Accumulation of E2F-4-DP-1 DNA Binding Complexes Correlates with Induction of dhfr Gene Expression during the G₁ to S Phase Transition*

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Previously genomic DNase I footprinting showed changes in protein binding to two overlapping E2F sites correlates with activation of dhfr gene expression at the G₁/S boundary of the Chinese hamster cell cycle (Wells, J., Held, P., Illenye, S., and Heintz, N. H. (1996) Mol. Cell. Biol. 16, 684–694). Here gel mobility and antibody supershift assays were used to relate changes in the components of E2F DNA binding complexes in cell extracts to repression and induction of dhfr gene expression. In extracts from log phase cells, E2F complexes contained predominantly E2F-4 and E2F-2 in association with DP-1, and DNA binding assays showed complexes containing E2F-2 preferentially interact with only one of the two overlapping E2F sites. In serum starvation-stimulation experiments, arrest in G₁ by low serum was accompanied by decreased levels of dhfr mRNA and the appearance of an E2F-4-DP-1-p130 complex. After serum stimulation, induction of dhfr gene expression was preceded by loss of the p130 complex in mid G₁ and coincided with marked increases in two free E2F-DP-1 complexes in late G₁, one of which contained E2F-4 and a second which contained an unidentified E2F. We suggest activation of dhfr gene expression after serum stimulation requires at least two temporally distinct processes, relief of p130-mediated repression and subsequent activation of transcription by free E2F.

The G₁ to S phase transition requires the periodic expression of several genes whose products are involved in nucleotide biosynthesis and DNA replication. Of these genes, one of the most extensively characterized is dihydrofolate reductase (dhfr), 1 an enzyme essential for de novo synthesis of glycine, purines, and thymidylate. In mammalian cells, dhfr message levels are low during quiescence, increase throughout G₁ and peak during late G₁ or early S phase (1–5). dhfr is transcribed from two overlapping, bidirectional, TATA-less promoters (reviewed in Refs. 6, 7). This unique promoter arrangement is highly conserved among humans, hamsters, and mice. Approximately 90% of dhfr transcripts originate from the 3’ (or major) promoter while the remaining 10% are generated from the 5’ (or minor) promoter (8–10). The major promoter contains several elements involved in regulating transcription, including four consensus Sp1 binding sites, two structural control elements which may act as transcriptional repressors (11), and two overlapping, inverted E2F binding sites (Fig. 1A).

In Chinese hamster ovary cells, binding to the overlapping E2F sites has been shown to be required for efficient dhfr expression in vitro and in vivo (12). Similarly, a single E2F site was shown to be sufficient to confer growth regulated promoter activity on a luciferase reporter gene in the absence of other regulatory elements (13, 14). Although the importance of the overlapping E2F binding sites in regulating dhfr transcription has been well documented, relatively little is known about which specific E2F protein complexes bind to these sites.

E2F is a family of closely related heterodimeric transcription factors that is capable of forming a variety of DNA binding protein complexes that include important cell cycle regulators. High affinity DNA binding and efficient transcriptional activation by E2F requires heterodimerization of one E2F-like protein with one DP-like protein (15–18). To date, five E2F-like subunits have been identified and cloned: E2F-1 (19–21), E2F-2 (22, 23), E2F-3 (23), E2F-4 (24–26), and E2F-5 (26, 27). cDNA clones for three DP-like proteins, DP-1 (15, 28), DP-2 (29, 30), and DP-3 (31), have also been described. Although both DP-1 and DP-2 are capable of dimerizing with each of the E2F proteins, association of E2F complexes with other cell cycle regulatory proteins appears to be directed solely by the E2F subunit (16, 18, 28–30). E2F-1, -2, and -3, for example, bind to the product of the retinoblastoma gene (pRB) (19–23, 32), whereas E2F-4 and -5 preferentially associate with the pRB-related proteins p107 and p130 (24–26, 33–35).

In addition to associating with different members of the retinoblastoma protein family, specific E2F species also differ in their association with cyclin A-Cdk2 and cyclin E-Cdk2 complexes. E2F-1, -2, and -3 bind to cyclin A directly through an amino-terminal domain (36–39). E2F-4 and -5 lack the terminal cyclin A binding domain and recruit cyclin A-Cdk2 and cyclin E-Cdk2 to promoter complexes through independent interactions with p107 and p130 (40–47).

Association of E2F with members of the pRB protein family is cell cycle-dependent and results in inhibition of E2F-dependent transcription. Characterization of E2F-1, -4, and -5 has
revealed that the transactivation domains of these proteins are masked by pocket protein binding domains, suggesting that pRB family proteins inhibit E2F-dependent transactivation through direct protein-protein interactions (21, 25, 26). During $G_1$, E2F associates with the hypophosphorylated forms of pRB (21, 25, 26). As cells pass through the $G_1$ to $S$ phase transition, pRB becomes phosphorylated and dissociates from E2F, thereby releasing “free” E2F that is capable of activating transcription (48–50). Similarly, E2F-p107 complexes are prominent during $G_1$ but disappear upon entry into the cell cycle (27, 35, 51). In contrast, E2F-p107 complexes are present throughout the cell cycle but contain different cyclin-Cdk partners. During the transition from $G_1$ to $S$ phase, cyclin E/Cdk2 bound to p107 is replaced by cyclin A/Cdk2 (33, 35, 51). In contrast, E2F-p107 complexes are present throughout the cell cycle but contain different cyclin-Cdk partners.

Previously we used a high resolution genomic foot printing technique to show that the nuclease protection patterns of each of the overlapping E2F sites at the $dhfr$ gene promoter behave differently during the mid $G_1$ to $S$ phase transition (54). The nuclease protection pattern of the E2F site on the transcribed DNA strand remained invariant, whereas changes in the cleavage pattern across the E2F site on the nontranscribed strand correlated with induction of $dhfr$ transcription. In the $dhfr$ gene promoter the two overlapping and inverted E2F sites are not identical; one site has the sequence TTTGGCGC while the other site has the sequence TTTGGCAGC. We refer to these sites as the CG and GG sites, respectively. Initial gel shift analysis with probes containing only one or the other of the two overlapping E2F sites showed that protein binding to each of the two sites was not equivalent (54).

To further our understanding of the relationship between E2F DNA binding activity and regulation of $dhfr$ gene expression, we have used a panel of antibodies to identify the components of at least five distinct protein complexes that bind to the overlapping E2F sites. In the presence of both binding sites, E2F-2 preferentially bound to the GG site while E2F-4 was the predominant CG binding activity, suggesting that different E2F complexes recognize the individual binding sites in a sequence-specific manner. In synchronized cells entrance into $S$ phase was preceded by the loss of an E2F-4- DP-1-p130 complex and accompanied by increases in the abundance of two free E2F complexes, one containing DP-1 and E2F-4 and the second containing DP-1 and an unidentified component. Our results support a model in which induction of $dhfr$ gene expression in serum response experiments is the result of two separate processes, relief of p130-mediated repression and subsequent activation of transcription through free E2F.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Synchrony Conditions**—For these experiments, we used CHOC 400 cells, a Chinese hamster ovary cell line that contains approximately 1000 copies of the $dhfr$ gene (54). CHOC 400 cells were grown in 5% CO$_2$ at 37°C in Dulbecco’s modified Eagle’s medium (Mediatech, Inc.). Cells were plated at low density and harvested at approximately 90% confluency for asynchronous whole cell extract. Cells were synchronized in $G_1$ by plating cells at approximately 50% confluency for asynchronous whole cell extract. Cells were plated at low density and harvested at approximately 90% confluency for asynchronous whole cell extract. Cells were synchronized in $G_1$ by plating cells at approximately 50% confluency for asynchronous whole cell extract. Cells were plated at low density and harvested at approximately 90% confluency for asynchronous whole cell extract. Cells were plated at low density and harvested at approximately 90% confluency for asynchronous whole cell extract. Cells were plated at low density and harvested at approximately 90% confluency for asynchronous whole cell extract. Cells were plated at low density and harvested at approximately 90% confluency for asynchronous whole cell extract.

**Gel Mobility Shift Analysis**—Gel mobility shift assays were performed with whole cell extracts and end-labeled double-stranded oligonucleotide probes. In assays using asynchromous cell extract, equal amounts of extract were incubated for 10 min with 2 μg of herring sperm DNA and 100 ng of specific competitor DNA (if used) as described previously (54). One ng of labeled probe was then added to each reaction, and the reactions were incubated at room temperature for an additional 20 min. Gel shift assays of extracts from synchronized cells contained equal amounts of total protein. For supershift experiments, either 1 μl of polyclonal antibody or 4 μl of monoclonal antibody were added during the first 10-min incubation.

**RESULTS**

Identification of E2F DNA Binding Complexes in Chinese Hamster CHOC 400 Cells—In order to identify the constituents of the protein complexes that bind specifically to the overlapping E2F sites of the $dhfr$ promoter in CHOC 400 cells, whole cell extract was prepared from asynchronously growing cultures and used in gel shift mobility assays. Gel mobility shift assays were conducted using a series of double-stranded oligonucleotide probes. The 2-site probe contained the two overlapping E2F sites of the $dhfr$ promoter but with different flanking sequences. The CG and GG site probes differed by a single bp within the E2F binding site (Fig. 1B). The mutant 2-site probe was identical to 2-site probe except that it contained a single G to T substitution within the overlapping E2F binding sites that altered the last bp of the GG site and the 4th bp of the CG site (Fig. 1B).

When 2-site probe was used in gel mobility shift assays, five distinct complexes were detected (Fig. 2, lane 8, labeled A–E). Five protein complexes with the same apparent migration were also detected when either the CG site or GG site was used as probe, although complexes C and E were faint in this exposure (Fig. 2, lanes 2 and 5). In contrast, only one protein-DNA complex, with the same mobility as complex E, was observed with the mutant 2-site probe (Fig. 2, lane 1F). Competition experiments in which binding to labeled probe was competed for by a 100-fold excess of unlabeled probe showed that binding of complexes A–E to all four probes is specific (Fig. 2, lanes 3, 6, 9, and 12).

Previous competition experiments showed that binding of complexes A–E to the 2-site probe was completely eliminated in the presence of a 100-fold excess of unlabeled 2-site or GG site oligonucleotides (54). Sequence preferences of specific complexes for either the GG or CG site were revealed under two binding conditions and only when the binding sites were present in the context of the 2-site probe. First, in the presence of a...
100-fold excess of unlabeled CG site, only complex E remained bound to the 2-site probe (54). Second, when the mutant 2-site oligonucleotide was used as competitor, only complexes A–D and a small fraction of complex E remained bound to the 2-site probe (54). Therefore, complex E appears to be a composite of at least two different binding activities, and only one of these binding activities binds the mutant 2-site probe.

The Predominant E2F Binding Activities in Extracts from Log Phase Cells Contain E2F-2 and E2F-4—To determine the identity of E2F DNA binding complexes A–E in CHOC 400 cells, we first transfected cells with pCMV expression vectors for human E2F subunits 1–4 alone or in combination with pCMV-DP-1 or pCMV-DP-2. Expression of E2F subunits 1–4, DP-1, or DP-2 alone had little effect on the spectrum of specific E2F DNA binding complexes that formed on the 2-site probe (data not shown). Cotransfection of E2F expression vectors with pCMV-DP-1 resulted in the accumulation of E2F complexes with distinct migration patterns (Fig. 3, lanes 2–5). Expression of DP-1 with E2F-1, -2, or -3 resulted in complexes that migrate at the position of endogenous complex E (Fig. 3, lanes 2–4). E2F-DP-1 complexes containing E2F-2 or E2F-3 migrated slightly faster than those containing E2F-1 (compare lanes 3 and 4 to lane 2). Expression of E2F-4 and DP-1 resulted in the accumulation of a complex that comigrated with complex D (lane 5). Coexpression of E2F subunits 1–4 with DP-2 resulted in gel shift complexes identical to those seen with the expression of DP-1 (data not shown).

The transfection experiments provided information about
the relative mobility of different combinations of E2F and DP proteins. We next used antibody supershifts to identify the components of individual endogenous complexes. To test the specificity of a variety of antibody preparations, extracts from transfected cells were incubated with antibody for 10 min prior to the addition of probe in gel shift experiments. As shown in Fig. 3B, only antibody to E2F-4 shifted complex D (Fig. 3B, lanes 2–5). Note that all of complex D, which in this extract was composed of both the endogenous complex and that derived from the ectopic expression of E2F-4 and DP-1, was shifted by the antibody to E2F-4 but not by antibodies to E2F-1, -2, or 3 (lanes 2–3). Thus, endogenous complex D contains E2F-4.

Similar experiments were used to test the specificity of polyclonal antibodies, monoclonal antibodies, or mixtures of monoclonal antibodies for each of the E2F and DP proteins (Fig. 3C, lanes 1–6). Some antibody preparations, such as the mixture of monoclonal antibodies KH20 and KH95 against E2F-1, resulted in clear supershifts of free E2F-DP complexes (Fig. 3C, compare lanes 1 and 2). Others, such as the polyclonal antibody to E2F-2, disrupted DNA binding (Fig. 3C, compare lanes 3 and 4). The specificity of the immune reagents under these binding conditions also was indicated by the observation that the antibodies did not alter the mobility of all the endogenous hamster E2F complexes but rather only influenced the mobility of specific complexes.

Using these reagents, the E2F constituents of hamster complexes A–E were identified by antibody supershift experiments using several DNA probes. Complexes A and D were shifted by an antibody against E2F-4 in gel shift experiments using 2-site probe (Fig. 4, lane 6). Similar results were obtained when the CG or GG sites were used as probes (data not shown). Complex A was supershifted by antibody to p107 (Fig. 4, lane 10) but not by antibody to pRB (lane 9) or p130 (see below). Thus, complex A contained E2F-4 and p107. In a similar manner, the electrophoretic mobility of complex E on 2-site probe was only altered by the addition of an antibody specific for E2F-2 (Fig. 4, lane 4), although no supershift was observed in this instance. The addition of antibodies against either E2F-1 or E2F-3 did not affect the migration of any of the complexes (Fig. 4, lanes 3 and 5). While in this experiment the antibody to DP-1 only partially supershifted all of the complexes (lane 7), other experiments showed DP-1 is the only DP protein in CHO C400 E2F binding complexes (see below). Although antibodies against human cyclins A and E recognized hamster cyclins A and E in Western blots (data not shown), these reagents did not influence the migration of complexes A–E (Fig. 3, lanes 11 and 12). We did not test the effect of adding purified cyclins to the whole cell extract on the gel shift patterns.

To better identify the components of complex E, the complex was isolated by adding a 100-fold excess of the CG site oligonucleotide to binding reactions that contained the 2-site oligonucleotide as probe (Fig. 5A, lanes 3–8). Only the antibody to E2F-2 had an effect on the migration of complex E, and this antibody disrupted rather than shifted the complex (Fig. 5A, lane 6). A fraction of complex E binding activity was also isolated by using the mutant 2-site as probe (Fig. 5B). Once again, when antibodies against E2F2’s 1–4 were added to the gel mobility shift assays, only the antibody against E2F-2 affected protein binding (Fig. 5B, lane 4). Since the CG site of the mutant 2-site probe contains a G to T transition at a critical nucleotide of the consensus E2F binding site, and the CG single site competitor did not compete for complex E, any binding to this probe is presumed to be due to the presence of the GG binding site, of which only the last nucleotide is affected by the mutation. However, E2F binding to the GG site of the mutant 2-site probe is not equivalent to GG site probe, for complexes A–D are not observed when the mutant 2-site is used as probe (Figs. 2 and 5B). From these and other experiments, we conclude that hamster E2F complexes containing E2F-2 and E2F-4 are capable of binding to both the CG and GG sites. However, in the presence of native flanking sequences, com-

**Fig. 3.** Identification of E2F binding complexes in CHO C400 cells. A, to compare the mobility of hamster E2F complexes with their human counterparts, CHO C400 cells were transfected with pcMV expression vectors for E2F subunits 1–4 and DP-1 as indicated. Whole cell extract was prepared, and binding activity was assayed with the 2-site probe. B, whole cell extract from cells transfected with pcMV-E2F-4 and pcMV-DP-1 was incubated with the indicated antibody for 10 min prior to the addition of probe. Gel shift complexes were visualized as before. C, whole cell extract from cells transfected with pcMV-DP-1 and pcMV-E2F-1 (lanes 1–2), pcMV-DP-1 and pcMV-E2F-2 (lanes 3–4), and pcMV-DP-1 or pcMV-E2F-3 (lanes 5–6) were incubated with the indicated antibodies for 10 min prior to the addition of 2-site probe. Gel shift complexes were visualized as before.
E2F Complexes at the dhfr Promoter

Changes in E2F DNA Binding to the dhfr Promoter During the G to S Phase Transition—In order to determine how binding of the individual E2F protein complexes change during induction of dhfr gene expression, gel shift analysis was performed with whole cell extracts prepared from synchronized cells. CHOC 400 cells were arrested in G1 by incubation in low serum (0.1% FBS) for 48 h and then were stimulated to re-enter the cell cycle by the addition of complete media containing 5% FBS. Greater than 70% of the cell population remained in G1 until 16 h after the addition of complete media with 5% FBS, at which time cells began to enter S phase. By 24 h after stimulation to reenter the cell cycle, greater than 60% of cells had entered S phase and less than 15% remained in G1. Northern blot analysis revealed that dhfr message levels increased almost 10-fold during the G1 to S phase transition and reached peak levels 18 h after release into complete media (Fig. 7B).

Gel shift analysis of whole cell extracts from synchronized cells detected E2F DNA binding complexes A, B, D, and E during transition of G1 and entry into the S phase (Fig. 8). All four complexes were observed with the CG, GG, and 2-site probes (Fig. 8, panels A–C), although complexes A and B were relatively faint with the GG site probe (Fig. 8C). As shown in Fig. 8, transition through G1 and entrance into the S phase was accompanied with several changes in the spectrum of E2F DNA binding activity. In serum-starved cells, complex B was prevalent during the first 8 h after release from the serum block (Fig. 8A, lanes 1–5). Complex B disappeared between 10 and 12 h after the addition of complete medium (Fig. 8, A and B, lanes 6–13). Complex E was not detectable during the first 8 h of the G1 to S phase transition, first appeared at 12 h (Fig. 8, A–C, lanes 1–6), and then increased in abundance from 12 to 24 h (Fig. 8, A–C, lanes 7–13). Similarly, the abundance of complex D remained relatively unchanged during the first 8–10 h after addition of medium with serum but increased dramatically from 12 to 24 h (Fig. 8, A–C, compare lanes 1–6 to lanes 7–13). By 24 h after release from arrest in low serum, most of the detectable E2F DNA binding activity was present in complexes D and E. The relative abundance of complex A remained constant throughout the entire course of the experiment (Fig. 8, A–C, lanes 1–13).

Loss of p130 from an E2F-4-DP-1 DNA Binding Complex Precedes Induction of dhfr Gene Expression—Gel shift analysis indicated induction of dhfr gene expression occurred after the disappearance of complex B and concomitant with increased abundance of complexes D and E. Using a panel of antibodies in supershift experiments, the constituents of complexes A, B, D, and E from synchronized cells were identified. Because many different hamster E2F complexes migrate similarly on neutral polyacrylamide gels, samples from three different time points were analyzed. Antibody supershift analysis of samples from the 0- and 8-h time points gave identical results (data not bated in medium containing 0.1% FBS for 48 h contained a G1 DNA content (time 0, Fig. 7A). Greater than 70% of the cell population remained in G1 until 16 h after the addition of complete media with 5% FBS, at which time cells began to enter S phase. By 24 h after stimulation to reenter the cell cycle, greater than 60% of cells had entered S phase and less than 15% remained in G1. Northern blot analysis revealed that dhfr message levels increased almost 10-fold during the G1 to S phase transition and reached peak levels 18 h after release into complete media (Fig. 7B).
When added alone or in combination with E2F-2 or E2F-4, antibodies against E2F-4 shifted all of complex D but did not alter the mobility of complex E (Fig. 10B, lanes 2–11). Moreover, the mobility of complex E was not shifted or disrupted by the addition of antibodies against E2F-1, E2F-2, or E2F-4, either when added alone or in combination (Fig. 10B, lanes 3–11).

To relate changes in E2F DNA binding complexes to induction of dhfr gene expression, the radioactivity in gel shift complexes B, D, and E was quantified with a phosphorimager and plotted against dhfr mRNA levels as determined by Northern blotting (Fig. 11). Comparison of these plots showed that p130 was lost from a complex containing E2F-4-DP-1 approximately 6–8 h before entry into the S phase and that free E2F complexes containing E2F-4-DP-1 and an unidentified E2F (or other protein) in association with DP-1 accumulated to high levels during late G1. Note that the free E2F complexes reached high levels 2–4 h before peak levels of dhfr mRNA were observed and that these complexes persisted in S phase when dhfr mRNA levels had declined precipitously.

**DISCUSSION**

E2F DNA binding activity can be attributed to the collective activities of multiple heterodimeric complexes, each containing one E2F-like subunit and one DP-like subunit. E2F activity, in turn, is regulated through association with the pRB family of pocket proteins, by direct or indirect interactions with Cdk 2 and cyclins A and E, and by phosphorylation. In an effort to determine which E2F species may participate in the regulation of dhfr gene expression, we first used gel mobility shift and antibody supershift assays to identify the constituents of at least five distinct protein complexes which bind to the conserved overlapping E2F sites found in the dhfr promoters of hamsters, humans, and mice. Because polyclonal antibodies were added to extract prior to the addition of probe, and may react with portions of E2F complexes involved in DNA binding, we considered both disruption and supershifts of specific complexes as evidence that a complex harbored the antigen in question. Once the constituents of the hamster complexes were identified, we examined their DNA binding activity and documented changes in specific forms of E2F DNA binding activity to induction of dhfr message levels during the G1 to S phase transition.

DNA Binding Preferences of Hamster E2F Complexes That Contain E2F-2 and E2F-4—Almost all of the detectable dhfr E2F DNA binding activity in extracts from asynchronously growing CHOC 400 cells was attributable to complexes containing two E2F species, E2F-2 and E2F-4. All of the E2F DNA binding complexes in CHOC 400 cells contained DP-1 as the E2F heterodimeric partner. In context of the native flanking sequences of the dhfr promoter, complexes containing E2F-2-DP-1 preferentially bound to the GG site, whereas complexes containing E2F-4-DP-1 were the predominant CG site binding activity. The preference of complex E for the GG binding site was revealed by competition studies using the 2-site oligonucleotide as probe and the CG site as competitor or by examining binding to the mutant 2-site probe (Fig. 5). Under these binding conditions, only complex E bound to the E2F probe, and only antibodies to E2F-2 and DP-1 affected the mobility of the complex.

While complexes containing E2F-2 displayed clear preference for the GG site, complexes containing E2F-4 appeared to bind either the CG or GG sites. When binding to a probe containing the single CG site was competed for by oligonucleotides containing only the GG site, no binding activity was observed. Moreover, in the absence of any competitors, the same spectrum of E2F complexes A–D bound to each of the single CG and GG site probes. However, E2F-4-DP-1 complexes were able to bind to the 2-site probe in the presence of the mutant 2-site oligonucleotide as competitor, conditions that abolish binding of complexes containing E2F-2. Therefore the CG and GG sites in the native dhfr promoter are not equivalent and most likely bind specific endogenous E2F-DP-1 complexes. Because the difference in binding site specificity was observed with complexes that each contained DP-1, the apparent specificity of these interactions is most likely contributed by the E2F subunits.

**Two Separate Processes May Be Required for Induction of dhfr mRNA**

*E2F Complexes at the dhfr Promoter*
dhfr Gene Expression—In serum starvation-stimulation experiments, distinct changes in E2F DNA binding complexes were detected in CHOC 400 cell extracts. After serum starvation, three complexes were detected, E2F-4zDP-1 (complex D), E2F-4zDP-1 in association with p130 (complex B), and E2F-4zDP-1 in association with p107 (complex A). Based upon previous genomic footprinting studies and the apparent preference of complexes containing E2F-2 for sequences containing GG nucleotides at positions 4 and 5 of the E2F binding site, we originally suggested that E2F-2 was constitutively bound to the GG binding site at the dhfr promoter and, in conjunction with Sp1, formed a basal transcription complex (54). We therefore expected to observe a significant amount of DNA binding activity that contained E2F-2 in extracts from cells after synchronization by serum starvation and stimulation. However, complexes containing E2F-2 were essentially undetectable in extracts from serum-starved cells and in cell extracts for at least 24 h after serum stimulation. Thus, complexes containing E2F-2 do not appear to play a role in repression or activation of dhfr gene expression under serum starvation conditions. These observations suggest that regulation of dhfr gene expression in cycling cells may involve different forms of E2F than those that regulate expression after synchrony by serum starvation. By comparing the effect of different growth conditions on the spectrum of E2F DNA binding activities in CHOC 400 cell extracts and relating these differences to dhfr gene expression and the genomic footprint of the dhfr promoter, it may be possible to identify those complexes that repress and activate dhfr gene expression transcription under specific growth conditions.

In serum-starved cells, the E2F-4zDP-1-p130 complex accumulated as message levels declined, suggesting a role for both E2F-4 and p130 in repression of dhfr transcription. Upon serum stimulation, flow cytometry showed that entry into the S phase occurred about 16–18 h after the addition of medium with 5% FBS. Maximum dhfr mRNA levels coincided with S phase entry. By 10–12 h after serum stimulation, p130 had disappeared from the E2F-4zDP-1-p130 complex, and two free E2F complexes began to accumulate, one of which was composed of E2F-4zDP-1, whereas the second complex contained DP-1 and an unidentified E2F. The 6–8-h lag phase between disappearance of p130 and the appearance of peak dhfr mRNA levels suggests that relief of p130-mediated repression alone is not sufficient for induction of dhfr gene expression.

Rather, at least one subsequent event in addition to relief of p130-mediated repression appears to be required for induction of dhfr gene expression. This second activating event is not known but may be related to the marked increases in the DNA binding activity of free E2F complexes containing E2F-4 or the unidentified E2F. During the first 10 h after serum stimulation the abundance of free E2F-4zDP-1 did not change. Between 12 and 16 h after addition of medium with serum, the abundance of two complexes, E2F-4zDP-1 and complex E containing the unidentified E2F, increased dramatically (Fig. 11). Although dissociation of the E2F-4zDP-1-p130 complex during G1 likely contributed to the pool of free E2F-4zDP-1, this alone seems incapable of accounting for the observed increase in the binding activity of E2F-4zDP-1 complexes, as quantitation showed that the total amount of free E2F-4 was significantly greater than the combined amounts of E2F-4zDP-1-p130 and E2F-4zDP-1 complexes detected in early G1 (Fig. 11). Increased free E2F-4zDP-1 binding activity could result from increased protein synthesis, decreased protein turnover, modification of previ-
ously or newly synthesized protein, or increased availability of DP heterodimeric partner proteins. Since Northern blot analysis showed that E2F-4 mRNA changed less than 2-fold during the G1 to S phase transition (data not shown), increased E2F-4 binding activity is not likely to be solely the result of increased E2F-4 transcription.

Similarly, complex E was essentially undetectable until 10 h after addition of complete medium and steadily increased until the 24-h time point. In contrast to E2F-4, the increased DNA binding activity of complex E cannot be due to the dissociation of other complexes, as no other DNA binding complexes containing this unidentified E2F were detected before mid-G1. Using several different approaches, we were unable to identify the E2F constituent of complex E that increased during induction of dhfr gene expression. Clearly, this complex contained DP-1. Although DP-1 possesses a DNA binding domain, it binds DNA only weakly in the absence of an E2F partner. It is therefore unlikely that this complex represents a DP-1 homodimer. Potential candidates as the E2F moiety of this complex include a form of E2F-1 not recognized by the antibodies to E2F-1 that were used, E2F-5 (a possibility which we did not test), or an as yet undescribed sixth E2F. Interestingly, a recent study using stimulated human T cells also identified an E2F DNA binding activity that was not affected by antibodies specific for E2F-1, E2F-2, E2F-3, or E2F-4 (60). As for complex E, the DNA binding activity of the unidentified E2F was absent in early G1, increased in late G1, peaked during S phase, and declined thereafter. Until this species of E2F is identified, its relationship to dhfr gene expression will be difficult to establish.

Studies in a variety of cell types have shown that E2F-1 mRNA levels increase at the G1/S phase boundary and peak during early S phase (reviewed in Ref. 61), supporting the possibility that the unknown E2F may be E2F-1. However, in separate studies we have shown that ectopic expression of E2F-1 does not induce dhfr gene expression in CHOC 400 cells.2

The accumulation of free E2F complexes D and E 4–6 h before maximal dhfr mRNA levels were attained raises the possibility that induction of dhfr gene expression is not simply a consequence of the presence of free E2F complexes D and E. Moreover, these complexes persisted well into the S phase well after dhfr mRNA levels had declined, suggesting the presence of free E2F is not sufficient to maintain dhfr mRNA levels. Thus, other signals must participate in the regulation of dhfr gene expression. For example, Sp1 has recently been reported to functionally interact with E2F-1 and synergistically activate dhfr transcription (62). Because the interaction between E2F-1 and Sp1 is regulated during the cell cycle (62), phosphorylation of Sp1 or other components of the promoter complex also may affect the time of activation of dhfr transcription. The events that regulate the decline in dhfr mRNA levels during the CHOC 400 S phase are not known.

The Role of Pocket Proteins in dhfr Gene Expression—Similar to CHOC 400 cells, the major E2F activity in resting human T cells was identified as E2F-4, and E2F-4 was associated with p130 in extracts from cells collected in G₀ and early G₁. As cells passed through G₁ and entered into S phase, p130 disappeared from an E2F-4-DP-1-p130 complex, the DNA binding activity of free E2F-4-DP-1 increased, and p107 and pRB became associated with E2F-4-DP-1 (60). In our studies, we were unable to detect pRB in association with E2F-4-DP-1 with either polyclonal antiserum to pRB (C-15) or a mixture of two monoclonal pRB-specific antibodies (XZ91 and XZ77). Western blot analysis of CHOC 400 cells revealed that endogenous pRB levels are quite low in these cells (data not shown). However, when CHOC 400 cells are induced to express high levels of E2F-1, endogenous pRB levels increase 30-fold within 8 h, and a single complex containing ectopic E2F-1, endogenous pRB, and endogenous DP-1 accumulates. This complex migrates at the position of endogenous complex C. Thus, CHOC 400 cells are capable of expressing pRB, but this protein does not appear to associate with complexes containing E2F-4. While it is possible that different forms of E2F in association with different pocket proteins may regulate the repression and expression of dhfr and other E2F-responsive genes in different cell types, and under different growth conditions in the same cell type, it appears that p130 is sufficient to repress dhfr gene expression in cells deprived of serum. Complexes containing p107 (complex A) did not appear to play a role in repression because they did not change during conditions in which dhfr gene expression was repressed or induced. The complexity of the regulatory lattice that controls the expression of E2F subunits and the pRB family of pocket proteins in different cell types is also revealed by the broad spectrum of cellular defects that occur in mice lacking E2F-1 (63, 64).

Why Does the dhfr Promoter Contain Two Different, Overlapping E2F Binding Sites?—The promoters of several genes, including dhfr, E2F-1, DNA polymerase α, thymidine kinase, p107 and n-myc, contain multiple E2F binding sites, in either a tandem or overlapping arrangement (reviewed in Ref. 61). Although the reason for binding site duplication is presently unknown, in the human p107 promoter a promoter proximal site is critical for basal transcription, whereas the upstream site is required for repression (65), suggesting each site acts through different E2F complexes. It is interesting to note that the organization of multiple E2F binding sites within a promoter is conserved among different species. For example, the organization of the two overlapping E2F sites contained within the dhfr promoter is conserved among humans, hamsters, and...
mice. In these species, the transcribed DNA strand contains the GG E2F site, the nontranscribed DNA strand contains the CG E2F site, and the two E2F sites overlap by exactly four nucleotides. Thus, both the sequence and spacing of the individual E2F binding sites are highly conserved. Similarly, the E2F-1 gene contains two sets of overlapping E2F sites, and their organization and sequence are identical between humans and mice (66, 67).

We are interested in the reason why the architecture of the overlapping E2F binding sites within the dhfr promoter is conserved and suggest a possible explanation. Because the sites are inverted and offset by about half a helical turn of B DNA, it is possible that both sites might be occupied by E2F complexes simultaneously. Our genomic footprinting studies support this notion, for both sites are protected from nucleosome cleavage at the same time during late G1 and early S phase (54). Moreover, the asymmetric nature of the footprint on each strand indicates the proteins bound to each site may be oriented on DNA in opposite directions. In this view, binding of one E2F-DP heterodimer to the one site early in the cell cycle may mediate repression of transcription and also provide a protein-DNA complex that dictates which E2F-DP complexes are able to bind the second site later in the cell cycle. Currently, we are using bacterially expressed E2F and DP proteins to test the binding specificities of different complexes and to determine how the binding of one complex to the overlapping E2F sites influences binding of another to the same DNA probe.

Once the nucleotides that contribute to sequence-specific interactions of E2F-DP complexes with the GG and CG sites in the context of the native dhfr flanking sequences are identified, site-directed mutagenesis can be used to assess the role of individual E2F complexes in repression and activation of dhfr gene expression.

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REFERENCES

Accumulation of E2F-4·DP-1 DNA Binding Complexes Correlates with Induction of dhfr Gene Expression during the G1 to S Phase Transition

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