Nuclear factor E2F mediates basic transcription and *trans*-activation by E1a of the human *MYC* promoter

Karin Thalmeier,¹ Heidi Synovzik,¹ Ronald Mertz,² Ernst-Ludwig Winnacker,^{1,2} and Martin Lipp^{1,3}

¹Institut für Biochemie, Ludwig-Maximilians-Universität, D-8000 Munich 2, FRG; ²Laboratorium für Molekulare Biologie—Genzentrum, Am Klopferspitz, D-8033 Martinsried, FRG

Transcription from one of the two initiation sites, P₁ and P₂, of the dual human *MYC* promoter seems to be essential in all proliferating cells. To identify proteins and target structures for *MYC* regulation, a DNA region was analyzed that is critical for P₂ promoter activity. Here, we show that a nuclear factor binds to a DNA element within P₂, which is conserved perfectly between mouse and man and displays a striking homology to the E1a-inducible E2 promoter of adenovirus type 5 (Ad5). We demonstrate that the same transcription factor, defined recently as E2F, which plays an essential role in the activation of adenovirus early promoters and enhancers, also interacts as a dominant nuclear factor with the *MYC* promoter. The presence of an intact E2F binding site is required for basic expression and for *trans*-activation of the P₂ promoter by E1a proteins. The human *MYC* promoter is the first cellular target described for E2F. The results suggest that expression of *MYC* might be regulated via modulation of E2F by cellular 'E1a-like' factors.

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Although the knowledge of transcriptional control in eukaryotic cells has advanced during recent years by the identification of elementary DNA sequence and nuclear factors interacting with these elements, the individual mechanisms leading to tissue-specific regulation during differentiation or to a characteristic deregulation during malignant transformation of certain genes are understood poorly. Recent studies suggest that multiple factors must cooperate and interact for enhancer- and tissue-specific transcriptional regulation and that different factors may recognize the same consensus sequence (for review, see Jones et al. 1988). The detection of a single yeast protein that has the ability of binding to two distinct DNA sequences has highlighted the puzzling complexity of transcriptional control (Pfeifer et al. 1987).

Viral systems have been analyzed extensively because their control elements are organized tightly. Recently, interest has focused also on the regulation of cellular oncogenes, some of which are deregulated consistently and expressed highly in certain types of tumors. Among the examples best analyzed are human and mouse *MYC* proto-oncogenes. They are activated by characteristic chromosomal translocations in Burkitt's lymphomas and mouse plasmacytomas, respectively (Klein and

Klein 1985). Several studies have attempted to elucidate the regulation of the *MYC* gene by 5' deletions, in vivo competition, and nuclear run-on transcription elements. The results suggest that multiple negative and positive regulatory elements, including transcriptional arrest within the first noncoding exon, are responsible for the steady-state level of *MYC* RNAs (Bentley and Groudine 1986; Chung et al. 1986; Eick and Bornkamm 1985; Yang et al. 1986; Hay et al. 1987; Lipp et al. 1987; for review, see Piechaczyk et al. 1987). Nevertheless, it is far from being understood which cellular transcription factors mediate repression or activation from the two *MYC* promoters (P₁ and P₂) and how expression is modulated during differentiation, immortalization, and malignant transformation. In this study, we describe a cellular transcription factor binding efficiently to an element within the second promoter (P₂), which possesses binding specificities nondistinguishable from a nuclear protein, recently defined as E2F (Kovesdi et al. 1987). This factor plays a fundamental role in the activation of early promoters and enhancers of adenoviruses by interacting with the virus-encoded early E1a proteins. We demonstrate that binding of this factor to the second *MYC* promoter increases during adenovirus infection and is essential for its *trans*-activation by E1a. Furthermore, our results suggest that the activity of P₂ can be modulated by other cellular 'E1a-like' proteins (Imperiale et al. 1984) via the E2F transcription factor.

³Corresponding author.

Results

Cell-type-specific binding pattern of nuclear extracts to a MYC promoter fragment

The two promoters of the human *MYC* gene (P_1 and P_2) are used differentially in certain cell types. P_2 dominates P_1 in most cell lines or tissues analyzed so far. However, in Burkitt's lymphomas, *MYC* becomes activated by consistent chromosomal translocations, and P_1 is used preferentially. We have shown recently by S1 analysis that transcription initiates efficiently from P_2 in 293 cells, a cell line constitutively expressing the *Ela* proteins of adenovirus type 5 (Ad5) (Lipp et al. 1989). Because these differences might reflect the presence or absence of distinct transcription factors, nuclear extracts of 293 cells and of the Burkitt lymphoma line BL64 were prepared and tested in gel retardation experiments with a 188-bp DNA fragment from the *MYC* promoter, denoted f-mycP₂, encompassing position -141 to +47 with respect to the transcriptional start site of P_2 . As shown in Figure 1, crude nuclear extract of 293 cells gave rise to two specific DNA-protein complexes (A and B), whereas the extract of BL64 cells revealed only one complex band (C) with an intermediate mobility

compared to bands A and B. The specificity of the DNA-protein complexes A, B, and C was demonstrated by competition analysis with a 50-fold excess of unlabeled homologous promoter fragment (data not shown). However, the weak double band E (Fig. 1) did not disappear even with a 200-fold excess of f-mycP₂, and therefore was considered provisionally as 'unspecific.'

To define more precisely the binding site of nuclear proteins within the 188-bp *MYC* promoter fragment, we took advantage of synthetic oligonucleotides to compete for particular sequences. Oligonucleotide myc-9/10 (Fig. 2; origin, see Fig. 1, bottom) was chosen, because its sequence is highly conserved between human and mouse *MYC* promoters. Of 38 bp of myc-9/10, 34 bp are identical to the mouse sequence. As shown in Figure 1, bands A, B, and C disappeared completely when a 60-fold molar excess of unlabeled double-stranded oligonucleotide myc-9/10 was added. The pattern of complex bands did not alter in the presence of unrelated oligonucleotides A1/B1, EW-1/2, K-1/2 (Fig. 2). These results demonstrate that crude nuclear extracts from 293 cells and from Burkitt lymphoma cells form different DNA-protein complexes by recognizing a short *MYC* promoter sequence of 38 bp represented by the oligonucleotide

Figure 1. Gel retardation assay of the fragment f-mycP₂ with crude extracts of 293 and BL64 cells. In the presence of 2 μ g poly[d(A-T)], 16 fmole of 5' end-labeled f-mycP₂ (-141 to +47) were incubated with 6 μ g of 293 extract (lanes 1-5) or BL64 extract (lanes 6-10). Competition experiments were done by adding a 60-fold molar excess of unlabeled double-stranded oligonucleotides (for sequence, see Fig. 2), as indicated above each lane. (A-E) DNA-protein complex bands; (D) the unbound fragment. (Bottom) The location of oligonucleotide myc-9/10 within fragment f-mycP₂. (*) Homology to the core consensus sequence of transcription factor Sp1; (Δ) homology to the consensus sequence of nuclear factor NF1.

Figure 2. Synthetic oligonucleotides and DNA fragments used in gel retardation and competition experiments. Known binding sites for nuclear factors are indicated.

Interference of nuclear factors with a conserved DNA sequence of the second MYC promoter

Analogy in binding specificities of the MYC promoter fragment and an enhancer region of the adenovirus E1a gene

Keeping in mind that the *MYC* promoter can be *trans*-activated efficiently by E1a proteins, we compared the DNA sequences of fragment f-mycP₂ with sequences from adenovirus early promoters known to be *trans*-activated by E1a. We found a striking homology to a region of the E2 promoter of Ad5 that is essential for *trans*-activation and is protected in footprint analysis (Kovesdi et al. 1986). Therefore, we proposed that a transcription factor with a similar recognition sequence should bind also to the *MYC* promoter. This hypothesis was proved by gel retardation competition experiments using the left-hand terminal *Hind*III fragment of Ad5 (f-Ad5H3-G) carrying two binding sites for a transcription factor that also binds to the E1a enhancer region included in the *Hind*III fragment, as well as to an upstream element of the E2 promoter. Recently, this factor has been defined as E2F (Kovesdi et al. 1987). As shown in Figure 4B, f-Ad5H3-G competed to a similar extent as the oligonucleotide myc-9/10 for nuclear factors forming bands A and B. Because the terminal fragment of Ad5 carries recognition sites for multiple nuclear factors (Jones et al. 1988), the specificity of competition was demonstrated by destroying the proposed consensus binding site for E2F (TTTCGCGC) with the restriction enzyme *Fnu*DII (Fig. 4D). f-Ad5H3-G digested with *Fnu*DII did not compete for complex A and B (Fig. 4A), but the same fragment cut with *Hpa*II (CCGG) competed efficiently. To prove that E2F, or a related transcription factor, binds to the *MYC* promoter, similar competition experiments were performed using the labeled fragment f-E2F originating from the E1a enhancer of Ad5 carrying two E2F sites (Fig. 2). As shown in Figure 4C, the Mono-Q fraction, screened and identified with f-mycP₂, also had a strong binding activity to f-E2F, resulting in the double complex bands A' and B'. Bands A' and B' could be competed very efficiently by myc-9/10 by undigested or *Hpa*II-digested f-Ad5H3-G but not by f-Ad5H3-G cut with *Fnu*DII and not by control DNA sequences or the presence of EDTA. These results prove convincingly that E2F or a transcription factor with a similar recognition sequence binds as a dominant nuclear factor to a P₂-specific fragment of the human *MYC* gene.

Figure 3. Footprint analysis of fragment f-mycP₂ by methylation interference. (Lanes A–D) DNA–protein complex (+) or free fragment (–) bands indicated in Fig. 1. (▲) G residues interfered with protein binding after methylation. For experimental details, see Methods.

Figure 4. Gel retardation competition analysis of the MYC promoter (f-mycP₂) and Ad5 E1a enhancer (f-E2F) fragments. End-labeled f-mycP₂ (A,B, 100 fmole) or end-labeled f-E2F (C, 50 fmole) were incubated with a Mono-Q fraction purified from HeLa cells, without (–) or with a 50-fold molar excess of competitor DNA (for sequence and origin, see Fig. 2). Fragment f-Ad5H3-G was either cut by *Fnu*DII (Fnu) destroying the E2F binding site (D), or by *Hpa*II leaving the E2F site intact. For explanation of complex bands A, B, E, A', and B', see text.

The sequence of G residues protected by DNA methylation (Fig. 3) displays some similarities with the core binding sequence of transcription factor Sp1 (see sequence marked by asterisks at bottom of Fig. 1). A recent report of microinjection experiments with MYC deletion constructs into frog oocytes suggested that Sp1 should bind to this particular sequence and mediate the observed transcriptional activity (Nishikura 1986). Using *in vitro* gel retardation assays, we could not obtain any evidence for the binding of Sp1 to f-mycP₂. Neither Sp1-specific fragments (f-Sp1, Fig. 2) nor oligonucleotides containing a Sp1 consensus sequence (K-1/2 and AP-1/2, Fig. 2) were able to compete for any of the f-mycP₂-specific complex bands (Fig. 1; Fig. 4B, last lane). The DNA–protein complex formed with labeled f-Sp1 and crude or partially purified HeLa nuclear extracts could not be competed by the fragment f-mycP₂ or with any P₂-specific oligonucleotide (data not shown).

Increasing levels of the MYC binding factor during adenovirus infection

Because it was shown previously (Kovesdi et al. 1986) that the level of the E2F factor that can bind to the E2 promoter increases in Ad5-infected cells, a similar effect should be observed with the MYC promoter fragment if both factors are identical and have the same properties. Therefore, we prepared nuclear extracts from HeLa cells infected with adenovirus type 12 (Ad12) for increasing time periods and performed gel retardation assays with

fragment f-mycP₂. As shown in Figure 5, the complex bands A and B are detected readily in uninfected cells, but a large increase in binding activity is seen at 16 hr after Ad12 infection. Because of the prolonged replication cycle of Ad12 compared with Ad2/Ad5, early viral proteins are synthesized in significant amounts ~12–15 hr postinfection. This result confirms again the prior

Figure 5. Induction of nuclear factor binding to the MYC promoter during adenovirus infection. Gel retardation analyses were carried out with 100 fmole end-labeled f-mycP₂ fragment incubated with nuclear extracts that were prepared from HeLa cells after infection with Ad12 (25 pfu/cell).

conclusion that the factor binding to the *MYC* promoter is identical to E2F.

Detection of additional nuclear factors by synthetic oligonucleotides

In addition, we examined whether oligonucleotides myc-7/8 and myc-9/10 (4 bp longer, Fig. 2), being part of f-mycP₂ and competing efficiently for E2F, would reveal an identical binding pattern in gel retardation assays as observed with the P₂-specific fragment. Surprisingly, upon incubation of labeled myc-9/10 with crude extracts or a Mono-Q fraction from HeLa cells, in addition to complex bands A and B, three further complex bands with higher mobilities, denoted F, G, and H, could be detected by gel retention analysis (Fig. 6A). Complex H was observed rather clearly with crude extracts but not with the particular Mono-Q fraction. In contrast, complexes F and G were obtained significantly only with the purified Mono-Q fraction (first lane). Competition experiments with a 50-fold molar excess of unlabeled homologous or unrelated oligonucleotides demonstrated the specificity of binding (Fig. 6A). Complex bands A and B disappeared exclusively by competition either with

the E1a enhancer fragment f-E2F or with oligonucleotide EF-5/6 (Fig. 2) containing the two E2F binding sites of the Ad5 E2a promoter. Oligonucleotide EF-3/4 (Fig. 2), containing only the intact ATF binding site of the E2a promoter but mutated E2F binding sites, did not compete for complex bands A and B. In the reverse experiment (Fig. 6A, last 4 lines) using labeled oligonucleotide EF-5/6, two complex bands, A' and B', were formed, which disappeared by competition with myc-9/10 and EF-5/6 but not with EF-3/4. Thus, the results obtained with the promoter fragment f-mycP₂ were confirmed, indicating again that transcription factor E2F binds specifically to P₂. The reduced binding of all nuclear factors, especially F and G to myc-9/10 in the presence of f-Sp1, can be explained by the high GC content of this fragment (Fig. 6A).

To investigate a possible cell type specificity of complex bands F, G, and H and to study the identity of involved components, we incubated labeled oligonucleotide myc-7/8 with crude nuclear extracts from Burkitt lymphoma cell line BL64 without or in the presence of a 50-fold and 250-fold molar excess of an unlabeled homologous oligonucleotide of A1/B1. This oligonucleotide originated from the left-hand terminal repeat of

Figure 6. Gel retardation competition analysis of P₂-specific oligonucleotides with extracts from HeLa (A) and BL64 (B) cells. (A) 10 fmole of oligonucleotide myc-9/10 (lanes 1–12) or EF-5/6 (lanes 13–16), labeled by the Klenow fragment of DNA polymerase I, was incubated with a Mono-Q fraction purified from HeLa cells or with crude extract (CE). Competition experiments were done by adding a 50-fold molar excess of the unlabeled competitor DNA indicated above each lane. (B) 10 fmole of oligonucleotide myc-7/8 (lanes 1–5) or tk-1/2 (lanes 6–10), labeled as in A, were incubated with 5 μ g of crude nuclear extract from BL64 cells. The molar excess of unlabeled competitor oligonucleotides added to the binding reaction is indicated above each lane. For explanation of complex bands A, B, F, G, H, A', and B', see text. (D) Position of unbound DNA.

adenovirus type 2 (Ad2) containing a binding site of NFI (Fig. 2). A1/B1 was chosen as competitor because a stretch of bases of myc-7/8 (GCCAA) is homologous to one half of the NFI consensus sequence, which is also an incomplete CCAAT box (see also sequence marked by open triangles at bottom of Fig. 1). As shown in Figure 6B (left), complex band H can be competed efficiently by the homologous, as well as by the NFI-specific oligonucleotide. Interestingly, complex bands F and G, which can be recognized only weakly with the crude extract, suddenly appeared in the presence of an excess of A1/B1. This suggests that binding of F- and G-specific factors was inhibited by the H-specific component. The control experiment outlined in Figure 6B (right) confirmed the assumption that complex H belongs to the NFI or CCAAT families of transcription factors, because oligonucleotide tk-1/2 (Fig. 2), containing the canonical CCAAT box of the *tk* gene of herpes simplex virus type 1 (HSV1), specifically recognized the same factor. In conclusion, a synthetic oligonucleotide representing 38 bp of P₂ could form three distinct complexes with overlapping binding sites in addition to the two E2F-specific DNA-protein complexes also observed with the MYC promoter fragment. One of these factors recognized both NFI- and CCAAT-specific sequences and was found to be highly stimulated in the B-cell line BL64 in comparison to HeLa cells.

Functional significance of the E2F binding site for E1a-mediated trans-activation

We asked whether the E2F-specific binding site, identified *in vitro* by gel retardation assays and footprint analysis, would also be critical for *in vivo* promoter activity and *trans*-activation by E1a. For that purpose, 5'-deletion constructs were generated by *Bal31* digestion, which contained portions of the P₂ promoter, the complete first noncoding exon, some intron sequences, a synthetic splice acceptor oligonucleotide, and the chloramphenicol acetyltransferase (CAT) gene (Fig. 7). The MYC-*cat* hybrid plasmids were either transfected into

293 and HeLa cells or cotransfected with an E1a-expressing plasmid (pAd5H3-G) into HeLa cells. The enhancement of CAT activity by E1a in HeLa cells and CAT activity in HeLa and 293 cells was determined from cell extracts prepared 40 hr upon transfection. As shown in Figure 7, deletion constructs encompassing position -2489 bp (p2-2489) down to -66 bp (p2-66), with respect to the transcriptional start site of P₂, could be *trans*-activated significantly by E1a (about six- to sevenfold) and expressed high levels of CAT activity in 293 cells. Deletion of two additional G residues, which were identified as the outermost contact points by the methylation interference analysis (marked by asterisks in Fig. 7), abrogated *trans*-activation by E1a completely and diminished CAT activity in 293 cells to <10%. This activity was reduced further considerably to ~5% by transfecting plasmid p2-20, which lacks the TATA box.

The analysis of the behavior of promoter deletions in HeLa cells in the absence of E1a proteins confirmed previous observations of a slight negative upstream element (Chung et al. 1986; Hay et al. 1987; Lipp et al. 1987) but also revealed the importance of an intact E2F binding site for full promoter activity of P₂ (Fig. 7). The CAT activity was diminished to <50% by transfecting plasmid p2-64, which lacks a complete E2F site. The negative element was not detectable in 293 cells, indicating that the efficient activation of P₂ can abolish this effect. From these results, we conclude that the E2F binding site contributes to the entire basic activity of the human MYC promoter and is the critical target for its *trans*-activation by E1a.

Discussion

Irrespective of the mechanisms by which nuclear oncogenes might exert their capacity for cell transformation, either directly or possibly through transcriptional control of essential genes involved in differentiation and proliferation, the deregulation of their own expression frequently seems to be one crucial event during multistage tumorigenesis. In the present study we have at-

Figure 7. *Trans*-activation of 5'-deletion constructs of the MYC promoter by E1a and promoter activity in 293 cells. Deletion constructs (15 µg HeLa or 5 µg 293) were either transfected (HeLa, 293) or cotransfected with 1.5 µg pAd5H3-G (HeLa) into the recipient cells. The basic activity and the enhancement of CAT activity by E1a in HeLa cells or the activity in 293 cells was determined from 500 µg (HeLa) or 50 µg (293) cellular extract prepared 40 hr upon transfection. The relative CAT activities of 1.0 in HeLa and 293 cells correspond to 9500 cpm and 140,000 cpm, respectively. (*) G residues protected in the methylation interference assay (Fig. 3); the TATA motif is boxed.

tempted to identify nuclear factors, which are able to modulate transcriptional activity of the human *MYC* proto-oncogene. We could demonstrate that a nuclear protein binds efficiently and predominantly to an upstream region of the second *MYC* promoter (−58 to −67). This protein has binding properties identical to those of a transcription factor, recently defined as E2F, which plays an essential role in activating early promoters (E2a) and enhancers (E1a) of adenoviruses by interacting with the virus-encoded E1a proteins (Kovesdi et al. 1987). Most importantly, the P_2 promoter of *MYC* is the first cellular target described for the E2F factor. P_2 also can be *trans*-activated efficiently by E1a proteins, depending on the presence of an intact E2F binding site. Our results suggest that the activity of P_2 can be altered by cellular E1a-like proteins via the E2F transcription factor.

In agreement with experiments reported recently (Kovesdi et al. 1987; Yee et al. 1987), we also obtained two complex bands with a fragment derived from the E1a enhancer and with an oligonucleotide from the E2a promoter of Ad5 carrying both of the two distinct binding sites for E2F. By footprint analysis and by extensive competition experiments, we clearly could establish that the three *MYC*-specific and the two E1a/E2a-specific protein complexes are recognizing the identical DNA sequence. As summarized in Figure 8, a comparison of the critical DNA sequence within P_2 with known binding sites for E2F within the E2a promoter and the E1a enhancer of Ad5 and Ad12 identifies a common element in all sites with the 9-bp consensus sequence 5'-(C/C)CG(C/C)-GAAAA-3'. Yee et al. (1987) recently proposed the more simple consensus recognition site, 5'-GCGCGAAA-3', for E2F. It is not known yet whether the outermost A residue of the consensus sequence displayed in Figure 8 is necessary for the binding of E2F, because this A is missing in the second binding site within the E2a promoter. However, the G residue next to the consensus sequence within P_2 interfered clearly with E2F binding after methylation (Fig. 3), indicating that this contact point contributed to the stability of the DNA-protein complex.

Surprisingly, the usage of short double-stranded synthetic oligonucleotides in gel retardation assays instead of longer DNA fragments led to the detection of three additional DNA-protein complexes bound to the same

Figure 8. Comparison of the E2F binding sites in E1a-inducible promoters. (*) Sequence homologies between the P_2 promoter of the human *MYC* gene and the E2a promoter of Ad5. The human *MYC* (P_2) sequence is identical to the murine promoter sequence, except one nucleotide exchange (C to G, •). The E2F consensus sequence is boxed.

region of P_2 . The binding of one of these nuclear factors could be significantly competed with oligonucleotides carrying a canonical CCAAT box or a NFI consensus binding site (TGGN₅GCCAA). This suggests that the sequence TTC₇GCCAA of the *MYC* promoter can be recognized by one of the various CCAAT or NFI binding factors described recently (for review, see Jones et al. 1988). We have determined the equilibrium binding constants of purified and cloned NFI from porcine liver to the NFI site of Ad2, to the CCAAT motif of the *tk* gene of HSV1, and to the *MYC* promoter sequence, being 2×10^{10} , 1×10^7 , and 2×10^7 M, respectively (Meisterernst et al. 1988a,b). These low binding constants argue against an involvement of NFI in the control of *MYC*. Thus, the functional significance of the particular interactions of nuclear proteins with *MYC*-specific oligonucleotides remains to be determined experimentally.

However, our results demonstrate directly a functional role of the E2F factor in modulating the activity of the *MYC* promoter. Based on our finding that the second *MYC* promoter can be *trans*-activated efficiently by E1a proteins of human and mouse adenoviruses (Lipp et al. 1989), we demonstrated by a series of 5'-deletion constructs that an upstream promoter element, now identified as an E2F binding site, was essential for basic transcription, *trans*-activation by E1a, and for the high CAT activity in 293 cells (Fig. 7). This finding is in agreement with a recent report proposing that *trans*-activation of early adenovirus promoters is mediated through a transcription factor that binds to the E2 promoter of Ad5 and was therefore denoted E2F (Kovesdi et al. 1987). However, other studies failed to identify another target sequence beside the TATA box necessary for *trans*-activation of several viral and cellular promoters by E1a (for review, see Berk et al. 1986). This puzzling subject was reexamined very recently, and evidence was presented that only a particular TATA motif, TATAA, is responsible for the *trans*-activation effect by E1a (Simon et al. 1988). However, the same TATA motifs are present in both *MYC* promoters (P_1 and P_2). Under our experimental conditions using a 10-fold smaller amount of a plasmid expressing E1a with respect to the reporter CAT plasmid in the cotransfection assay, we observed a non-significant 2- to 3-fold enhancement of CAT activity compared to the substantially higher 6- to 25-fold *trans*-activation mediated through the E2F elements (Lipp et al. 1989). Recently, it was shown that the human *FOS* gene is stimulated about sevenfold by E1a, depending on the presence of enhancer upstream sequences (Sassone-Corsi and Borelli 1987). However, another study demonstrated that the *FOS* TATA element is indeed responsive to E1a (Simon et al. 1988). Sassone-Corsi and Borelli (1987) included a *MYC*-CAT plasmid containing a large promoter fragment (−2325 to +36) in their cotransfection analysis. Although these authors observed a stimulation of CAT activity comparable to our results, no further data demonstrating the specificity of this effect were presented.

Two possible mechanisms are discussed as to how E1a can *trans*-activate: stimulation of the synthesis of a cellular protein or modification of a preexisting factor. Evi-

dence for the latter possibility was presented recently. These data suggest that activation of E2F occurs as a post-translational event (Reichel et al. 1988). It is of particular importance to see whether transcription of the *MYC* gene can be regulated due to modification of E2F by cellular factors with similar activities as E1a. The existence of such an E1a-like factor was proposed for F9 undifferentiated teratocarcinoma cells, because an adenovirus E1a mutant can be complemented in those cells (Imperiale et al. 1984). Interestingly, a binding activity of a cellular protein to the E2 promoter of Ad5 was detected in undifferentiated, but not in differentiated, F9 cells (Reichel et al. 1987). It also was shown that *MYC* mRNA levels decrease when F9 cells are induced to differentiate (Campisi et al. 1984). Recently, on the basis of nuclear run-on analyses, it was proposed that a post-transcriptional control mechanism might be responsible for the observed down-regulation (Dony et al. 1985; Dean et al. 1986). Currently we are testing whether the binding pattern of E2F to the *MYC* promoter or its activity is changing during differentiation of F9 teratocarcinoma cells.

Methods

Cells

Monolayer cultures of HeLa cells and 293 cells (Graham et al. 1977) were grown in Dulbecco's modified Eagle's medium, and suspension cultures of HeLa cells in Spinner medium supplemented with 5% fetal calf serum. BL64 cells (Hartl and Lipp 1987) were cultivated in RPMI medium supplemented with 10% fetal calf serum.

Preparation and purification of nuclear extracts

Nuclear extracts were prepared by the method of Dignam et al. (1983). Purification of crude extracts by DEAE-cellulose and Mono-Q chromatography has been described recently (Meisterernst et al. 1988a). Fractions were monitored by gel retardation assays using end-labeled synthetic oligonucleotides or isolated DNA fragments.

Gel retardation assays

Gel retardation assays were performed as described by Schneider et al. (1986). Briefly, a reaction volume of 40 μ l usually contained 10–100 fmole of double-stranded oligonucleotides or DNA fragment end labeled by Klenow fragment of *Escherichia coli* DNA polymerase I or T4 polynucleotide kinase, 2 μ g poly[d(A-T)], and up to 6 μ g of protein. Extracts were preincubated with the carrier DNA for 10 min on ice, and the binding reaction was carried out at room temperature for 5 min. DNA–protein complexes were separated from free DNA, either on 5% polyacrylamide gels if labeled fragments were tested or on 11% polyacrylamide gels for oligonucleotides.

Methylation interference footprint analysis

The 188-bp *NotI/NaeI* fragment of *MYC* (–141 to +47 with respect to P₂) was isolated, methylated partially with dimethylsulfoxide (DMSO), as described by Maxam and Gilbert (1977), and incubated with crude nuclear extracts of 293 and BL64 cells as described above. After separation of DNA–protein complexes on a 5% polyacrylamide gel, complex bands and free

DNA were identified by short exposure to an X-ray film, cut out from the gel, melted into a 0.8% agarose gel, and transferred electrophoretically to NA45 paper (Schleicher & Schuell). Then the DNA was eluted and purified as described by the manufacturer, treated with piperidine, and analyzed on a 5% urea sequencing gel.

Plasmids

For cloning of the 5'-deletion constructs of the *MYC* promoter, a special CAT vector, pUC-CAT-SA, was constructed: A 25-bp synthetic oligonucleotide

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ctagaGCTCCAGCAGCCTCCCGCGA
tCGAGGTCGTGCGAGGGCGCTctag
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containing the original splice acceptor site of exon two and a 5' *XbaI* and a 3' *BglII* site, was inserted into the *XbaI* and *BglII* site of vector pUC-CAT2 (Lipp et al. 1987). To construct p2-2489, the 3507-bp *HindIII-XbaI* fragment cloned from the nontranslocated *MYC* allele of cell line BL64 (Hartl and Lipp 1987) was inserted into vector pUC-CAT-SA, digested with *HindIII* and *XbaI*. The deletion constructs p2-104 to p2-20 were obtained by opening a pUC12-derived plasmid carrying the 3507-bp *HindIII-XbaI* fragment with *SmaI*, followed by *Bal31* digestion, fill-in reaction, and ligation after adding *HindIII* linkers. This mixture was digested with *HindIII* and *XbaI*; fragments of different size classes were isolated from an agarose gel and ligated into pUC-CAT-SA. Deletion constructs within P₂ were identified by colony hybridization with oligonucleotide myc-7/8 and increasing stringency during the washing procedure.

DNA transfection and CAT assays

Transfection with a total amount of 20 μ g superhelical plasmid DNA (pUC12 was used as carrier) per 10⁶ cells in 9-cm tissue culture dishes was performed by the calcium phosphate coprecipitation technique, and determination of CAT activity was carried out essentially as described previously (Lipp et al. 1987). The amounts of the monoacetylated forms of chloramphenicol were measured and computed with a thin-layer scanner (TCL-510, Berthold, Bad Wildungen).

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K Thalmeier, H Synovzik, R Mertz, et al.

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