Volume 17 Number 2 1989 Nucleic Acids Research

Pleiotropic derepression of developmentally regulated cellular and viral genes by c-myc proto-oncogene products in undifferentiated embryonal carcinoma cells

R.Onclercq, A.Lavenu and C.Cremisi

Unité de Génétique des Mammifères, Institut Pasteur, 25 rue du Docteur Roux, 75015 Paris, France

Received August 12, 1988; Revised and Accepted September 14, 1988

ABSTRACT

We show here in mouse embryonal carcinoma (EC) cells that the endo A gene is negatively regulated and shares negative transacting factors with the Py and SV40 viruses. The products of the proto-oncogene c-myc derepress at the transcriptional level the appropriately initiated expression of the endo A gene and activate the Py early promoter in EC stem cells. C-myc products also activate the endo A and the Py early promoters in TDM epithelial cells, and the Py early promoter in 3T6 cells in which the two genes are already expressed or can be expressed. Furthermore we show that the myc exon 1 is essential for activation and that this activation might be mediated by AP1 family factors.

INTRODUCTION

Little is known about gene regulation during early murine embryogenesis and the molecular approach to the study of this regulation presents many difficulties. Mouse embryonal carcinoma (EC) cell lines derived from teratocarcinoma stem cells provide an attractive *in vitro* system for studying the regulation of gene expression because these cells have many features in common with the cells of preimplantation embryos (1, 2). Several cellular and viral genes are silent in EC stem cells, but are expressed upon differentiation (for review see 3, 4).

We recently showed that the Py T antigen is negatively regulated in PCC4 cells by one or several labile repressor molecules which might be partly responsible for the absence of Py DNA expression in EC cells (5). We also showed, using cycloheximide, that one or several labile inhibitors block endo A gene expression in undifferentiated PCC4 cells (6). In the present study, we show that in the same cells, this gene is negatively regulated and has negative trans-acting factors in common with Polyoma and SV40 viruses.

Given that nuclear oncogene products like c-myc may influence gene regulation (7, 8, 9), we tested the effect of these products on endo A gene and Py antigen expression. We found

that human c-myc gene products derepressed these two genes in undifferentiated EC cells, superactivated both of them in TDM and 3T6 cells. We also show that the myc exon 1 is necessary for activation and that this activation might be mediated by AP1 family factors.

EXPERIMENTAL PROCEDURES

Cells and transfection

PCC4, F9, F9MTmyc, TDM, and 3T6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum. EC cells were replated in a 1 to 10 dilution every two days for maintenance and used for 10 passages at most. F9 and F9MTmyc cells were cultured on gelatinized plastic culture dishes. 60 mm dishes containing 30-40% confluent cells fed with fresh medium 2-4hr earlier, were used for transfection using the calcium phosphate coprecipitation technique (10). The amount of transfected DNA was kept constant by adding pUC18 plasmid DNA. As internal control, one µg of the βactβgal construct (pHβALacZ) was cotransfected with the other plasmids in the same transfection in each sample. The cells were exposed to the precipitate 16-18 hr and harvested 7-24 hr later for the CAT assay. Preparation of cell extracts, CAT assays and thin-layer chromatography were performed according to Gorman et al. (10). After chromatography, the acetylated and non acetylated forms of chloramphenicol were cut out and counted. CAT activity was calculated as the percentage of chloramphenicol converted to acetylated forms. Each transfection was reproduced at least three times with different plasmid preparations.

Plasmids

pCmyc (11) contains the entire human genomic c-myc gene, a 12 kb EcoR1 fragment composed of three exons and all the regulatory region (-6700 bp). Thus, this plasmid contains the coding information for all the c-myc products especially the p67 and p64 proteins. pCmyc Δ was constructed by inserting the myc DNA fragment Hind III-XbaI (3510 bp) in Puc 18 at the same sites. This fragment contains a part of the regulatory region, the first exon and a part of the first intron. pSB1 is a pML_2 derived plasmid containing the CAT gene (kindly provided by P. Herbomel).

pPyCAT (kindly provided by P. Herbomel) contains the Polyoma early promoter fused to the CAT gene. pendo CAT contains the endo A promoter fused to the CAT gene. pendo Aα contains the entire genomic endo A gene (-2000, +1300) cloned in PBR

(12). pendo A (-900, +1300) contains 900 bp of the regulatory region and the entire coding sequence of endo A gene (a gift of P. Duprey). pPy contains the viral Polyoma DNA cloned in PBR. TKCAT is the pBLCAT2 plasmid described by Luckow and Schütz (13) containing the Herpes TK promoter (-109 +55) fused to the CAT gene. PK3 contains three AP1 binding sites (TGAGTCA) fused to the TK promoter (-109 +55) and CAT gene (kindly provided by J. Piette and M. Yaniv). pJun (kindly provided by B. Wasylyk (14)) contains v-jun gene under the control of SV40 early promoter. pJI (14) contains v-jun sequences in the inverse orientation. The pHβALacZ plasmid contains the βactin promoter fused to the LacZ gene and was kindly provided by Andreas Püschel (Max-Planck Institut, Göttingen). All plasmid DNA was purified by ethidium bromide -CsCl gradient centrifugation performed twice.

RNA preparation

Total cytoplasmic RNA was prepared from cultured cell lines, as described by Maniatis et al. (15).

S1 nuclease analysis

S1 protection assays were performed as described in Duprey et al. (16). All the single strand probes were already described in Cremisi and Duprey (6) and Onclercq et al. (submitted, 1988).

Nuclear run off

Nuclear run off experiments were performed as described in Cremisi and Duprey (6). The following DNA were applied to nitrocellulose paper under vacuum using a Schleicher and Schull apparatus: 1 µg of SV40 viral DNA, 4 µg of a 2500 bp EcoRI fragment containing the endo A first exon, 4 µg of a 850 bp PvuII fragment containing the human c-myc first exon and 2 µg of a 1120 bp PstI fragment derived from a mouse β actin cDNA (a gift from M. Buckingham).

RESULTS

Negative regulatory trans-acting factors common to the endo A gene, and Py and SV40 viruses in PCC4 cells

We have previously proposed that the endo A gene and Py virus, which are not expressed in EC cells (17, 18), share negative regulatory trans-acting factors in PCC4 cells (6). To test this hypothesis, we infected EC cells with Py and also SV40 virus, extracted cytoplasmic mRNA 48 hours later and determined the level of endo A transcripts using an S1 protection

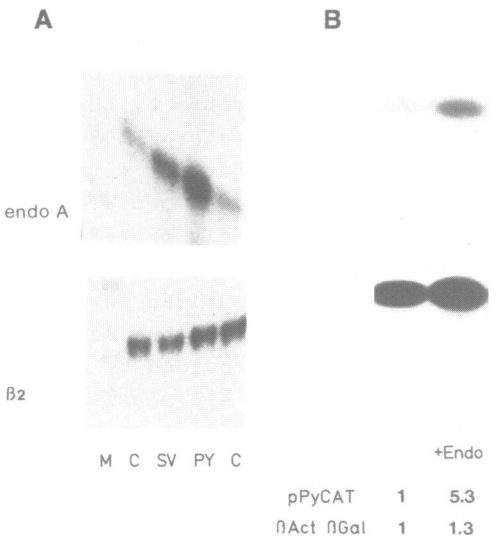

Figure 1 : Panel A : Activation of endo A gene expression in PCC4 cells after Py and SV40 infection. PCC4 cells were mock infected (c) or infected at a multiplicity of 50 to 100 PFU per cell by Polyoma (Py) and SV40 viruses. Cytoplasmic RNA was prepared 48 h after infection and analyzed by S1 nuclease mapping with endo A and β2 microglobulin probes. 50 µg of cytoplasmic RNA were run in each lane. M-markers; ^{32}P-labeled HpaII-digested pBR322 DNA was used as a size marker. Panel B: Activation of the early Py promoter in EC cells when cotransfected with pEndo A DNA. F9 cells were transfected with 10 µg of the pPy-CAT recombinant plasmid and 20 µg of Puc 18, or 20 µg of pEndo A plasmid. As internal control, 1 µg of pHβALacZ was cotransfected with the other plasmids in all the samples.

assay. As shown in Fig.1 (panel A), the amount of appropriately initiated endo A transcripts was significantly greater after Py and SV40 infection. Thus, the viral DNA presumably captured negative regulatory trans-acting factors permitting induction of endo A gene expression. We used northern blots to verify that the size of the endo A transcripts was appropriate (data not shown). The use of a β2 microglobulin probe as a control showed that the amount of mRNA was the same in each sample, and that viral infection had a specific effect on endo A gene expression (Fig.1).

With regard to viral infection of these cells, it is important to remember that neither Py nor SV40 virus is expressed after such infection (for review see 19). We found that only about 0.01 % of EC cells expressed T antigen after Py and SV40 infection (5). Consequently, the observed activation of endo A gene expression is probably due to an interaction between nega-

tive trans-acting factors and the regulatory regions of Py and SV40 viruses, and not to a trans-acting effect exerted by Py or SV40 T antigen. Nevertheless, in order to rule out this possibility completely we performed transient cotransfection experiments. For this purpose, we used a plasmid pPyCAT (a gift of P. Herbomel), in which the regulatory region of the early Py promoter was fused with the reporter gene chloramphenicol acetyltransferase (CAT), and compared the level of CAT activity under different conditions. When this plasmid pPyCAT was transfected with Puc18 DNA into undifferentiated EC cells, its expression was very slight, but when cotransfected with twice amount of pEndo A DNA (a gift of P. Duprey) its expression was activated (Fig. 1, panel B). Pendo A plasmid contains 900 bp of the 5' regulatory region and the entire coding sequence of endo A gene (1300 bp). pHβALacZ plasmid was included as internal control in these experiments. These two sets of results for infection and transfection prove that in EC cells, the cellular endo A gene, and the Py virus have common negative regulatory factors acting on their regulatory regions. Although we cannot formally exclude, it is very unlikely that the cytokeratin endo A transactivates the early Py promoter.

Derepression of the Py early promoter by c-myc gene products

EC cells therefore contain negative regulatory factors which influence the expression of cellular and viral genes (our present results, 5, 20, 21). This raises the important question of identifying the protein(s) or gene(s) participating in this negative regulation. It has been shown that i) nuclear viral and cellular oncogenes participate in the regulation of gene expression (for review, see 8, 9), ii) adenovirus E1a gene products can act either positively or negatively as transcriptional regulators, and negatively on Py and SV40 enhancers (22, 23), iii) EC cells contain a E1a-like cellular activity (26), and iv) c-myc and E1a genes have functional and structural homologies (8, 25). For all these reasons, we tested the effect of c-myc gene expression on the early Py promoter which was fused with the reporter gene chloramphenicol acetyltransferase (CAT). We performed transient cotransfection experiments and compared the levels of CAT activity. A human genomic clone of c-myc (11) containing the three c-myc exons and its regulatory region was used in these experiments. Human c-myc expression was tested by S1 nuclease mapping with cytoplasmic RNA extracted (28-40 h) after transfection (data not shown). When the

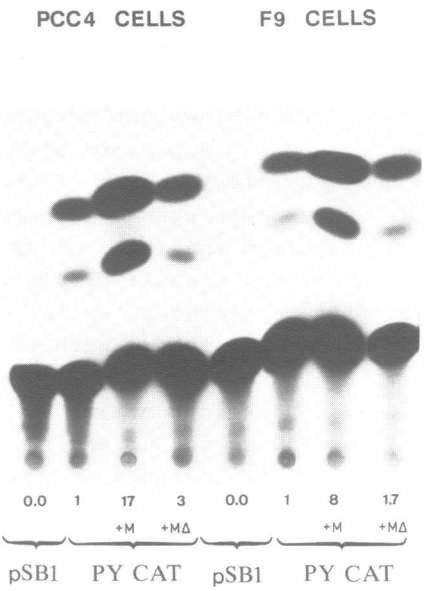

Figure 2 : Trans-activation of the early Py promoter in EC cells. PCC4 and F9 cells were transfected with 10 µg of the pPy-CAT recombinant plasmids and 10 µg of PUC 18, pc-myc (+M) or pc-myc (+MΔ). As a negative control, the cells were transfected with 10 µg of the bacterial plasmid pSB1. The numbers under each lane represent the relative CAT Activity.

plasmid pPyCAT was cotransfected with the same amount of plasmid pc-myc, which is able to code for all the c-myc proteins, the expression of the early Py promoter was activated (Fig. 2). The negative control pSB1, a pML2-CAT plasmid, was included in each experiment (Fig. 2). This control shows that the basal activity of pPyCAT exceeds the background level significantly and is not due to a cryptic bacterial promoter. Twelve transfection experiments were analyzed and a mean activation factor of 12 was obtained for PCC4 and F9 cells.

To ascertain that this activation was due to c-myc products, we used a construct containing a large part of the 5' regulatory region and the first exon with no poly A tail. Consequently, this truncated c-myc construct (pc-mycΔ) was not able to code for any functional c-myc products.When pc-mycΔ was used in the transient cotransfection experiments, the early Py promoter was not activated. We also used a plasmid containing exons 2 and 3 (pSVc-myc) (26). This construct has no effect (see Fig. 9). In the same way, a plasmid containing all the 5'

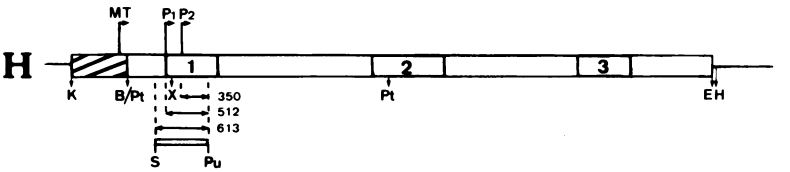

Figure 3 : Structure of the chimaeric MTmyc gene and of the human c-myc probes used for S1 nuclease digestion. The hatched box represents the mouse metallothionein I sequences, containing the heavy metal-sensitive region. The boxes (1, 2, 3) indicate the Exons of human (H) c-myc gene. MT, P1 and P2 designate the promoters of these genes. The SmaI-PvuII fragment of the human c-myc gene was used as a human-specific probe and gave rise to three protected fragments indicated by arrows. B: BamHI; E: EcoRI; K: KpnI: Pt: PstI; Pu: PvuII; S: SmaI; X: XhoI.

myc sequences (RI to BglII) fused to the third exon (BglII to RI) has also no effect (data not shown). Hence the activation of the early Py promoter by the c-myc gene can not be attributed to the regulatory c-myc region. Thus, exogenous c-myc products activate the early Py promoter by a direct or indirect effect on the regulatory region of this gene. We did not find a negative effect of exogenous c-myc on Py early promoter.

Induction of transcriptional activation of the cellular endo A gene by c-myc gene products.

To test the effect of c-myc gene products on endo A gene expression, we used the F9MTmyc cellular clone which was derived from F9 cells and was transformed by a plasmid containing the human c-myc proto-oncogene controlled by the inducible mouse metallothionein I promoter (Onclercq et al., 1988, submitted, see figure 3 for the map of the MTmyc plasmid). Like F9 parental cells, F9MTmyc cells are undifferentiated and are comparable in all other respects. F9MTmyc cells do not constitutively synthesize detectable amounts of human c-myc transcripts (Onclercq et al., 1988, and Fig. 5 lane 3 Hc-myc). When treated with cadmium (cd) for eight hours, we observed using S1 analysis, significant induction of human c-myc transcripts, initiated on the P1, P2, and on the metallothionein promoters (Fig. 5 and Fig. 3 for the probes). Northern analysis using an human exon 3 c-myc probe cross hybridizing with the murine gene showed that the amount of human c-myc transcripts was three fold greater than that of the endogenous myc gene after 10 µM of cd, and even greater (six fold) after 20 µM (Fig. 4). The same RNA samples were used to determine the

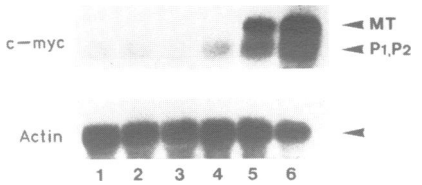

Figure 4 : C-myc expression after cadmium treatment in F9MTmyc cells. F9 (1, 2, 3) and F9MTmyc cells (4, 5, 6) were treated with 10 µM (2, 5) or 20 µM (3, 6) cd for 8 hr after which cytoplasmic RNA was prepared and analyzed by northern blot using a human exon 3 c-myc probe cross hybridizing with the murine gene and a mouse βactin probe (actin). 25 µg of cytoplasmic RNA were loaded in each lane. P1, P2 indicates RNA initiated at these promoters and MT at the metallothionein promoter.

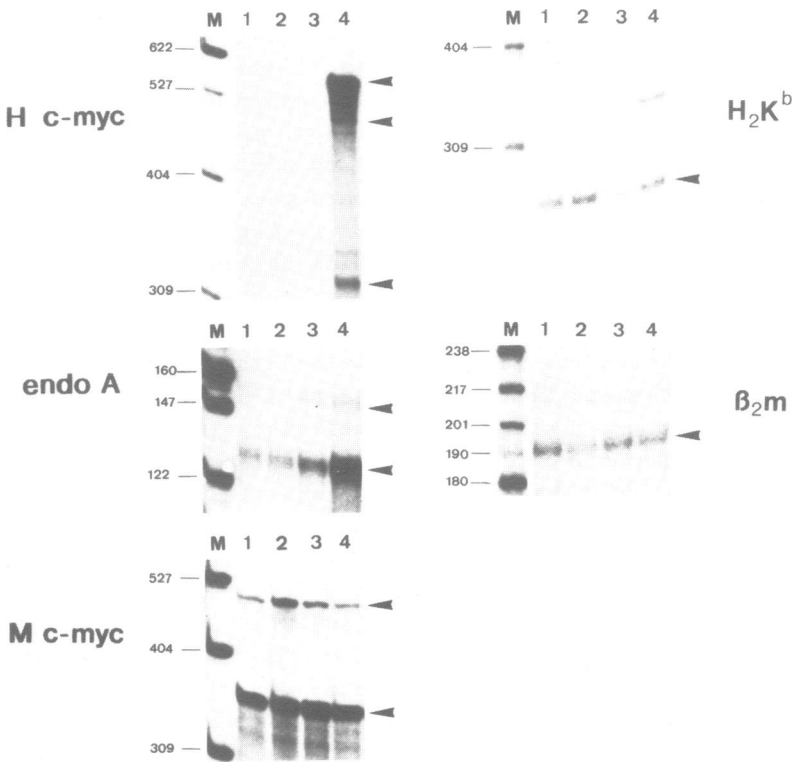

Figure 5 : Endo A gene derepression by the c-myc gene products. F9 (1, 2) and F9MTmyc cells (3, 4) were treated with 10 µM cd (2, 4) for 8 hr after which cytoplasmic RNA was prepared and analyzed by S1 nuclease mapping using a human exon 1 c-myc (H c-myc), endo A, mouse exon 1 c-myc (M c-myc), H2K^b and β2 microglobulin probes. The protected fragments are indicated by arrows: H-c-myc 613, 513 and 350 bp - endo A 153 and 130 bp - Mc-myc 497 and 357 bp - H2K^b 270 bp - β2m 200 bp. 35 µg of cytoplasmic RNA were loaded in each lane. M-markers.

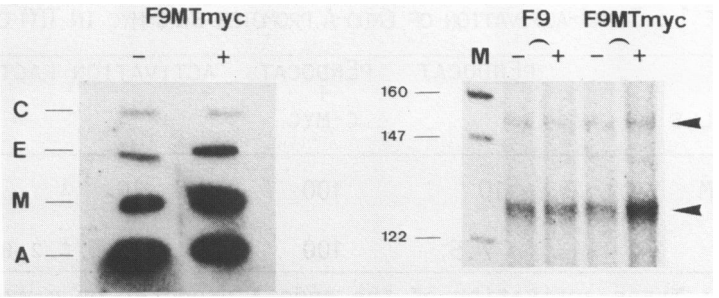

Figure 6 : Transcriptional activation of the endo A gene by c-myc gene products.Left Panel (A). Run off transcriptional analysis of the endo A (E), human c-myc (M) and βactin (A) genes in F9MT cells before (-) and after (+) treatment with 20 μM cd for 8 hr. (C)-SV40 DNA was used as a negative control. Right Panel (B). S1 nuclease analysis, using an endo A probe, on RNA extracted from F9 and F9MT myc cells before (-) and after (+) cd treatment. The protected fragments (153 and 130 bp) are indicated by arrows.

amount of endo A transcripts in F9MTmyc cells. As shown in Fig. 5, c-myc gene expression caused significant induction -about 3 to 6- of appropriately initiated endo A transcripts. Northern analysis indicated that the size of these transcripts was appropriate (data not shown). Figure 5 also shows that in F9 parental cells, cd in itself had no effect on the amount of endo A transcripts (lanes 1 and 2).

To establish whether or not activation of the endo A gene by c-myc products was specific to this gene, we tested the effect of these products on the expression of other cellular genes, again by S1 analysis. We observed that overexpression of the exogenous human c-myc gene did not affect the amount of endogenous murine c-myc transcripts, as we and others have shown previously (Onclercq et al., 1988, submitted; 27). The expression of two other genes -H2Kb and β2 microglobulin- which are silent in EC cells but coexpressed upon differentiation, also was not affected by the overexpression of c-myc (Fig. 5). We did not detect any effect on the expression of the p53 gene or the constitutive and inducible heat shock genes p68 and p70 (data not shown).

To establish whether endo A expression was induced by transcriptional activation, we performed nuclear run-off transcription analysis on nuclei from F9MTmyc cells before and after cd treatment (Fig. 6). In untreated F9MTmyc cells, as in other EC cells (6), the level of endo A transcription was very low (Fig. 6, panel A(E(-)). After cd treatment, we observed a si-

TABLE 1 : TRANS-ACTIVATION OF ENDO-A PROMOTER BY C-MYC IN TDM CELLS

CELLS		pENDOCAT	pENDOCAT + C-MYC	ACTIVATION FACTOR
TDM	1	10	100	10 ± 1
	2	7.5	100	13.5 ± 2.8

Table 1 : Trans-activation of the endo A promoter by c-myc in TDM cells.TDM cells were transfected with 10 µg of the pendoCAT recombinant plasmid and 10 µg of PUC 18 or pc-myc. The results of two independent experiments are shown. The numbers represent relative CAT activity.

gnificant induction of endo A gene transcription (three fold) and also, as expected, an increase in exogenous human c-myc gene transcription (Fig. 6, M). In contrast, the transcription of the cellular βactin gene did not vary significantly after cd treatment (Fig. 6, A). Inclusion of SV40 DNA as a negative control showed that the background level of hybridization was low (Fig. 6, C). Panel B of figure 6 illustrates the results of an S1 analysis using an endo A probe hybridized with cytoplasmic RNA extracted from the same F9MTmyc cell culture as in panel A. The magnitude of endo A gene induction in run off and S1 analysis was comparable. Panel B also shows that cd treatment had no effect on endo A gene expression in F9 parental cells.

From the results of these experiments, we conclude that human c-myc gene products specifically induce transcriptional activity of the cellular endo A gene.

We also tested the trans-acting effect of c-myc products in some differentiated cells. In transfection assays, c-myc gene products stimulated the endo A promoter in a teratocarcinoma epithelial cell line TDM in which the cellular endo A gene is expressed (Table 1). Similarly, c-myc gene products activated the Py early promoter in TDM cells and in mouse 3T6 fibroblasts,a cell line permissive for Py virus infection (Table 2).

C-myc activation might be mediated by a factor of the AP1 family

How does the c-myc gene regulate gene expression ? Would c-myc products activate endo A and Py directly, or indirectly as do E1A gene products ? The c-myc protein is a DNA-binding protein but binding specificity has not yet been demonstrated (28, 31). C-myc products may not bind to DNA directly, but

TABLE 2 : TRANS-ACTIVATION OF EARLY PY PROMOTER IN TDM AND 3T6 CELLS

CELLS		PY CAT	PY CAT + C-MYC	ACTIVATION FACTOR
TDM	1	6	100	17.5 ± 0.3
	2	3	100	25 ± 5
3T6	1	20	100	5 ± 0.3
	2	22	100	4.5 ± 0.1

Table 2 : Trans-activation of the early Py promoter in TDM and 3T6 cells. TDM and 3T6 cells were transfected with 10 µg of the pPyCAT recombinant plasmid and 10 µg of PUC 18 or pc-myc. In each case, the results of two independent experiments are shown. The numbers under each lane represent relative CAT activity.

require the formation of a complex with other nuclear proteins. Moreover we have shown that c-myc products activate E4 adenovirus promoter by probable activation of ATF factor (32). So we tested the involvement of a transcriptional factor in myc activation and more specially of AP1 or PEA1 factor.

Indeed it has been shown that PEA1 activity, the murine equivalent of an AP1 factor, is involved in Py expression during F9 differentiation (33) and we have found an AP1 consensus binding site in the beginning of the coding sequence of the endo A gene (+96, +102 TGAGTCA). We do not yet know if this site is functional. Nevertheless, we tested if c-myc activation might be mediated by the use of AP1 binding sites. We used a plasmid termed PK3 containing three AP1 binding sites fused to the thymidine kinase (TK) promoter and linked to the CAT gene (kindly provided by J. Piette and M. Yaniv, manuscript in preparation). One microgram of PK3 was cotransfected into F9 cells with PUC 18, pc-myc or pc-myc ▲ DNA. Figure 7 shows significant activation (13 to 16) of PK3 only when it is cotransfected with pc-myc DNA. When the TK promoter fused to the CAT gene was cotransfected into F9 cells with pc-myc, about twofold activation of this promoter occured, suggesting that PK3 activation for the most part (at least by a factor 10) is due to a direct or indirect interaction between c-myc products and AP1 sites. The same experiments were done using 3T6 (see Fig. 9) and Hela cells (data not shown). In these experiments, we included the plasmid DNA pHβALacZ as an internal control. We observed a very

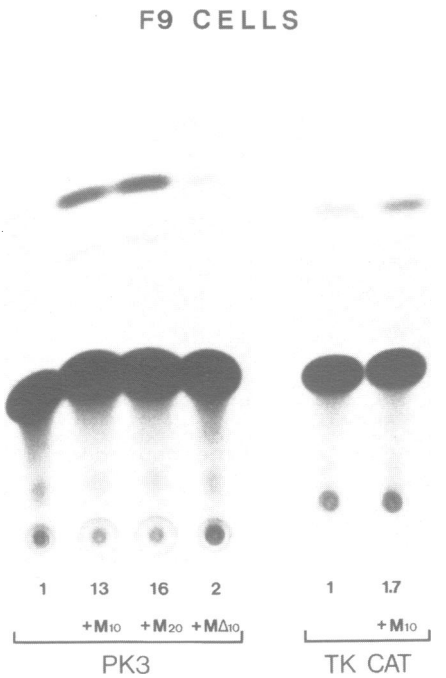

Figure 7 : Trans-activation of the PK3 plasmid by c-myc products. Left Panel. F9 cells were transfected with 1 µg of PK3 recombinant plasmid and 10 µg of PUC 18, 10 or 20 µg of pc-myc (+M10, +M20), or 10 µg of pc-myc▲ (+M▲10). Right Panel. As a control, F9 cells were transfected with 1 µg of the TK-CAT recombinant plasmid and 10 µg of PUC 18 or pc-myc (+M10). As internal control, 1 µg of pHβALacZ was cotransfected with the other plasmids in all the samples. The numbers under each lane represent relative CAT activity.

reproducible if small activation of the βactin promoter, by a factor of 3 (data not shown). Taking into account the constant activation of the βactin promoter, this control allowed us to estimate the transfection efficiency in all the samples.

These results are compatible with either a direct or an indirect myc effect. In order to distinguish between the two possibilities, we used a v-jun expression vector (kindly provided by B. Wasylyck, 14) in the cotransfection assay in addition to pc-myc. It has been shown that viral jun and the human proto-oncogene c-jun encode DNA binding proteins with the same functional properties of as the transcription factor AP-1 (34). As control, we used v-jun sequences in the inverse orientation (pJI (14)) to verifie that the activation was not due to the titration of negative factors.

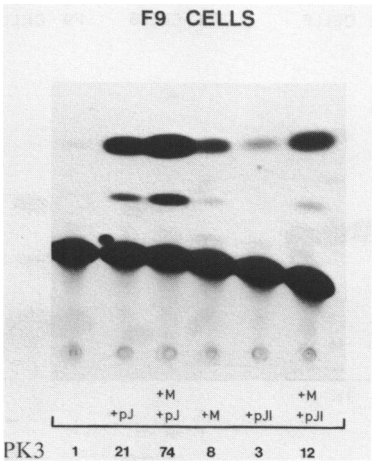

Figure 8 : Additive effect of jun and c-myc products in the trans-activation of PK3 plasmid. F9 cells were transfected with 2 µg of PK3 recombinant plasmid and 4 µg of pJun (+pJ) or 4 µg of pJun inverse (+pJI), and 10 µg of pc-myc (+M). The amount of DNA was kept constant in each sample by adding PUC 18 DNA and 1 µg of pHβALacZ was added in all the samples as internal control. The numbers under each lane represent relative CAT activity.

If c-myc acts indirectly on DNA, through AP1 factor, one should observe an additive effect. Figure 8 shows that the effects of v-jun and c-myc on PK3 activation are additive, demonstrating that c-myc acts indirectly through AP1 factor possibly by activating it. We did not observe an increase of AP1 mRNA by c-myc (data not shown).

Is a c-myc plasmid without the first exon able to trans-activate?

To test if the c-myc exon 1 was necessary for activation, we used pSVc-myc containing only the exons 2 and 3 of a mouse myc gene under the control of the simian virus 40 early promoter (26). It has been shown that this myc construct has oncogenic properties (26). pSVc-myc is able to code only for the p64 myc protein, since the p67 myc protein is initiated at the end of the first exon by a non conventional codon (35). We compared in the transfections assays, the trans-activation effect of pc-myc and pSVc-myc on PK3 and PyCAT in 3T6 and F9 cells. In all cases, only pc-myc in contrast to pSVc-myc was able to transactivate significantly PK3 and PyCAT (see Fig. 9). The failure of pSVc-myc to activate is not artefactual since this construct, which was previously used in several other

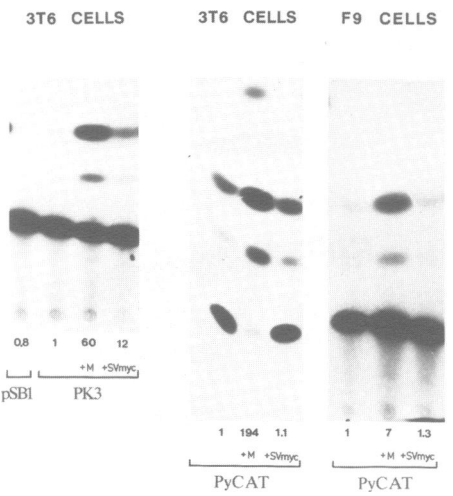

Figure 9 : c-myc exon 1 is necessary for trans-activation. First panel: 3T6 cells were transfected with 2 µg of PK3 recombinant plasmid and 10 µg of PUC 18, pc-myc (+M), or pSVc-myc (+SVmyc). As negative control, the cells were transfected 10 µg of the bacterial plasmid pSB1. Second and third panel: 3T6 and F9 cells were transfected with 5 µg of pPyCAT and 10 µg of PUC 18, pc-myc (+M), or pSVc-myc (+SVmyc). as internal control, 1 µg of pHβALacZ was cotransfected with the other plasmids in all the samples. The numbers under each lane represent relative CAT activity.

studies (26, 36, 37) was shown to be functional. However we compare by CAT assays in our experimental systems F9 and 3T6 cells, the c-myc and SV40 promoters. We used a construct H-cmyc CAT which contains the regulatory region of the human c-myc gene, HindIII-PVUII (-2329 +518) linked to the CAT gene and the plasmid pSV2CAT which contains the SV40 promoter linked to the CAT gene. We found in both cells types F9 and 3T6 that SV40 promoter is not less expressed than c-myc promoter (data not shown). Consequently the failure of pSVc-myc to activate can not be explained by a smaller amount of c-myc products produced by this construct. These results show that the c-myc exon 1 is essential for activation.

DISCUSSION

Derepression by c-myc products

We have previously shown that the Py virus, which cannot be expressed in EC stem cells, is negatively regulated by labile trans-acting factor(s) in these cells, and we suggested that both negative and positive regulators control early Py

gene activity in these cells (5). We then showed that a labile inhibitor blocks endo A gene transcription in EC cells and proposed the same model, involving negative and positive regulators, for endo A gene expression before and after EC cell differentiation (6). Here, we demonstrate that the endo A gene is negatively regulated in these cells, and that this gene shares negative trans-acting regulatory factor(s) with Py and SV40 viruses.

We also show that the products of the c-myc proto-oncogene derepress the endo A gene and the Py early promoter. This oncogene induces at the transcriptional level appropriately initiated endo A gene expression. To the best of our knowledge, this is the first example of derepression of a cellular gene by the products of the c-myc proto-oncogene. We underline that during mouse embryonic development, endo A and Py T antigen start to be expressed at the same time in the first differentiated cells, i.e. the trophectoderm layer of the blastocyst (16, 19).

We also show that c-myc gene products are able to activate the endo A and Py early promoters in TDM and 3T6 cells respectively i.e. cells in which these genes are already expressed. We also found an overexpression of the endo A gene in epithelial cell cultures derived from human breast tumors (Crémisi and Brison, unpublished results), in which the c-myc gene is amplified and over expressed (38).

How does the c-myc gene regulate gene expression ?

We showed that AP1 binding sites and AP1 proteins (Vjun proteins) are involved in c-myc activation. This is shown by cotransfection experiments involving the PK3 plasmid (containing three AP1 binding sites fused to the TK promoter) and c-myc DNA (Fig. 7). The use of a v-jun expression vector simultaneously with pc-myc in co-transfection experiments in the presence of the reporter gene PK3 shows that the effects of v-jun and pc-myc are additive in PK3 transactivation. Thus, c-myc products act indirectly probably by activating AP1 family factors (Fig. 8). C-myc might act like the c-fos protein and require the formation of a complex with other nuclear proteins, in particular transcriptional factors (39, 40, for review see 41).

It is noteworthy that TPA activates the c-myc gene and the AP1 factor (42, 43, 44). Thus, the involvement of factors of the AP1 family in c-myc mediated activation is not surprising since AP1 is influenced by cellular signals implicated in

growth control and neoplasia (42, 43). It is also important to recall that AP1 is homologous to the proto-oncogene c-jun (34), and that v-jun induces fibrosarcomata in chickens (45, 46). Interestingly, it has been shown that the Py enhancer is also activated in EC cells by the Ras oncogene (47). This activation is mediated by AP1 family factors (48).

It was already shown that AP1 family factors are involved in Py expression (33, 49) and we found an AP1 consensus binding site in the beginning of the coding sequence of the endo A gene (+96 +106), but we do not know yet if this site is functional. However we carried out competition experiment between PK3 in one hand and endo A and Py DNA in the other and we found in this case an activation of PK3 (unpublished observations). These results strongly suggest that AP1 family factors are involved in endo A expression and even also as a negative regulator.

How to explain that exogenous c-myc products trans-activate endo A gene and Py T antigen,whereas endogenous c-myc gene is already expressed. This activation could be due to a global increase of c-myc products and/or the additionnal amount of p67 myc protein synthetized by exogeneous c-myc (MTmyc or pC-myc) might produce a subtle difference in the ratio of $p\frac{64}{67}$ proteins producing a shift in myc activity in favour of activation. This latter possibility is more probable since we found that only a c-myc plasmid coding for the two p67 and p64 proteins was able to trans-activate, whereas a plasmid coding only for the p64 protein was not. These results might suggest that the two myc proteins have different functions and that the p67 protein contains a specific domain having strong activating properties absent or truncated in the p64 protein. Furthermore, it was already suggested that the two myc proteins p67 and p64 might have different functions, since the amount of p64 protein is much higher in tumor cells and in Burkitt's lymphoma cells (35, 50).

It might appear paradoxal that in undifferentiated EC cells, exogenous c-myc expression derepresses precisely the two genes (endo A and Py T antigen) which are negatively regulated by common factors, whereas there is an apparent antagonism between the expression of the endogeneous c-myc gene and that of endo A and the PyT antigen, since these genes are expressed during F9 differentiation, when c-myc is no more expressed. We had proposed a model (51) in which we speculate that the endo-

genous c-myc participates in the repression of the endo A gene and the Py virus by inhibiting the activity of AP1 factor. A subtle difference in the ratio of $p\frac{64}{67}$ myc proteins would produce a shift in my activity. This hypothesis could explain the results obtained in the competition experience between PK3 and Py and endo A.

It was already shown, that the c-myc gene might act either positively or negatively on cellular and viral genes (36, 52). The trans-acting effect of the pSVc-myc construct on other promoters has already been studied in differentiated 3T3 fibroblasts (36). It has been shown that the pSVc-myc construct stimulates the human heat shock protein 70 promoter, inhibits the mouse metallothionein I promoter, and has very little effect on SV40 and E2A adenovirus promoters in 3T3 cells (36). In this study, the myc gene was a murine gene containing only the second and third exons (26), and as already mentioned, this construct is only able to code for the p64 myc protein (35). It has recently been shown that pSVc-myc is unable to activate the PEA1 factor (54). In this sense, this study is in agreement with our work which shows that only the proto-oncogene containing the three exons, is able to trans-activate a vector containing AP1 binding sites.

In summary, we have shown that exogenous c-myc products act as positive transcriptional regulators by acting on the cellular endo A gene and on viral Py DNA and that this activation might be mediated by AP1 family factors. We have also previously shown that the c-myc gene can activate the E4 and E2A promoters (adenovirus E1A inducible promoters) in EC stem cells using the same target sequence as that used by E1A i.e., the binding site of the transcriptional ATF factor (32). Thus the interaction of the c-myc proto-oncogene with transcriptional factors leading to positive regulation might be a general mechanism.

ACKNOWLEDGMENTS

We would like to thank P. Duprey for stimulating discussions during the experimental work; P. Herbomel, J. Piette, A. Püschel, M. Yaniv and B. Wasylyck for plasmids ; C. Babinet, P. Duprey, A. Kahn and J. Piette for critical reading of the manuscript; I. Fleurance for secretarial assistance and all the staff of the photography laboratory for their kindness.

REFERENCES

1. Martin, G.R. (1980) Science 209, 768-776.
2. Evans, M.J. and Kaufman, M.H. (1981) Nature 292, 154-156.
3. Goodfellow, P.N. (1984) Cell differentiation 15, 257-267.
4. Hogan, B.L.M., Barlow, D.P. and Tilly, R. (1983) Cancer survey 2, 115-140.
5. Crémisi, C. and Babinet, C. (1986) J. Virol. 59, 761-763
6. Crémisi, C. and Duprey, P. (1987) Nucl. Acid. Res. 15, 6105-6116.
7. Bishop, J.M. (1983) Ann. Rev. Biochem. 52, 301-310.
8. Kingston, R.E., Baldwin, A.S. and Sharp, P.A. (1985) Cell 41, 3-5.
9. Varmus, H.E. (1987) Science 238, 1337-1339.
10. Gorman, C.M., Moffatt, L.F. and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044-1051.
11. Gazin, C., Dupont de Dinechin, S., Hampe, A., Masson, J.M., Martin, P., Stehelin, D. and Galibert, F.(1984) Embo J. 3, 383-387.
12. Vasseur, M., Duprey, P., Brûlet, P. and Jacob, F. (1985) Proc. Natl. Acad. Sci. USA 82, 1155-1159.
13. Luckow, B. and Schütz, G. (1987) Nucl. Acid. Res. 15,5490.
14. Imler, J.L., Ugarte, E. and Wasylyk, B. (1988) Nucl. Acid. Res. 16, 3005-3012.
15. Maniatis, T., Fritsch, E. and Sambrook, J. (1982) Cold Spring Harbor, New York.
16. Duprey, P., Morello, D., Vasseur, M., Babinet, C., Condamine, H., Brûlet, P. and Jacob, F. (1985) Proc. Natl. Acad. Sci. USA 82, 8535-8539.
17. Brûlet, P., Babinet, C., Kemler, R. and Jacob, F. (1980). Proc. Natl. Acad. Sci. USA 77, 4113-4117.
18. Swartzendruber, D.E. and Lehman, J.M. (1975) Physiol. 85, 179-188.
19. Kelly, F. and Condamine, H. (1982) Biochim. Biophys. Acta 651, 105-141.
20. Gorman, M.C., Rigby, P.W.J. and Lane, D.P. (1985) Cell 42, 519-526.
21. Sassone-Corsi, P., Fromental, C. and Chambon, P. (1987) Onc. Res. 1, 113-119.
22. Borelli, E., Hen, R. and Chambon, P. (1984) Nature 312, 608-612.
23. Velcich, A. and Ziff, E. (1985) Cell 40, 705-716.
24. Imperiale, M.J., Kao, H.T., Feldman, L.T., Nevins, J.R. and Strickland, S. (1984) Mol. Cell. Biol. 4, 867-874.
25. Ralston, R. and Bishop, J.M. (1983) Nature 306, 803-806.
26. Land, H., Parada, L.F. and Weinberg, R.A. (1983) Nature 304, 596-602.
27. Keath, E.J., Caimi, P.G. and Cole, M.D. (1984) Cell 39, 339-348.
28. Abrams, H.D., Rohschneider, L.R. and Eisenman, R.N. (1982) Cell 29, 427-439.
29. Donner, P., Greiser-Wilke, I. and Moelling, K. (1982) Nature 296, 262-266.
30. Alitalo, K., Ramsay, G., Bishop, J.M., Pfeifer, S.O., Colby, W.W. and Levinson, A.D. (1983) Nature 306, 274-277.
31. Persson, H. and Leder, P. (1984) Science 225, 718-721.
32. Onclercq, R., Lavenu, A. and Crémisi, C. (1988) J. Virol. 62, in press.
33. Kryszke, M.H., Piette, J. and Yaniv, M. (1987) Nature 328, 254-256.
34. Bohmann, D., Bos, J.T., Admon, A., Nishimura, T., Vogt, K.P. and Tjian, R. (1987) Science 238, 1386-1392.
35. Hann, R.S., King, W.M., Bentley, L.D., Anderson, W.C. and Eisenman, R.N. (1988) Cell 52, 185-195.
36. Kaddurah-Daouk, R., Green, M.J., Baldivin, S.A. and Kingston, E.R. (1987) Genes and Dev. 1, 347-357.
37. Wasylyk, C., Imler, J.L. and Wasylyk B. (1988) EMBO J. 7, 2475-2483.
38. Modjtahedi, N., Lavialle, C., Poupon, M.F., Landin, R.M., Cassingena, R., Monier, R. and Brison, O. (1985) Cancer Res. 45, 4372-4379.
39. Distel, R.J., Ro, H.S., Rosen, B.S., Groves, D.L. and Spiegelman, B.M. (1987) Cell 49, 835-844.
40. Rauscher III, F.J., Sambucetti, L.C., Curran, T., Distel, R.J. and Spiegelman, B.M. (1988) Cell 52, 471-480.
41. Verma, M.I. and Sassone-Corsi, P. (1987) Cell 51, 513-514.

42. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987) Cell 49, 729-739.
43. Lee, W., Mitchell, P. and Tjian, R. (1987) Cell 49, 741-752.
44. Kelly, K., Cochran, B.H., Stiles, C.D. and Leder, P. (1983) Cell 35, 603-610.
45. Cavalieri, F., Ruscio, T., Tinoco, R., Benedict, S., Davis, C. and Vogt, P.K. (1985) Virology 143, 680-683.
46. Maki, Y., Bos, T.J., Davis, C., Starbuck, M. and Vogt, P.K. (1987) Proc. Natl. Acad. Sci. USA 84, 2848-2852.
47. Wasylyk, C., Imler, J.L., Perez-Mutul, J. and Wasylyk, B. (1987) Cell 48, 525-534.
48. Imler, J.L., Shatz, C., Wasylyk, C., Chatton, B. and Wasylyk, B. (1988) Nature 232, 275-278.
49. Piette, j. and Yaniv, M. (1987) Embo J. 6, 1331-1337.
50. Hann, S.R. and Eisenman, R.N. (1984) Mol. Cell. Biol. 4, 2486-2497.
51. Onclercq, R. (1988) Thèse de l'Université Pierre et Marie Curie de Paris VI.
52. Versteeg, R., Noordermeer, I.A., Krüse-Wolters, M., Ruiter, D.J. and Schrier, I.P. (1988) Embo J. 7, 1023-1029.