Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 3594–3598, May 1989
Biochemistry

E1A-dependent trans-activation of the human *MYC* promoter is mediated by the E2F factor

SCOTT W. HIEBERT*, MARTIN LIPP†, AND JOSEPH R. NEVINS*

*Howard Hughes Medical Institute, Department of Microbiology and Immunology, Duke University Medical Center, P.O. Box 3054, Durham, NC 27710; and †Institut fuer Biochemie, der Ludwig-Maximilians-Universitaet, Karlstrasse 23, D-8000 Munich 2, Federal Republic of Germany

Communicated by W. K. Joklik, February 13, 1989

ABSTRACT **E2F is a cellular transcription factor that binds to two sites in the adenovirus E2 promoter. Previous experiments have implicated E2F in the E1A-dependent trans-activation of the E2 gene since levels of active E2F increase markedly during adenovirus infection in parallel with the increase in E2 transcription, and an E2F binding site can confer E1A inducibility to a heterologous promoter. Here we show that E2F binds to two sequence elements within the P2 promoter of the human *MYC* gene which are within a region that is critical for promoter activity. The *MYC* promoter can be trans-activated in an E1A-dependent manner and site-directed mutagenesis demonstrates that these E2F elements are essential for trans-activation. Finally, we also find that adenovirus infection of quiescent cells results in a stimulation of the endogenous *MYC* gene. We conclude that the activation of the E2F factor, which is likely responsible for the activation of viral E2 transcription, is also responsible for the E1A-dependent induction of *MYC* transcription.**

Trans-activation of adenovirus transcription, dependent on the action of the 289-amino acid E1A gene product, clearly involves the use of cellular transcription factors. A variety of cellular proteins have been identified that interact with the sequences within viral promoters that are essential for transcription (1–3). Of particular interest with respect to the E1A-dependent transcription activation process is a factor termed E2F that appears to mediate the activation of the viral E2 promoter. Several lines of evidence support the idea that E2F is important for E2 transcription activation. E2F binds to two sequence elements in the promoter (4, 5) that are critical for transcription (6–9), E2F levels increase 30- to 50-fold during a virus infection dependent on expression of the 289-amino acid E1A gene product (4, 10), and an E2F binding site can confer E1A inducibility to a heterologous promoter (11).

Since the E2F factor is of cellular origin and indeed can be regulated in a cellular context independent of the viral E1A gene (12), we presume that it must be responsible for the control of transcription of certain cellular genes. Recent experiments have demonstrated that in transient transfection assays, expression of the E1A gene products induces the transcription of the human *MYC* gene (13), a finding of particular interest given the connection between E1A function and *MYC* function in the process of oncogenesis (14, 15). Here we show that the E1A-dependent trans-activation of the *MYC* promoter is mediated through the E2F factor.

METHODS

Cells and Virus. Suspension cultures of HeLa cells, grown in minimal essential medium (Joklik-modified) with 5% calf serum, were used for the preparation of nuclear extracts. Transfection assays used monolayer cultures of HeLa cells (S3) grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. Human 143 cells were grown in DMEM with 5% fetal calf serum. The growth and purification of wild-type (wt) adenovirus 5 (Ad5) and the E1A-deficient adenovirus mutant dl312 have been described (16).

E2F Preparation. E2F was prepared from nuclear extracts (17) of adenovirus-infected HeLa cells as described (5). Briefly, nuclear extract was applied to a heparin-agarose column, washed with 250 mM KCl, and then E2F eluted with a KCl gradient of 0.25–1 M. The peak of E2F activity, eluting at ≈450 mM KCl, was pooled, dialyzed, and applied to a FPLC Mono Q column. The column was eluted with a 100–550 mM KCl gradient and E2F activity was recovered at ≈350 mM KCl. Active fractions were pooled, dialyzed, and used for most of the binding assays.

DNA Binding Assays. *MYC* and *MYC* mutant promoter oligonucleotides −70 to +7 were cloned between the *Xba* I and the *Bgl* II restriction sites 5′ to the chloramphenicol acetyltransferase (CAT) gene in the plasmid pCAT3M. A 95-nucleotide DNA fragment was excised from each plasmid with *Hin*dIII and *Bgl* II and used for competition studies as described (5). Oligonucleotides containing single E2F binding sites (see Fig. 1) were cloned into the *Xba* I/*Bgl* II sites of the pGEM-2 plasmid and an *Eco*RI/*Pvu* II DNA fragment was used in competition assays. Preparation of the E2 promoter ^{32}P-labeled DNA probes and E2 competitor DNA was done as described (5). Mobility shift assays were performed with 0.1 ng of probe in a 25-μl reaction mixture. The products were separated on a 4% polyacrylamide gel in 0.25× TBE as described (5). A preparation of E2F purified through the Mono Q step as described above was used for the assays.

Methylation Interference Assays. Affinity-purified E2F was prepared by passing partially purified E2F (Mono Q fraction) over a nonspecific DNA-agarose column followed by chromatography over an E2F-specific DNA-agarose affinity column as described (18). E2 and *MYC* probes were 3′-end-labeled with ^{32}P and methylated with dimethyl sulfate. Binding assays used affinity-purified E2F. Methylated DNA complexed with E2F was separated from methylated DNA not complexed by a mobility shift gel. The free probe and bound probe were eluted from the gel, cleaved with piperidine, and analyzed in an 8% sequencing gel (19, 20).

Trans-Activation Assays. pCAT3M-*MYC* plasmids were transfected by the calcium-phosphate procedure into HeLa monolayers, previously infected with 2000 virus particles per cell of Ad5 or dl312 and maintained in the presence of cytosine arabinoside 25 μg/ml as described (21). CAT assays were performed as described (22).

Infection of Human 143 Cells and Assay of *MYC* RNA. Confluent monolayers of human 143 cells were maintained in DMEM with 0.5% fetal calf serum prior to infection with 5000 virus particles per cell of wt Ad5 (16). Primer-extension

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: wt, wild type; Ad5, adenovirus 5; CAT, chloramphenicol acetyltransferase.

analysis of the *MYC* mRNA species was performed with a synthetic oligonucleotide complementary to nucleotides 49–68 of the *MYC* mRNA.

RESULTS

Two E2F Binding Sites in the *MYC* Promoter. Deletion analysis of the *MYC* promoter has demonstrated that sequences within 66 nucleotides 5′ of the P2 start site are essential for *MYC* transcription (23, 24). Interestingly, the sequence immediately 3′ to the −66 border (GCGGGAAA) displays a high degree of similarity (only a single G/C difference) with two promoter sequences previously shown to be involved in the induction of transcription from the E2 gene of adenovirus (see Fig. 1*A*). These E2 sequences, as well as two similar sequences in the enhancer of the E1A gene, interact with a cellular transcription factor termed E2F, which is activated upon adenovirus infection and whose activation is dependent on expression of the 289-amino acid E1A gene product (4, 10). In addition to the sequence at −65 to −58 of the *MYC* promoter, a similar sequence is found at −42 to −35. This sequence differs at two positions (GATCGCGC) from the E2 promoter E2F site. The sequence similarity of the *MYC* sequences to the E2F binding sites in the adenovirus genome (Fig. 1*B*) along with the spatial relationship of the two sites as compared with the E2 promoter (see Fig. 1*A*) prompted us to examine whether E2F binds to the *MYC* promoter and is involved in the transcription of the *MYC* gene.

To measure an interaction of the E2F factor with this region of the *MYC* promoter, complementary oligonucleotides were synthesized encompassing nucleotides −70 to +7 of the *MYC* P2 promoter along with oligonucleotides containing site-specific mutations in the two putative E2F binding sites (Fig. 1*C*). Using the mobility shift assay, the ability of a partially purified preparation of E2F to bind to these sequences was determined by their ability to compete for binding of E2F with radioactively labeled E2 promoter DNA (Fig. 2*A*). Although the *MYC* promoter region does not compete as well as the homologous E2 competitor, E2F does clearly interact with the *MYC* sequences, whereas the mutations in the E2F binding sites eliminate binding (Fig. 2). Because the two E2F binding sites in the *MYC* promoter are not homologous, we compared the relative affinity of each site for E2F. Complementary oligonucleotides encoding each of the *MYC* E2F binding sites, as well as the E2F site from the E2 promoter and an E2F site with a single point mutation (Fig. 1*D*) were synthesized, cloned, and used in mobility shift competition assays. It should be noted that the single sites compete less efficiently than the double site, presumably because of a lack of cooperation. The distal *MYC* site (−65 to −58) competes as well as the E2 sequences for binding of E2F (Fig. 2*B*, compare E2 and *MYC* II), indicating that it is a fully competent E2F binding site. In contrast, the proximal *MYC* site (*MYC* I) binds considerably less efficiently (compare *MYC* I with *MYC* II), although an interaction is evident when compared with the mutant competitor, which does not compete for E2F binding (E2 null).

We have also examined the interaction of a preparation of highly purified E2F with the *MYC* promoter by methylation interference. Highly purified E2F was prepared by DNA affinity chromatography, yielding a preparation that is nearly homogeneous for a 54-kDa polypeptide and that is free of other detectable DNA binding proteins (18). This preparation of E2F binds strongly to both sites in the E2 promoter (Fig. 3). An analysis of E2F binding to the *MYC* promoter revealed an interaction with the strong distal site (site II) but no evidence of an interaction at the proximal site (site I). This result clearly documents the interaction of E2F with the E2-like element at −65/−56 (site II) and, consistent with the results of Fig. 2, suggests that site II is a much stronger binding site for E2F than site I.

E1A Trans-Activation Requires E2F Binding. Previous experiments have shown that the *MYC* promoter is activated in an E1A-dependent fashion but did not define sequences essential for this activation (9). More recent experiments

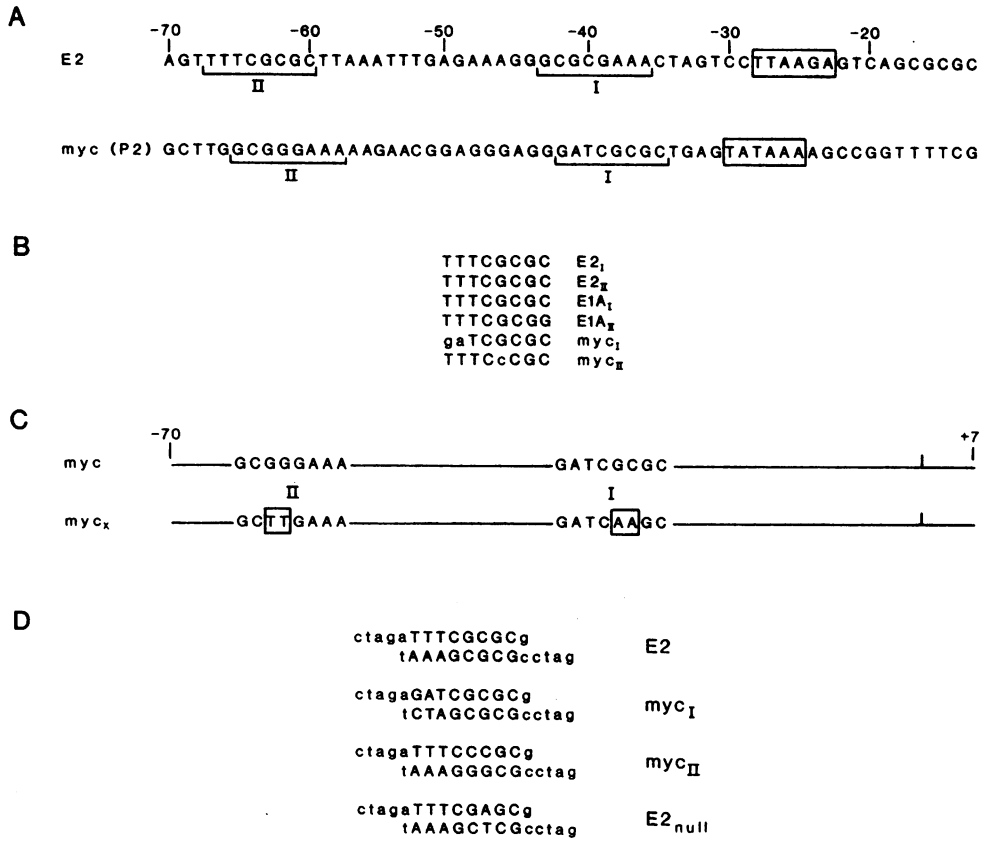

FIG. 1. E2F binding sites in the human *MYC* and the adenovirus E2 promoters. (*A*) Sequence of the human *MYC* promoter and the adenovirus E2 promoter. E2F sites in the E2 promoter are indicated as well as the pseudo-TATA element. Related sequences in the human *MYC* promoter are indicated. (*B*) Sequence of known E2F binding sites in the E2 promoter and the E1A enhancer as well as the potential *MYC* sites. (*C*) Synthesized oligonucleotides containing the normal human *MYC* promoter and a mutant with alterations in each of the proposed E2F binding sites. (*D*) Synthesized oligonucleotides representing an E2–E2F binding site, the two potential *MYC* E2F sites, and a point mutation suggested to be null in DNA binding.

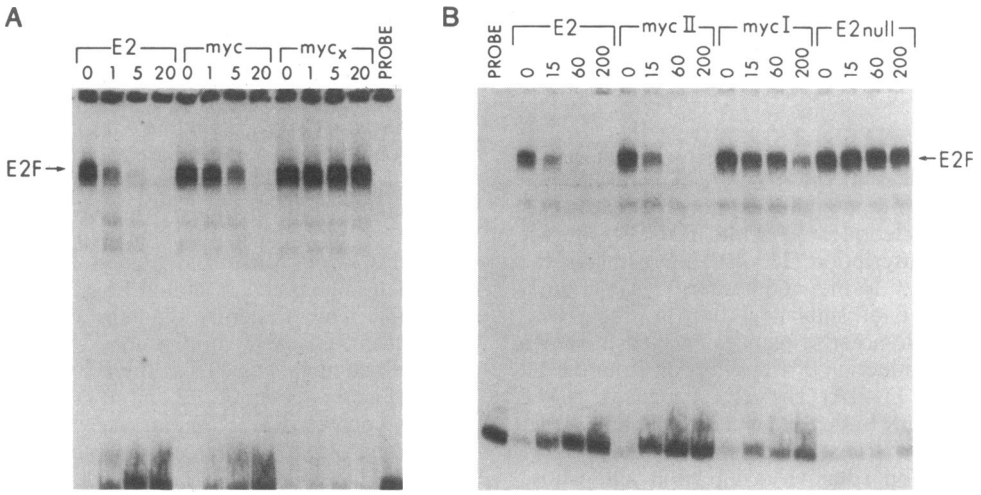

FIG. 2. Competition assays for the interaction of E2F with the human *MYC* promoter. Mobility shift assays using a partially purified E2F preparation and a labeled E2 promoter probe were performed in the presence of the competitor indicated above each set of lanes. The amount of specific competitor DNA used is shown (in ng) above each lane. (*A*) Competition using DNA fragments (see Fig. 1) from the Ad5 E2 promoter (nucleotides −98 to −21), the *MYC* promoter (−70 to +7), or the *MYC* promoter containing mutations within the E2F binding sites (MYC_X). Lane Probe, binding assay without added E2F. E2F, position of the E2F–DNA complex. (*B*) Oligonucleotides containing a single E2F binding site from the E2 promoter (nucleotides −67 to −60), the *MYC* promoter (*MYC* II, nucleotides −65 to −58; *MYC* I, nucleotides −42 to −35), and a mutant E2F binding site (E2 null, see Fig. 1) were cloned and a 95-base-pair DNA fragment was used in mobility shift competition assays.

have demonstrated that E1A-dependent activation of *MYC* utilizes the P2 promoter (25). To address the role of the E2F binding sites within P2, the *MYC* promoter and the *MYC* promoter containing mutant E2F binding sites used earlier for mobility shift competition assays (Fig. 1*B*) were cloned 5′ to the CAT gene and these plasmids were then assayed for E1A-dependent trans-activation. The two plasmids were transfected into HeLa cells that were infected with either wt adenovirus or the mutant dl312. As shown in Fig. 4*A*, the wt *MYC* promoter was indeed induced dependent on E1A expression, whereas the mutation of two nucleotides within each of the two E2F binding sites severely impaired the activation of the *MYC* promoter.

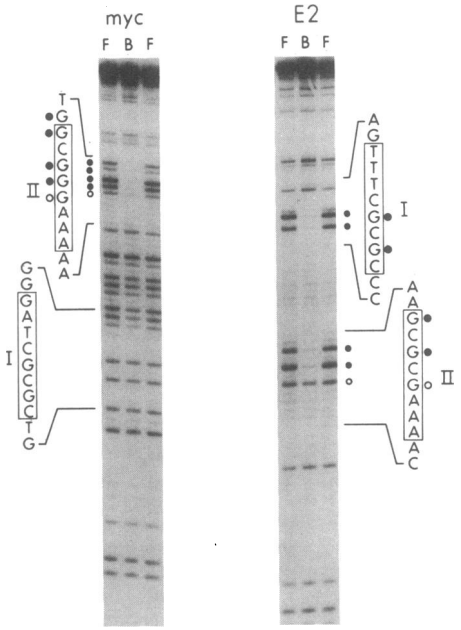

FIG. 3. Methylation interference analysis of the interaction of affinity-purified E2F with the E2 and *MYC* promoters. Sequences of the E2F binding sites are shown. Methylated G residues that prevent E2F binding are indicated by solid circles. Methylated residues showing partial inhibition are indicated by open circles. Lanes F, free DNA; lanes B, bound DNA.

Two additional constructs, harboring the mutations in either the distal site alone or the proximal site alone, were assayed to address the relative importance of the two E2F sites. As shown in Fig. 4*B*, the distal site mutation ($MYC_{x\text{-}II}$) was as defective as the double site mutant (MYC_x). The proximal site mutant ($MYC_{x\text{-}I}$) was also impaired but not as severely as the distal site mutant. Quantitation of the CAT activity in this experiment revealed that the wt *MYC* promoter activity was increased 57-fold in an Ad5 infection relative to a dl312 infection. In contrast, the double site mutant reduced the response to a 3-fold activation as did the single mutation in the distal site. The mutation of the proximal site was reduced to a 7-fold activation. We conclude that in addition to the demonstration that the cellular E2F factor does bind to the *MYC* promoter, we have shown that the sequences to which E2F binds are important for E1A-dependent activation. It would also appear that although the distal site (site II) is sufficient for activation, the proximal site does contribute since a mutation of this site alone did impair the response to E1A. It is also interesting to note that the murine *MYC* promoter, while containing an exact copy of site II of the human promoter, does not contain the second binding site (site I) (26), once again suggesting that, depending on the context, two sites may not be necessary for activation.

Activation of the Endogenous *MYC* Gene. The assays presented thus far demonstrate that E2F can bind to the *MYC* promoter and that this allows E1A-dependent trans-activation in a transfection assay. To determine whether E1A might activate the endogenous *MYC* gene, we have assayed for activation of the human *MYC* gene upon infection with adenovirus. For this experiment, we used human 143 cells that had been rendered quiescent by serum starvation. We found this to be essential to observe an effect on adenovirus infection, presumably due to an already activated state of the *MYC* gene in proliferating cells. As shown in Fig. 5, assay for *MYC* RNA by primer extension revealed a low level in the mock-infected cells but then a 3- to 4-fold increase upon adenovirus infection. We therefore conclude that the endogenous *MYC* gene is a target for trans-activation and, based on the analyses described in this paper, this is likely brought about through the E2F factor.

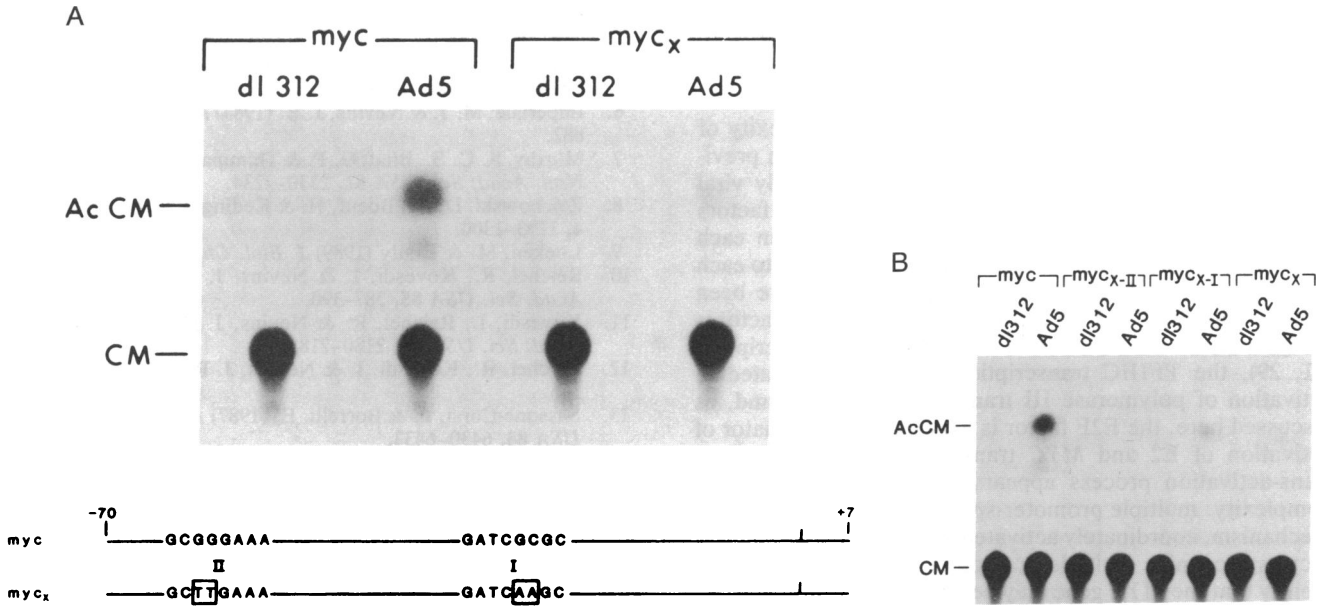

FIG. 4. Effect of E2F binding site mutations on E1A-dependent induction of *MYC* transcription. The wt and mutant promoters were linked to the CAT gene and these constructs were introduced into monolayer HeLa cultures previously infected with either wt (Ad5) or dl312 adenovirus. The level of induced CAT activity was determined by the acetylation of [^{14}C]chloramphenicol (26). Ac-CM, acetylated chloramphenicol; CM, chloramphenicol. (*A*) Assays of the wt *MYC* promoter (myc) and the double E2F site mutant promoter (myc_x). The structure of the two constructs is depicted at the bottom. Each is fused to the CAT gene at +7. (*B*) Assays of the wt promoter (myc), the double site mutant (myc_x), and the two single site mutants ($myc_{x\text{-}I}$ and $myc_{x\text{-}II}$). The single site mutants are wt at one site and contain the same mutation as shown for the double mutant at the indicated site.

DISCUSSION

The E2F factor was originally identified as a DNA binding activity that recognized two specific sequence elements in the adenovirus E2 promoter and that increased dramatically upon adenovirus infection. Given the cellular origin of the factor, we presumed that there were cellular promoters that utilized and were regulated by the factor. The experiments described in this report clearly demonstrate that the E2F factor can bind to the *MYC* promoter and that the E1A-dependent activation of the *MYC* promoter requires the E2F binding sites. These findings, together with the fact that an adenovirus infection results in a 30- to 50-fold increase in E2F activity (4), strongly argue for a role of E2F activation in the E1A-dependent stimulation of the *MYC* promoter. Furthermore, the analysis of the expression of the *MYC* gene in virus-infected human 143 cells demonstrates that this activation is not limited to the transfection assays. Rather, under the appropriate conditions of cell quiescence, in which the activity of the *MYC* gene is reduced to a low basal level, there is an activation of *MYC* gene expression upon infection with adenovirus. We thus conclude that the E1A-dependent control of E2F activity, which is clearly important for the activation of the transfected *MYC* promoter, also has an influence on cellular gene activity.

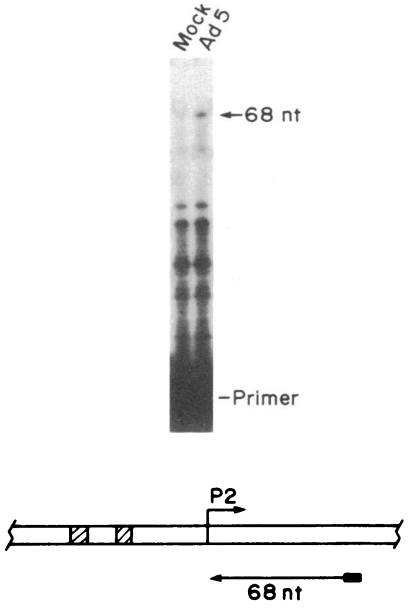

FIG. 5. Activation of the endogenous human *MYC* gene during adenovirus infection of 143 cells. A quiescent culture of 143 cells was mock-infected or infected with Ad5 for 10 hr. RNA was extracted and equal amounts of poly(A)$^+$ RNA were analyzed by primer extension (27). nt, Nucleotides.

The fact that E2F is a cellular factor raises the broader question of whether this factor is involved in the normal regulation of *MYC* transcription apart from a virus infection. Recent experiments have shown that the activation of E2F is a posttranslational event (10) and assays of E2F activation in cell-free systems have demonstrated a role for phosphorylation in the activation (28). Various lines of evidence suggest that it is unlikely that the E1A gene product or another early viral protein is the kinase that mediates the activation; rather, a more likely scenario would involve the activation of a cellular kinase system that then leads to the increase in active E2F factor. We would speculate that such a kinase may be involved in the normal regulation of the E2F factor in the uninfected cell and, given the results described here, may function normally in the control of *MYC* transcription. In this context, two observations are of interest. First, previous analyses of *MYC* promoter sequences have shown that the distal E2F binding site is important for *MYC* promoter activity, independent of E1A control (23, 24). Second, our recent experiments have demonstrated that E2F levels increase upon serum stimulation of quiescent NIH 3T3 cells with kinetics similar to that shown for the activation of *MYC* transcription (M. Mudryj, S.W.H., and J.R.N., unpublished data). It is thus possible that E2F is in fact involved in the normal control of *MYC* transcription, dependent on the

proliferative state of the cell. The activation as a function of E1A expression might then simply constitutively activate the E2F factor, thus short-circuiting this normal control mechanism.

These results also provide insight into the complexity of the network of E1A-mediated trans-activation. From previous work, it is clear that the trans-activation of early viral transcription must involve multiple promoter specific factors since no single sequence element could be found in each regulated promoter and indeed no single factor binds to each promoter (1, 2). Furthermore, multiple factors have been directly implicated in E1A control. A TATA binding factor is likely involved in activation of E1B and hsp70 transcription (21, 29), the TFIIIC transcription factor is implicated in activation of polymerase III transcription (30–32), and, as discussed here, the E2F factor is very likely the mediator of activation of E2 and *MYC* transcription. Thus, the E1A trans-activation process appears to exhibit two levels of complexity: multiple promoter-specific factors are, by some mechanism, coordinately activated and at least some of these factors recognize multiple promoters. In addition, the possibility that the *MYC* gene may be activated by E2F suggests a further complexity to the system. Indirect evidence, including the capacity to bind to DNA (33–35) and the fact that a MYC–LEXA fusion protein can stimulate transcription (36), suggests the possibility that the MYC protein is a cellular transcription factor. Thus, the E1A-dependent activation of the various factors described above, including E2F, would not only activate transcription of the genes to which these factors bind but would also result in the production of new transcription factors (i.e., MYC protein), which would then presumably activate an additional set of genes.

Finally, these results also provide molecular detail to the link between E1A and *MYC* and the generation of a transformed cell. E1A and *MYC* can establish immortalized cell lines and each, in conjunction with the *RAS* oncogene, can transform cells to an oncogenic state (14, 15). One obvious possibility arising from these and past studies is a transcriptional activation link between *MYC* and E1A involving the E2F factor such that at least one role for E1A in the process is in the activation of *MYC* transcription. An analysis of the events leading to *MYC* activation, in relation to the E2F factor, should help to clarify this possibility.

We thank Laszlo Jakoi for providing the purified E2F. This work was supported by a Grant GM26765 from the National Institutes of Health. S.W.H. is a Fellow of the American Cancer Society.

1. Berk, A. J. (1986) *Annu. Rev. Genet.* **20,** 45–79.
2. Nevins, J. R. (1987) *Microbiol. Rev.* **51,** 419–430.
3. Jones, N. C., Rigby, P. W. J. & Ziff, E. B. (1988) *Genes Dev.* **2,** 267–281.
4. Kovesdi, I., Reichel, R. & Nevins, J. R. (1986) *Cell* **45,** 219–228.
5. Yee, A. S., Reichel, R., Kovesdi, I. & Nevins, J. R. (1987) *EMBO J.* **6,** 2061–2068.
6. Imperiale, M. J. & Nevins, J. R. (1984) *Mol. Cell. Biol.* **4,** 875–882.
7. Murthy, S. C. S., Bhat, G. P. & Thimmappaya, B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2230–2234.
8. Zajchowski, D. A., Boeuf, H. & Kedinger, C. (1985) *EMBO J.* **4,** 1293–1300.
9. Loeken, M. & Brady (1989) *J. Biol. Chem.*, in press.
10. Reichel, R., Kovesdi, I. & Nevins, J. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 387–390.
11. Kovesdi, I., Reichel, R. & Nevins, J. R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2180–2184.
12. Reichel, R., Kovesdi, I. & Nevins, J. R. (1987) *Cell* **48,** 501–506.
13. Sassone-Corsi, P. & Borrelli, E. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6430–6433.
14. Land, H., Parada, L. F. & Weinberg, R. A. (1983) *Nature (London)* **304,** 596–602.
15. Ruley, H. E. (1983) *Nature (London)* **304,** 602–606.
16. Nevins, J. R. (1980) *Methods Enzymol.* **65,** 768–785.
17. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
18. Yee, A. S., Raychaudhuri, P., Jakoi, L. & Nevins, J. R. (1989) *Mol. Cell. Biol.* **9,** 578–585.
19. Staudt, L. M., Singh, H., Sen, R., Wirth, T., Sharp, P. A. & Baltimore, D. (1986) *Nature (London)* **323,** 640–643.
20. Raychaudhuri, P., Rooney, R. & Nevins, J. R. (1987) *EMBO J.* **6,** 4073–4081.
21. Simon, M. C., Fisch, T. M., Benecke, B. J., Nevins, J. R. & Heintz, N. (1988) *Cell* **52,** 723–729.
22. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051.
23. Lipp, M., Schilling, R., Wiest, S., Laux, G. & Bornkamm, G. W. (1987) *Mol. Cell. Biol.* **7,** 1393–1400.
24. Nishikura, K. (1986) *Mol. Cell. Biol.* **6,** 4093–4098.
25. Lipp, M., Shilling, R. & Bernhardt, G. (1989) *Oncogene*, in press.
26. Stanton, L. W., Fahrlander, P. D., Tesser, P. M. & Marcu, K. B. (1984) *Nature (London)* **310,** 423–425.
27. Eisenberg, S. P., Coen, D. M. & McKnight, S. L. (1985) *Mol. Cell. Biol.* **5,** 1940–1947.
28. Bagchi, S., Raychaudhuri, P. & Nevins, J. R. (1989) *Proc Natl. Acad. Sci. USA* **86,** in press.
29. Wu, L., Rosser, D. S. E., Schmidt, M. C. & Berk, A. (1987) *Nature (London)* **326,** 512–515.
30. Hoeffler, W. K. & Roeder, R. G. (1985) *Cell* **41,** 955–963.
31. Gaynor, R. B., Feldman, L. T. & Berk, A. J. (1985) *Science* **230,** 447–450.
32. Berger, S. L. & Folk, W. R. (1985) *Nucleic Acids Res.* **13,** 1413–1418.
33. Persson, H. & Leder, P. (1984) *Science* **225,** 718–721.
34. Donner, P., Greizer-Wilke, I. & Moelling, K. (1982) *Nature (London)* **296,** 262–266.
35. Watt, R. A., Schatzman, A. M. & Rosenberg, M. (1985) *Mol. Cell. Biol.* **5,** 448–456.
36. Lech, K., Anderson, K. & Brent, R. (1988) *Cell* **52,** 179–184.