were performed on an EPICS 753 flow cytometer (Coulter Corp., Hialeah, FL), using UV laser excitation (351.1–363.8 nm, 70 mW) and simultaneous detection of fluorescence emission at 400 nm ± 20 nm and 480 ± 20 nm. The ratio of 480/400 fluorescence was recorded and displayed as a function of time. After establishing a stable baseline reading for each sample, either human recombinant CSF-1 (final concentration = 0.4 μg/ml), porcine PDGF (final concentration = 0.1 μg/ml) or the calcium ionophore, ionomycin (final concentration = 8.3 μg/ml; Behring Diagnostics, San Diego) was injected and sampling was continued. A shift in the INDO-1 fluorescence emission spectrum accompanies binding to calcium ions, resulting in a reduction of the recorded fluorescence ratio corresponding to the increase in intracellular calcium. The mean fluorescence ratio was computed as a function of time after factor addition.

Acknowledgements

We thank John Kayoma for excellent technical assistance, Dr Steven Clark of Genetics Institute, Cambridge, MA, for providing purified human recombinant CSF-1 and Dr E.Richard Stanley for providing BAC1.2F5 cells. This work was supported in part by NIH grants CA-01429 (J.R.D.) and CA-47064 (C.J.S.), by Cancer Center Core Grant CA-21765 and by the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital.

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Received on May 29, 1989; revised on July 10, 1989

The EMBO Journal vol.8 no.11 pp.3351 – 3355, 1989

Suppression of MHC class I gene expression by N-*myc* through enhancer inactivation

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Communicated by P.Borst

Amplification of the N-*myc* oncogene in human neuroblastoma is associated with increased metastatic ability. We previously found that over-expression of N-*myc* in rat neuroblastoma tumor cells causes a dramatic reduction in the expression of MHC class I mRNA. We show here that two distinct elements in the promoter render the MHC class I genes susceptible to N-*myc*-mediated suppression, one of which was identified as the MHC class I gene enhancer. Our data indicate that elevated N-*myc* expression is associated with reduced binding of a transcription factor that activates this enhancer. As a result, the activity of the MHC class I gene enhancer is greatly diminished. Elevated expression of the N-*myc* oncogene in human neuroblastomas and murine pre-B lymphoid lines also correlated with reduced factor binding to the MHC class I gene enhancer. Thus, an important effect of N-*myc* may be to impair the function of certain cellular enhancers by altering the levels of their cognate binding proteins.

Key words: enhancer/MHC class I antigens/N-*myc*/transcription factors

Introduction

The mechanism by which nuclear oncogenes act to transform cells is poorly understood. Most of our knowledge concerning their mode of action derives from studying the nuclear oncogenes of DNA tumor viruses. For example, the E1A gene products of andenovirus have the ability to both activate and suppress the expression of a number of genes (Jones and Shenk, 1979; Nevins, 1982; Schrier *et al.*, 1983; Borelli *et al.*, 1984; Hen *et al.*, 1985; Velcich and Ziff, 1985; Stein and Ziff, 1987). These data suggest that nuclear oncogenes alter the expression of certain key cellular genes whose aberrant expression is ultimately responsible for the transformed phenotype. The adenovirus E1A transforming functions have been shown to correlate more closely with the ability to repress gene expression than with transactivation (Lillie *et al.*, 1986; Velcich and Ziff, 1988).

We have previously shown that transfection of the N-*myc* gene in the rat neuroblastoma cell line B104 drastically reduces the cell surface expression of MHC class I antigens (Bernards *et al.*, 1986). The decrease in cell surface expression was shown to also be accompanied by a proportional decrease in the level of the mRNA encoding MHC class I antigens in the N-*myc*-transfected cells. This observation would appear to explain the phenotype of advanced childhood neuroblastomas in which N-*myc* gene amplification is accompanied by greatly reduced levels of MHC class I antigens (Bernards *et al.*, 1986). N-*myc* shares the ability to suppress MHC class I antigen expression with the c-*myc* oncogene, which has been shown to be responsible for reduced expression of MHC class I in human melanoma (Versteeg *et al.*, 1988).

We report here the further study of the interaction between the genes encoding the MHC class I antigens and the N-*myc* protein. Our findings suggest a mechanism by which the N-*myc* oncogene acts to deregulate cellular gene expression.

Results

N-myc represses the MHC class I gene promoter

To study the suppression of MHC class I gene expression by N-*myc*, we initially used a nuclear run-on assay (Greenberg and Ziff, 1984). However, as noted earlier for MHC class I (Vaessen *et al.*, 1987; Ackrill and Blair, 1988; Friedman and Ricciardi, 1988), we found the results of these experiments were variable (not shown). Therefore we analyzed MHC class I promoter function by using plasmids containing 2000, 190 or 138 bp of the murine H2-K^b promoter joined to the bacterial chloramphenicol acetyltransferase (CAT) gene (Figure 1; Kimura *et al.*, 1986; Baldwin and Sharp, 1987). It has previously been shown that transient transfection of these constructs results in the initiation of transcription at the proper start site (Kimura *et al.*, 1986). These plasmids were transfected into cells of the B104 neuroblastoma line and into an N-*myc*-expressing derivative of this cell line, B104 N-*myc*C7. The latter cells express the N-*myc* gene at a level that is comparable to that of a neuroblastoma tumor cell line that carries 50 copies of the N-*myc* gene (Bernards *et al.*, 1986). As an internal control, we co-transfected with pCH110, a plasmid in which the β-galactosidase gene is fused to the SV40 early promoter (Hall *et al.*, 1983). In each experiment CAT activity was normalized to β-galactosidase activity.

The results of these experiments (Figure 1) indicate that both the 2000 and 190 H2-K^b promoter constructs are ~12-fold more active in the parental B104 neuroblastoma cells than in the N-*myc*-transfected B104 cells. This effect closely parallels the 10- to 15-fold reduction in the steady-state mRNA level of the endogenous MHC class I antigen genes in these N-*myc*-transfected neuroblastoma cells (Bernards *et al.*, 1986). We conclude that the major elements required or the N-*myc*-mediated suppression of MHC class I mRNA are located within the first 190 bp of the promoter. Moreover, by comparing the activity of the 138 deletion mutant in the two cell types to that of the −190 construct we conclude that two elements within this 190-bp DNA fragment render the MHC class I gene promoter susceptible to suppression by N-*myc*: one located between bp −190 and

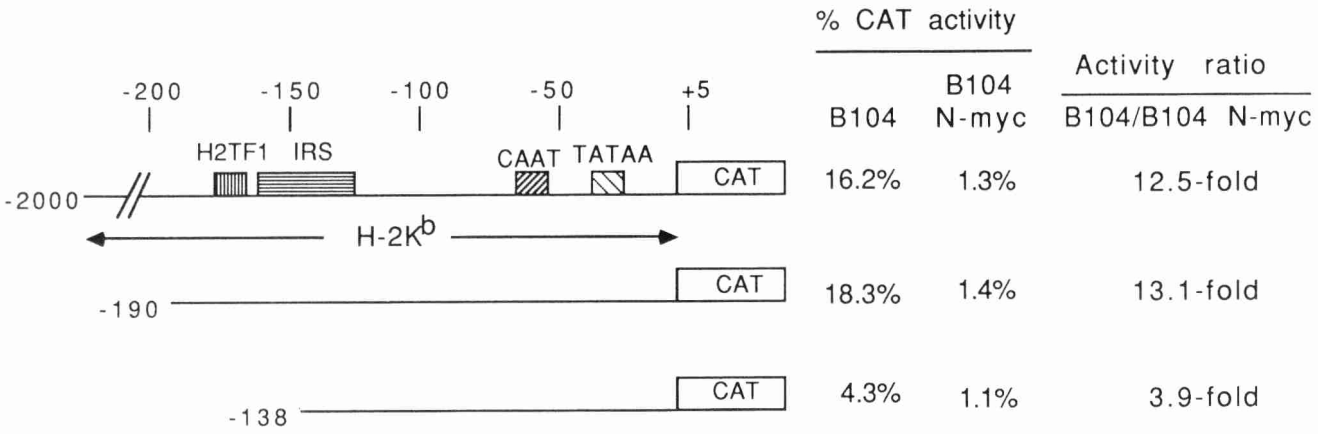

Fig. 1. Activity of H-2 promoter deletion mutants in neuroblastoma cells. B104 neuroblastoma cells and N-*myc*-transfected derivatives were transiently transfected with H-2K^b promoter deletion mutant plasmids linked to the CAT gene. The percentage chloramphenicol conversion obtained with extracts derived from each of the two cell types is indicated for each plasmid and represents an average value obtained in three independent transfection experiments. Values obtained in the three experiments differed by <30% from each other. A schematic representation of the H-2K^b promoter is indicated at the top: IRS, interferon response element; H2TF1, enhancer element and binding site of H2TF1 nuclear factor. The end points of the H-2 promoter deletions are indicated by the number on the left side, indicating nucleotide position upstream from the RNA cap site.

−138 and a second within the first 138 nucleotides of the promoter (Figure 1).

N-myc decreases transcription factor binding

An enhancer element is situated between nucleotides −172 to −160 of the H2-K^b promoter that binds the nuclear factor H2TF1 and possibly closely related factors (Kimura *et al.*, 1986; Baldwin and Sharp, 1987, 1988; Yano *et al.*, 1987). To determine if the H2-K^b enhancer-binding protein was affected by N-*myc*, we used a mobility shift assay to examine nuclear extracts from B104 cells and two N-*myc*-expressing derivatives (Lenardo *et al.*, 1987).

In B104 cells we observed a factor binding to a ^{32}P-labeled wild-type H2-K^b enhancer sequence, but not to the same sequence containing mutations that eliminate the binding of H2TF1 (Figure 2A), nor to the related NF-κB binding motif from the Igκ gene (data not shown). Significantly, a comparison of equal amounts of nuclear protein from parental B104 cells to its two N-*myc*-transfected derivatives revealed that the H2-K^b binding factor was greatly reduced in both N-*myc*-expressing cells (Figure 2A). Diminished binding was not due to non-specific degradation because binding of two other DNA-binding proteins was equivalent in all three extracts (Figure 2B and data not shown). Interestingly, reduced binding was not observed in extracts from a B104 derivative that expressed the adenovirus (Ad)5E1A oncogene at a high level and had normal levels of MHC class I antigens (Figure 2C and D). We therefore conclude that N-*myc* reduces binding of an H2TF1-like factor to the H2-K^b enhancer whereas at least one other nuclear oncogene, Ad5 E1A, is incapable of causing this reduction.

Enhancer suppression by N-myc

Since H2TF1 is required for the activation of the MHC class I gene enhancer, reduced binding of this factor should cause a decline in enhancer function (Kimura *et al.*, 1986; Baldwin and Sharp, 1987). To test this hypothesis, we fused three copies of the H2-K^b enhancer sequence to truncated, enhancerless promoters from either the c-*fos* or the herpes virus thymidine kinase (tk) genes, each linked to the bacterial CAT gene (Gilman *et al.*, 1986; Luckow and Schütz, 1987). To measure the effect of N-*myc* in the MHC class I gene enhancer, these plasmids were transiently transfected in both B104 neuroblastoma cells and one of the N-*myc*-transfected derivatives.

The results of these experiments (Table I) demonstrate that the presence of the MHC class I gene enhancer greatly stimulates the activity of the truncated c-*fos* promoter in the B104 cells, indicating that the H2TF1 binding motif is necessary and sufficient to exert enhancer activity. More importantly, the enhancer was much less active in B104 cells that expressed the N-*myc* gene (Table I, cf. pfosCATΔ56 and pMHCfosCATΔ56). In three independent experiments, the pMHCfosCATΔ56 plasmid was found to be on average 6.6-fold less active in the N-*myc*-tranfected neuroblastoma cells than in the parental B104 cells. This difference was not specific to the *fos* promoter because the H2-K^b enhancer linked to the tk gene promoter was also at least 10-fold less active in the N-*myc*-transfected B104 cells than in the parental B104 cells (Table I, cf. pBLCAT2 and pMHCBLCAT2). No significant difference in CAT activity was seen when the two types of cells were transfected with a plasmid in which the Moloney murine leukemia virus promoter drives the expression of the CAT gene, ruling out an effect of N-*myc* on the bacterial sequences (Table I, pMoECAT). Essentially the same results were obtained with a second, independently derived N-*myc* transfectant (not shown).

It has previously been shown that the mutant H2-K^b enhancer motif used in the gel retardation experiments described above has no biological activity (Baldwin and Sharp, 1987). This suggests that binding of the nuclear factor is required for enhancer activity of this element. In agreement with this, our data indicate that reduced binding of the nuclear factor in N-*myc*-transfected neuroblastoma cells specifically impairs the potency of the H2-K^b enhancer.

Reduced factor binding is also correlated with N-myc expression in other cells

To test whether reduced binding of the H2TF1 transcription factor to the MHC class I gene enhancer was also correlated with high expression of the N-*myc* gene in human neuroblastoma, we analyzed nuclear extracts from human neuroblastoma cell lines. Since H2TF1 is ubiquitously expressed, it is unusual to find a cell type with little or no

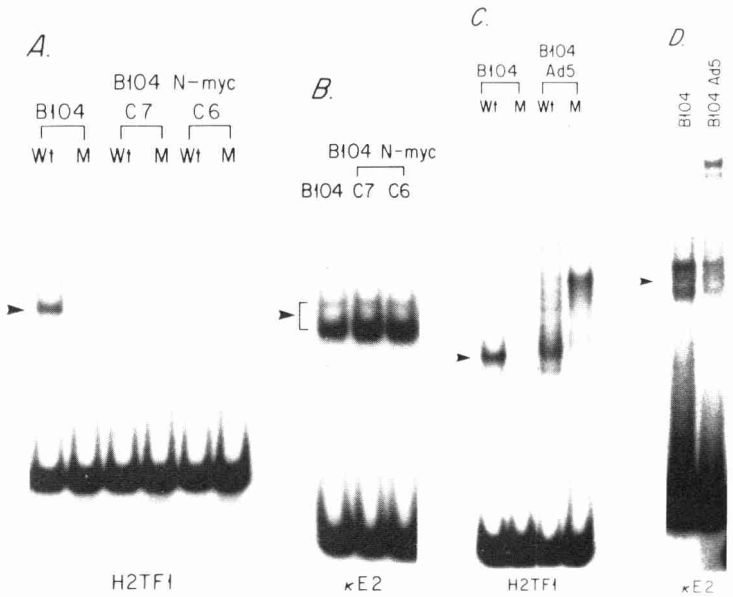

Fig. 2. Levels of an H2TF1-like enhancer activating protein in B104 neuroblastoma cells and N-*myc*-transfected derivatives. (**A**) Shown are the nucleoprotein complexes (indicated by an arrowhead) formed with ^{32}P-labeled oligonucleotides specifying the H-2K^b enhancer sequence (H2TF1) in either wild-type (Wt) or mutant (M) form. Radiolabeled fragments were incubated with 8 μg of nuclear extract from either B104 cells (B104) or from two independently derived lines expressing high levels of N-*myc* and low levels of MHC class I RNA (B104 N-*myc* C6 or C7). (**B**) Nucleoprotein complexes formed with 8 μg of the same nuclear extracts as in (A) incubated with a ^{32}P-labeled oligonucleotide specifying the E2 motif from the Ig κ gene enhancer which binds a factor which is expressed constitutively in all tissues, κE2 (Lenardo *et al.*, 1987). Multiple complexes indicated by the bracket are formed because the oligonucleotide had three copies of the binding site (Murre *et al.*, 1989). (**C**) Nucleoprotein complexes (arrowhead) formed using the same probes as in (A) with 8 μg of nuclear extract derived from B104 neuroblastoma cells or from B104 cells which express high levels of a transfected adenovirus 5 E1A gene (B104 Ad5). (**D**) Complexes formed using the extracts described in (C) incubated with the κ E2 oligonucleotide (κE2) described above.

H2TF1 binding activity (Baldwin and Sharp, 1987). However, consistent with our observations in the rat neuroblastoma cell lines we found that NGP, a human neuroblastoma line that expresses a large amount of N-*myc* and has low levels of MHC class I antigens, exhibited a dramatically reduced amount of the factor that binds to the H2-K^b enhancer compared with SKNMC neuroblastoma cells, which express to N-*myc* and high levels of MHC class I antigens (figure 3A and B) (Bernards *et al.*, 1986).

To ask whether a high level of N-*myc* expression also correlated with reduced binding of H2TF1 in cell types other than neuroblastoma, we examined murine pre-B lymphoid lines. These cells are unique in that they are one of the few cell types in the adult mouse that characteristically express high levels of N-*myc* (Zimmerman *et al.*, 1986). Again, in both 300-18 (Figure 3C and D) and in 38B9 (not shown), specific factor binding to the H2-K^b enhancer was substantially diminished compared with 70Z/3 cells, a late pre-B cell that has no N-*myc* expression (Figure 3) (Zimmerman *et al.*, 1986). We therefore conclude that the inverse relationship between N-*myc* expression and factor-binding to the H2-K^b enhancer extends beyond the rat neuroblastoma model.

Discussion

We show here that two elements in the promoter of the genes encoding the murine MHC class I antigens render these genes susceptible to N-*myc*-mediated suppression. One is located within the first 138 nucleotides from the RNA start site. The second element was identified as an MHC class I gene enhancer and has been the focus of our investigation. Our data indicate that the functional impairment of the MHC class I gene enhancer element by N-*myc* is caused by reduced binding of its cognate activating protein. High levels of N-*myc* in human neuroblastoma cells and pre-B lymphoid lines were also associated with reduced transcription factor binding to the MHC class I gene enhancer element.

We have previously shown that the ability of N-*myc* to suppress MHC class I gene expression is restricted to certain cell types. Thus, high expression of N-*myc* in Rat-1 fibroblasts did not alter the levels of MHC class I antigens in these cells (Bernards *et al.*, 1986). Our preliminary results indicate that N-*myc* does not reduce factor binding to the

Table I. Transient transfection of *fos* and tk promoter constructs in neuroblastoma cells

Plasmid	%CAT activity					
	Expt 1		Expt 2		Expt 3	
	B104	B104 N-*myc*	B104	B104 N-*myc*	B104	B104 N-*myc*
fosCAT	0	0	0	0	0	0
pMHCfosCAT	21	3	62	11	95	17
pBLCAT2	0.3	0.2	0.5	0.7	0.6	0.6
pMHCBLCAT2	8	0.7	11	1	20	2
pMoECAT	20	28	26	27	35	26

B104 cells and B104 N-*myc*-transfected cells were transiently transfected with pfosCATΔ56, pMHCfosCATΔ56, pBLCAT2, pMHCBLCAT2 and pMoECAT. The percentage conversion of chloramphenicol into acetylchloramphenicol obtained with extracts from each of these transfections in three independent experiments is indicated. pfosCATΔ56 contains 56 bp of the c-*fos* promoter linked to CAT (Gilman *et al.*, 1986), pBLCAT2 contains 105 bp of the tk promoter linked to CAT (Luckow and Schutz, 1987), MoECAT contains the Moloney leukemia virus LTR fused to the CAT gene.

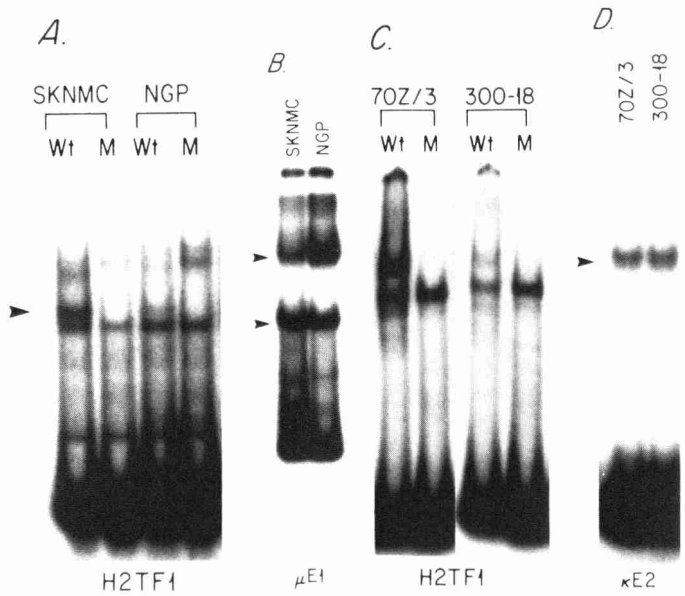

Fig. 3. Levels of the H2TF1-like enhancer factor in human neuroblastomas and in murine pre-B lymphoid lines. (**A**) Wild-type or mutant H2TF1 probe was incubated as described in the legend to Figure 2 with 12 μg of nuclear extract from either the human neuroblastoma cell line SKNMC or the human neuroblastoma cell line NGP. The specific complex is indicated by an arrowhead; the faster and slower migrating complexes that are evident are non-specific binding proteins. (**B**) 12 μg of extracts from the human neuroblastomas described in (A) incubated with a ^{32}P-labeled restriction fragment containing the E1 motif from the immunoglobulin heavy chain enhancer (Lenardo *et al.*, 1987). Complexes are indicated by arrowheads. (**C**) Nucleoprotein complexes obtained either with the wild-type or mutant H2TF1 probe indicated with 2.6 μg of nuclear extracts from 70Z/3 murine pre-B lymphoma cells or from 300-18 murine pre-B cells (Zimmerman *et al.*, 1986). (**D**) The arrowhead indicates the nucleoprotein complex formed with 2.6 μg of the same nuclear extracts described in (C) using the ^{32}P-labeled κE2 oligonucleotide described in the legend to Figure 2.

H2-K^b enhancer in these cells, indicating that the ability of N-*myc* to suppress MHC class I parallels its ability to suppress binding of the H2TF1 transcription factor to the MHC class I enhancer element (M.Lenardo and R.Bernards, unpublished obervations).

Recently, another nuclear oncogene, c-*fos*, has been found to associate in a heterodimeric complex with the transcription factor AP-1 (Rauscher *et al.*, 1988), leading to both positive and negative transcriptional effects (Distel *et al.*, 1987; Chiu *et al.*, 1988; Sassone-Corsi *et al.*, 1988). We found no evidence that N-*myc* inhibits enhancer function by direct association with H2TF1, using either dissociating agents or by mixing nuclear or cytosolic extract from B104 and B104 N-*myc*-transfected cells (unpublished observations). This suggests that the effect of N-*myc* on H2TF1 is fundamentally different from the c-*fos*/AP-1 interaction. One intriguing possibiliy is that N-*myc*, which is believed to be a DNA binding protein (Ramsay *et al.*, 1986), is able to alter the transcription of gene regulatory proteins such as H2TF1.

The adenovirus E1A oncogene can also suppress the enhancers of a number of genes by an as yet unknown mechanism (Borrelli *et al.*, 1984; Hen *et al.*, 1985; Velcich and Ziff, 1985), and the ability of the E1A oncogene to transform has been shown to be more tightly coupled to its ability to inhibit enhancer activity than to transactivation (Lillie *et al.*, 1986; Velcich and Ziff, 1988). This, together with our findings, suggests that impairment of enhancer function is an important means by which both viral and cellular nuclear oncogenes exert their effects on the biological characteristics of transformed cells.

The further study of the interaction between the N-*myc* protein and the H2TF1 transcription factor should provide valuable insights into the function of the *myc* family of oncoproteins.

Materials and methods

Cell culture and transfections

B104 neuroblastoma cells were cultured in Dulbecco's Modified Eagle's medium (DME) supplemented with 10% fetal calf serum. Transient transfections were performed essentially as described by Van der Eb and Graham (1980), with the exception that cells were treated 4 h post-transfection with a solution containing 15% glycerol in phosphate-buffered saline (PBS) for 1.5 min. In each transfection, 100 mm plates of neuroblastoma cells were transfected with a mixture of 30 μg of CAT construct and 30 μg of β-galactosidase plasmid. Cells were harvested 48 h post-transfection for anlaysis of enzyme activity.

Construction of plasmids

An 18 base duplex oligonucleotide 5′-GGCTGGGGATTCCCCATC-3′ containing the palindromic H2TF1 binding site found in the H2-K^b promoter with 4 bp *Sal*I overhanging ends was synthesized. Three copies of this oligonucleotide were inserted in the unique *Sal*I site upstream from the c-*fos* promoter in the plasmid pfosCATΔ56, yielding pMHCfosCATΔ56. To insert three copies of the same oligonucleotide upstream of the thymidine kinase promoter in pBLCAT2, we first inserted three copies of this oligonucleotide in the *Sal*I site of pUC12. This plasmid was then cleaved with restriction endonucleases *Bam*HI and *Hin*dIII and the fragment containing the pUC12 polylinker plus the three copies of the oligonucleotide were inserted in the *Bam*HI and *Hin*dIII digested vector pBLCAT2, yielding pMHCBLCAT2.

Enzyme assays

Two days post-transfection, cells were washed with PBS and incubated for 2 min at 37°C with 2 ml of a solution containing 10 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA. Cells were scraped off the plates, collected by centrifugation and resuspended in 100 μl of 0.25 M Tris pH 8.0. To obtain a cytoplasmic protein fraction, cells were freeze–thawed three times, cellular debris was removed by centrifugation and supernatants were used for enzyme assays. CAT activity was determined as described by Gorman

et al. (1982). Percentages of substrate conversion were determined by using a scintillation counter. β-Galactosidase activity was determined as described by Hall *et al.* (1983). In each experiment CAT activity was normalized to β-galactosidase activity. Two different plasmids were used to measure β-galactosidase activity, pCH110, a plasmid in which the SV40 early promoter is fused to the bacterial β-galactosidase gene, and pRSVβgal, a plasmid in which the Rous sarcoma virus LTR is fused to the β-galactosidase coding region. The results obtained in the CAT assays were essentially identical, irrespective of the type of control plasmid used.

Gel electrophoresis DNA-binding assays

The 70 bp DNA probes containing either wild-type or mutant versions of the H2TF1 site were previously described (Singh *et al.*, 1988). Nuclear extracts were prepared from various cell lines using essentially the procedure of Dignam *et al.* (1983) except that the protease inhibitors leupeptin (0.3 mg/ml) and antipain (0.7 mg/ml) were added. Cytosolic extracts were simultaneously prepared as described previously (Baeuerle and Baltimore, 1988). Mobility shift electrophoresis assays were carried out for 20 min at room temperature using 0.1 ng of radiolabeled probe, 2.5 μg of poly(dI−dC)·poly(dI−dC), and 8 μg of nuclear extract. Binding buffer contained 10 mM Tris−HCl pH 7.5, 50 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, 1 mM DTT and 1 mM EDTA. Electrophoresis was carried out on 4% polyacrylamide gels (30:1 crosslinking) containing 0.25 × TBE (1 × TBE = 89 mM Tris−borate pH 8.3, 1 mM EDTA).

Acknowledgements

We thank Robert Weinberg and David Baltimore, in whose laboratories part of this work was done, for their support, encouragement and critical reading of this manuscript. We also thank Albert Baldwin, Alain Israel, Drs Luckow and Schütz and Michael Gilman for the gift of various plasmids, Sue Blackmon for the synthesis of the oligonucleotides, and Harinder Singh, Albert Baldwin and Phillip Sharp for helpful discussions. This work was supported by a grant from the National Cancer Institute to R.A.Weinberg, a grant from the American Cancer Society to David Baltimore, an Institutional grant from the Massachusetts General Hospital and an NIH training grant. M.L. was supported by a Clinical Investigator Award from the National Cancer Institute.

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Received on April 27, 1989; revised on July 5, 1989