furthermore, for the htk probe, major protein contact points occurred on the noncoding strand at site 3 (Fig. 4A), correlating with the functional importance of site 3 in the htk promoter activity (Fig. 1B). In contrast, for the DHFR E2F probe, hypersensitivity was observed for the E2F site (site 1) located on the coding strand (Fig. 4B).

**Size Determination of the htk Complex I after Chromatographic Separation and Affinity Binding.** As a first step toward identifying the nuclear protein(s) that binds to the CCRU, NEs from exponentially growing CHO cells were size fractionated on a Bio-Rex 70 column. The eluates were assayed for DNA binding activity in EMSAs, using the wt(-110/-84) as probe. Complex I primarily eluted at 0.3 M KCl, and was resolved from the bulk of the slower migrating complexes (Fig. 5). Based on the success of this separation scheme, the fractionation on Bio-Rex 70 was repeated at a preparative scale, and fractions of the Bio-Rex eluate containing the majority of the complex I binding activity, with a minor amount of copurifying slower migrating complex, was pooled, then dialyzed, and finally applied batchwise onto a DNA affinity column.

The DNA affinity column was designed by coupling a shorter htk oligomer wt(-103/-84) such that only proteins binding with high affinity to the functional sites 2 and 3 of the CCRU would be retained (Fig. 2). The bound proteins were eluted with step gradients of increasing salt concentrations. The fractions collected were assayed both for the protein profile by silver staining (Fig. 6) and for htk binding activity by EMSAs (Fig. 7). A set of protein species of approximately 30–42 kDa were retained by the DNA affinity column and were eluted only by 0.3–0.5 M KCl, suggesting that they bound to the htk site with relatively high affinity (Fig. 6). In assaying for the htk binding activity, the majority of the binding activity for the top band of complex I eluted at the 0.3 M (Fig. 7, Lane B) and 0.4 M (Fig. 7, Lanes A and B) KCl column fractions. This binding activity correlated with the intensity of staining of the doublet protein species at 42 kDa and other minor bands at 35 and 30 kDa. The binding activity for the lower band of complex I was detected in the 0.4 M (Fig. 7, Lane B) and the 0.5 M fractions, correlating with the

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**Oligonucleotide**

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**Fig. 2.** Sequences of synthetic oligomers. Lowercase letters indicate linker DNA sequence; bold, italicized lowercase letters represent mutated bases; and the E2F-like sites are indicated by arrows.

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Fig. 3. Formation of complex I with the binding site spanning 
-110/-84. A. 2 ng of labeled binding site probe, wt(110/-84), were added to 1 μg of either CHO or HeLa cell NEs in the presence of 100 ng of poly(dI-dC). The complexes were separated on an 8% polyacrylamide gel. The positions of complex I (I) and unbound probe (F) are indicated. B, competition analyses of complex I in the presence of increasing quantities of binding site competitors: 5-, 10-, and 20-fold molar excess of self-competitor, LS(-109/-98) and LS(-97/-84) or 10-, 20-, and 40-fold molar excess of DHFR E2F, cdc2 E2F, and lacZ.

Fig. 4. Methylation interference analysis of the htk complex I (A) and the DHFR E2F complex (B). The free (F) and bound (B) methylated probes were electroeluted, piperidine digested, and separated on a 12% polyacrylamide gel with 8 M urea. The E2F-like sites for htk and DHFR are indicated. Arrows pointed inward, contact bases; arrows pointed outward, hypersensitive bases.

39 kDa protein doublet. Thus, these protein bands, collectively referred to as p40, are candidate proteins for the DNA binding factor for the htk site.

Lack of Immuno-crossreactivity of the Affinity-Purified Proteins with E2F. The specificity of the binding of p40 to the htk site was confirmed by competition of the complex formed with the affinity-purified fractions with homologous and heterologous oligomer competitors (Fig. 8A). The results showed that the binding specificity for the htk site was retained in the highly purified fractions containing p40, because the complex formation was selectively competed by molar excess of the homologous, but not the heterologous,
competitors. Having established the specificity of binding of p40 to the htk site, the effect of antibodies against E2F-4, E2F-5, and DP-2 on the formation of the htk complex was tested by EMSAs. The anti-E2F-4 antibody was raised against an epitope corresponding to amino acids 108–300 of human E2F-4. Based on sequence homology and tests on recombinant protein, this antibody was found to cross-react with E2F-1, E2F-2, and E2F-3.° The anti-DP-2 antibody was raised against the full-length DP-2 human protein and recognizes both DP-1 and DP-2 of mouse, rat, and human origin.° The antibodies were added to the NE and allowed to incubate briefly prior to the addition of the radiolabeled wt(−110/−84) probe. The results, as shown in Fig. 8B, revealed that none of the antibodies effectively blocked the formation of complex I, although the same antibody against E2F-4 was able to inhibit complex formation with the DHFR E2F site (data not shown).

Another approach to test whether p40 is immunologically related to E2F-1 through -4 is through Western blot analysis. As a positive control, bacterially expressed E2F-4 was run in parallel in SDS-PAGE with CHO NEs, the pooled postdialyzed Bio-Rex fraction containing complex I binding activity, and aliquots of column fractions from the DNA affinity chromatography. The Western blot was hybridized with an antibody against E2F-4, which was able to cross-react with E2F-1, E2F-2, and E2F-3. The results showed that the GST-E2F-4 was highly reactive with the antibody, as expected (Fig. 9). The NE from CHO, as well as the pooled Bio-Rex fractions applied to the DNA affinity column, revealed a 48-kDa protein species that was immunoreactive with the E2F-4 antibody (Fig. 9). However, none of the bound protein fractions from the DNA affinity column, including the 0.3 and 0.4 M fractions, which contained enriched amounts of p40, immunoreacted with the antibody. These results indicate that although p40 is similar in size to the known members of the E2F family, no immuno-crossreactivity can be detected with E2F-1 through -4 in two independent assays. Using the E2F-5 antibody in similar Western blots, we were unable to detect any immunoreactive band in either the unfractionated NE or in any of the purified fractions tested (data not shown).

**Discussion**

Members of the E2F protein family have been implicated as key regulators of the cell cycle (11). This is based on the wide occurrence of E2F or E2F-like sites on cellular promoters, the expression of which is regulated by the proliferative state of the cells (15, 16). In the case of the best characterized E2F-1, its expression profile coincides with the onset of DNA replication (17), and in transient transfection assays, it is capable of enhancing the promoter activities of several putative DNA replication-dependent target genes, including that of the htk promoter (12). This, coupled with the observation that the CCRU of the htk forms a complex containing cyclin A, cdk2, and p107 during S phase in synchronized cells (8), suggests

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that a strong candidate for the DNA binding component on the htk CCRU site is a member of the E2F protein family. Recently, E2F-4 has been shown to account for the vast majority of endogenous E2F DNA binding activity and is the likely partner protein for p107, one of the components identified in the htk complex (14). In the case of the mouse tk promoter, one of the regulatory sites, MT2, has been reported to bind E2F-1 (18).

Surprisingly, our investigations into the DNA binding component of the htk CCRU site reveals that the htk complex I in human and hamster cells has little affinity for consensus E2F sites added as competitors in EMSAs. Furthermore, the affinity-purified proteins of approximately 40 kDa, which bind with high affinity to the htk site, are immunologically distinct from E2F-1, E2F-2, E2F-3, and E2F-4. These results point to the possibility that a factor other than the above E2Fs may be interacting more specifically with the htk target gene.

In this study, we further investigated the relative contribution of the three GC-rich motifs within the CCRU to direct transcription. Our mutagenesis results indicated that site 1 and part of site 2 are dispensable, whereas the 14 bp that encompass part of site 2 and all of site 3 are critical. Interestingly, this 14-bp region resides immediately 3' to the hexamer GCGGCC sequence implicated in the cycloheximide-sensitivity response of the htk promoter (19). From the methylation interference studies, major protein contact points reside within site 3. The GC-rich sequence motif of site 3 is CCTGGGCGG. Although this site resembles an E2F binding site, it is divergent from the consensus sequence TTTTGGCCGG. Thus, a related but distinct E2F member may bind to this sequence instead. Possible candidates include a protein encoded by a newly cloned human gene. This protein is homologous to the E2F protein family but with only limited overall homology (11). Alternatively, because the 14-bp functional domain of the htk site contains sequences other than the E2F-like site, it is possible that a completely distinct nuclear factor is the major DNA binding component, and only during S phase will a minor complex containing cyclin A, cdk2, and p107 gain access to this site through protein-protein interactions. Recent investigations show that sequence elements distinct from E2F sites can mediate G1-S phase-specific activation (11). Thus, in the case of the cad gene, which controls pyrimidine biosynthesis and is needed for DNA replication, the activator protein for the G1-S transcriptional stimulation has been identified as c-Myc (20). As do the E2F family members, c-Myc also binds to DNA via a helix-loop-helix protein domain and interacts with pocket proteins such as p107.

The physiological role of various E2Fs and their related proteins in controlling endogenous proliferative gene expression is likely to be complex and may involve gene and tissue specificity. In the case of the best characterized E2F, in one study, E2F-1 overexpression in a stable cell line resulted in an increase in mRNA level of tk but was not sufficient to induce high-level expression of other targets, such as E2F-1, dhfr, and c-myc (21). Another study used recombinant adenovirus, containing E2F-1 cDNA, to infect quiescent cells to measure its activation of endogenous cellular genes as a consequence of E2F-1 production. The genes encoding DNA polymerase α, thymidine synthase, proliferating cell nuclear antigen, and ribonucleotide reductase were induced, as well as those encoding cdc2, cyclin A, and B-myb; however, several other S-phase-inducible genes, such as dhfr and tk, were only minimally induced (15). Nonetheless, an examination of the E2F site occupancy in vivo of the B-myb promoter showed that E2F-DNA interactions within this promoter occurred specifically in early G1, when the B-myb promoter was inactive (22), suggesting the existence of an E2F-site-directed transcriptional repression mechanism. Furthermore, in mice lacking E2F-1, tumor induction and tissue atrophy was observed, suggesting that E2F-1 functions to regulate apoptosis and to suppress cell proliferation.
(23). Our results are consistent with a factor other than the currently identified E2Fs as the major component of the complex that binds to the G1-S-regulatory motif of the htk promoter and lends support to the finding that overexpression of E2F-1 is insufficient to activate tk transcription at the G1-S border (15). It is tempting to speculate that other members of the E2F protein family or a novel class of proteins, with preferred binding affinity for the htk site, exerts control on the G1 to S regulation of the htk promoter through its associations with cyclins and kinases.

Materials and Methods

Cell Culture. Conditions for culturing and synchronization of the Chinese hamster K12 cell line have been previously described (24).

Plasmids. The construction of phtk474R and phtkLS(−97/−84)CAT has been described (8). The phtkLS(−112/−98)CAT was derived from phtk474R by PCR-directed site mutagenesis. The mutated sequence TAGTAAGTGTGGGC (residues −112 to −98), which contains a Spel site, was verified by restriction digestion and sequencing. The pCH110 plasmid, which contains the β-galactosidase gene under the control of the SV40 promoter, was described previously (25).

Transfections and CAT Assays. The transient transfections of DNA into K12 cells and subsequent assay for CAT activity were performed as described (25). The β-galactosidase activity for each cell extract was determined as described (25). Extracts containing equal β-galactosidase activities were assayed for CAT activity (26) and quantitated by either a phosphorimager (Molecular Dynamics) or by an AMBIS radioanalytic imaging system (AMBIS Systems).

NEs. NEs from HeLa, CHO, and K12 cells were prepared as described (27).

EMSAs. Fig. 2 shows the sequences of the binding site oligonucleotides. The htk probe was prepared by annealing the oligonucleotides and labeling the duplex with [α-32P]dATP and [α-32P]dCTP using the Klenow enzyme. Two ng of probe were added to 1 μg of CHO or HeLa NE, 100 ng poly(dI-dC), and 4 μg BSA in a total volume of 20 μl containing 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl2, 0.5 mM DTT, 0.5 mM EDTA, and 6% glycerol, and where applicable, 5 μl of the Bio-Rex 70 column fractions were used in the analyses. The reactions were performed at room temperature for 20 min. For the binding reactivities with the DNA affinity-purified fractions, 2 ng of probe were mixed with 6 μl of each fraction, 4 μg of BSA, and 50 ng of poly(dI-dC), and the salt concentration in the buffer was adjusted such that the final KCl concentration was 75 mM for the 0.1–0.5 mM eluates, 90 mM for the 0.6 mM eluate, 105 mM for the 0.7 mM eluate, and 120 mM for the 0.8 mM eluate. The final reaction volume for each fraction was 40 μl, and the reactions were incubated at 4°C for 20 min. For antibody interference studies, 2 μl of each antibody were added to the extract prior to the addition of the probe. The samples were resolved on 4–8% nondenaturing polyacrylamide (39:1 acrylamide: bisacrylamide) gels in 1X TBE buffer (90 mM Tris, 90 mM borate and 2 mM EDTA, pH 8.0). The gels were dried and exposed to film for autoradiography.

Methylation Interference Assay. The oligonucleotides wt(−110/−84) and DHFR E2F (Fig. 2) were end-labeled with [α-32P]dATP, and the major complexes formed with NEs from exponentially growing K12 cells were subjected to the methylation interference assay as previously described (24).
Fractionation Using htk DNA Affinity Column. The Bio-Rex fractions containing the majority of htk complex I binding activity were pooled and dialyzed against a modified 0.1 M NR B with pH 7.9 and 20% glycerol. The htk DNA affinity column (2 ml bed volume) was first washed and equilibrated with several washes of 0.1, 0.8, and 0.4 M and again with modified 0.1 M NR B. The respective samples (Bio-Rex pooled postdialyzed) were loaded onto the column and passed over twice before collecting fractions beginning with the flow-through. The bound proteins were eluted with a step gradient of increasing KCl concentrations from 0.1 to 0.8 M in modified NR B. Two-ml fractions were collected, and each fraction was assayed for DNA binding activity by EMSA. The protein profile of the fractions was analyzed by silver staining following SDS-PAGE.

Western Blot Analysis. The protein samples were mixed with equal volumes of 2X sample buffer (100 mM Tris, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromphenol blue, 20% glycerol), heated to 95°C for 5 min, and applied onto a 10% SDS-PAGE. The Western blots were probed using the Immun-Lite Chemiluminescent Kit (Bio-Rad). The E2F-4 antibody ([C-108 Gel Supershift reagent grade] is an affinity-purified rabbit polyclonal antibody raised against a GST fusion protein corresponding to amino acids 108–300 of human E2F-4, and is cross-react with E2F-1, E2F-2, and E2F-3.1 The E2F-5 antibody (E-19 X) is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 5–24 of human E2F-5 (Santa Cruz Biotechnology). DP-2 (FL-386 X) is an affinity-purified rabbit polyclonal antibody raised against the full length human DP-2 and recognizes both DP-1 and DP-2.2

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References