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# A STABLY TRANSFECTED c-myc CAT HYBRID GENE IS NOT REGULATED BY SERUM IN NIH3T3 CELLS

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A plasmid containing the CAT (chloramphenicolacetyltransferase) gene fused to the 5' adjacent sequences and first exon of the human c-myc gene was stably transfected into NIH3T3 cells. Single cell clones were grown and CAT activity was measured after serum starvation and stimulation. CAT activity of the hybrid construct remained unchanged in serum-deprived and serum-stimulated cells. In contrast the steady-state RNA level of the endogenous mouse c-myc gene was strongly elevated upon serum stimulation. The bona fide usage of the human c-myc promoter $P_1/P_2$ in mouse cells carrying the hybrid gene was revealed by S1 analysis. 

The position of target sequences regulating c-myc gene transcription from its dual promoter $P_1/P_2$ is presently under investigation in many laboratories (1-5). Three major DNAaseI hypersensitive sites are located upstream of the c-myc gene which are assumed to bind positively and negatively regulating transcription factors. Site I is present in chromatin of an expressed and unexpressed gene and was therefore proposed as a candidate for repressor binding (6,7).

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The presence of hypersensitive site II seems to be a requirement for c-myc expression, because all expressed c-myc genes investigated so far have an open chromatin configuration at this position. Transcriptional downregulation of c-myc expression is accompanied by disappearance of DNAaseI hypersensitive site II (7-9).

However, the correlation between accessibility of site II and transcriptional activity of c-myc is not absolute. Conditions have been described, where c-myc transcription from $P_1$ and $P_2$ is shut off while site II presents an open chromatin configuration (10,11). In these conditions the c-myc gene was found to be regulated by blocking RNA elongation near the end of the first exon (10, 12-14).

In resting or growth-arrested cells the c-myc RNA level can be increased by the addition of serum or growth factors (15,16). Serum induction of c-myc in quiescent mouse fibroblasts is regulated at the level of transcription initiation (17). Following the assumption that c-myc regulation by serum would be mediated by upstream regulatory sequences as shown in other systems (18-20) we used a DNA hybrid molecule with the CAT (chloramphenicol-acetyltransferase) gene fused to upstream and first exon sequences of the human c-myc gene and tested its activity and regulation by serum in mouse fibroblasts. Regulation of the human c-myc gene in mouse/man cell hybrids has already been demonstrated (21).

## MATERIALS AND METHODS

### Cell culture

Cells were cultured in Eagle's MEM supplemented with 5% fetal calf serum (FCS) as indicated. Proliferation of the cells was monitored by incorporation of 5-bromo-deoxyuridine (BUDR) followed by immunofluorescence with a monoclonal antibody against BUDR (22). DNA transfection followed the protocol of Graham and van der Eb (25).

Southern and Northern blot analysis
DNA extraction and Southern blot analysis was performed as described by Maniatis et al. (26).
RNA was isolated from cells cultured with 0.5% FCS (serum starvation) or 20% FCS (serum stimulation) by the lithium chloride/urea method (27). 20 µg total RNA was separated on a denaturing agarose-formaldehyde gel and transferred to nitrocellulose. Prehybridization, hybridization and washing was as described (26) with 400 µg/ml heat denatured t-RNA as a carrier and without dextran sulfate.

S1 analysis
A single stranded 862b PvuII-PvuII M13 probe specific for the first exon of the human c-myc gene (28) and labeled with [$^{32}$P] by primer extension (50.000 cpm) was hybridized with 30 µg total cellular RNA. Hybridization was carried out in 90% formamide, 400 mM NaCl, 40 mM PIPES, pH 6.5, 1 mM EDTA at 58°C for 12 hours followed by nuclease S1 treatment as described by Berk and Sharp (29) and separation of protected fragments on a 5% denaturating polyacrylamide gel.

Assay for CAT activity
Published procedures were followed (30) to assay CAT activity, with minor modifications. Cells were harvested with a rubber-policeman, suspended in 300 µl of 0.25 M Tris-HCl buffer (pH 7.8), and sonicated twice for 5 seconds each. Protein extract of 1.5 x $10^6$ cells corresponding to about 150 µl of the cell extract, was incubated with 20 µl of 4 mM acetyl coenzyme A (P-L Biochemicals) and 0.2 µCi of [$^{14}$C] chloramphenicol (CAM) (50 µCi/ml, 57.8 mCi/mmol; New England Nuclear Corp.) in 0.25 M Tris-HCl buffer (pH 8.7) at a final volume of 175 µl for 1h at 37°C. The [$^{14}$]C-labeled CAM was extracted twice with 400 µl ethylacetate, dried, resuspended in 10 µl ethylacetate, and spotted onto a thin-layer silica gel plate (0.25 mm, Merck).

## RESULTS AND DISCUSSION

NIH3T3 cells were stably transfected with a recombinant plasmid containing the human c-myc promoter-leader region, the bacterial chloramphenicolacetyltransferase gene (CAT) and the gene for neomycin resistance (NEO). The physical map of the construct pmycCAT-NEO is illustrated in Figure 1b.
Cells were transfected with the described construct, with pSV2CAT-NEO and with pSV0CAT-NEO, respectively. Neomycin resistant single cell clones were isolated and tested for CAT activity. High CAT activity was measured in cells containing the pSV2CAT-NEO construct, no activity in cells with the pSV0CAT construct (data

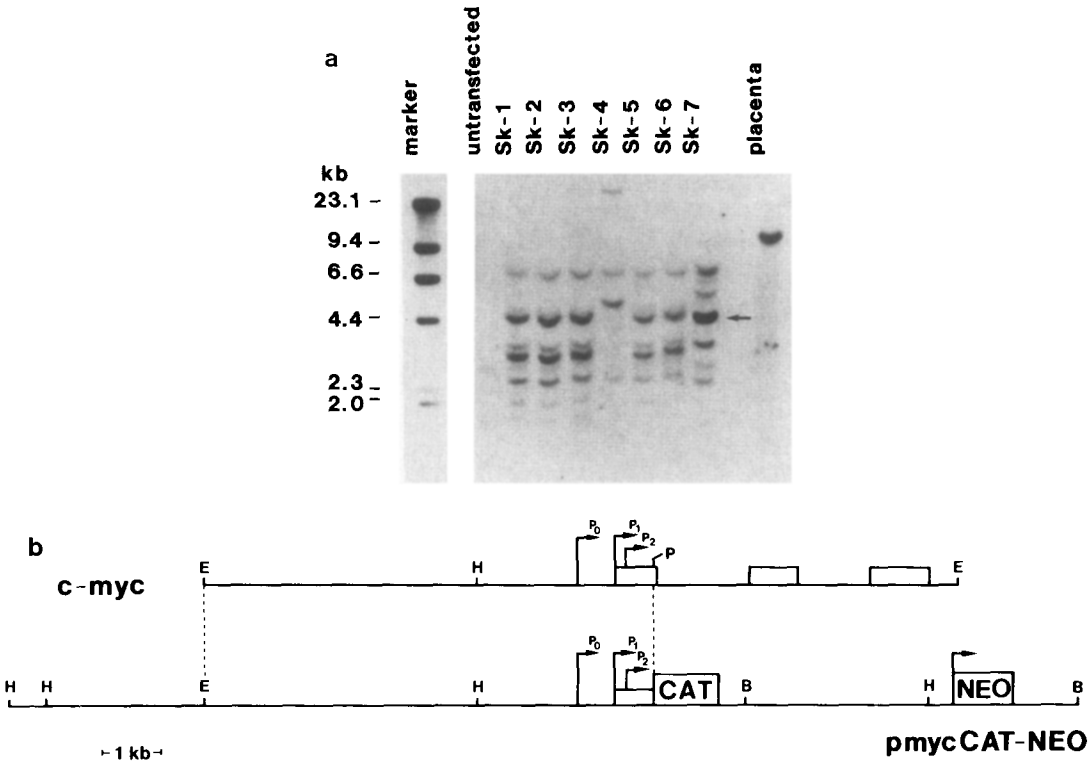


Figure 1. (a) Integration pattern of pmycCAT-NEO DNA in stably transfected and single cell cloned NIH3T3 cells.
DNA of clones Sk-1 to Sk-7, of untransfected NIH3T3 cells and of human placenta (10 µg each) was digested with HindIII and BamHI. The fragments were separated in a 0.6% agarose gel, blotted onto Zeta-Probe and hybridized with [$^{32}$P]-multi-prime labeled (31) pmycCAT-NEO derivate (missing the 4.5 kb EcoRI-HindIII fragment 5' of the c-myc gene containing repetitive sequences). The 4.4 kb HindIII-BamHI fragment, designated by an arrow, indicates intact integration of the CAT gene and 2800 bp of 5' adjacent c-myc sequence. A molecular size marker of HindIII-digested lambda DNA is shown at the left hand side.
(b) Schematic representation of the hybrid molecule of the human c-myc gene and the bacterial CAT gene.
The upper line represents the germ-line configuration of the human c-myc gene, located on a 12.5 kb EcoRI fragment. The hybrid molecule pmycCAT-NEO is constructed of a 7 kb EcoRI-PvuII fragment containing the dual c-myc promoter $P_1/P_2$ with upstream sequences, the bacterial CAT gene and the gene for neomycin resistance. Abbreviations for restriction enzymes: E, EcoRI; B, BamHI; H, HindIII; P, PvuII.

not shown). Cells transfected with the pmycCAT-NEO plasmid showed an intermediate CAT activity of about 3-30% of that found in pSV2CAT-NEO transfected cells. A severalfold higher activity of the SV40 promoter compared to the c-myc promoter was also detected in transient transfection assays (4).

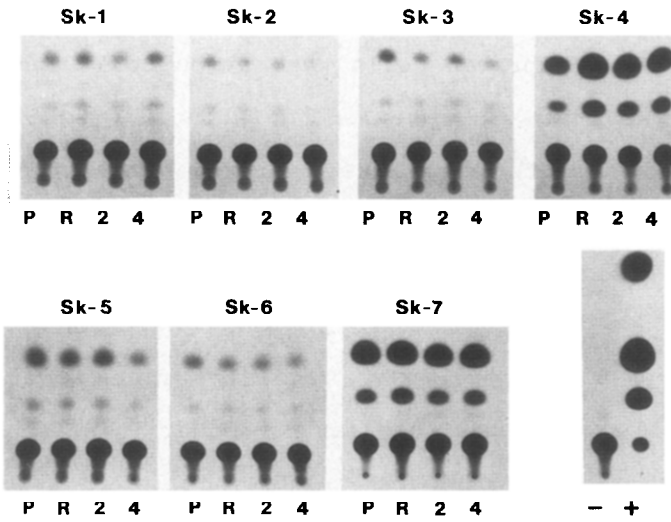


Figure 2. CAT activity of the pmycCAT-NEO construct in stably transfected and single cell cloned NIH3T3 cell lines. Extracts were prepared from proliferating cells (P), or from cells serum deprived for 36h (R) and subsequently stimulated for 2 and 4h by addition of 20% serum. Controls were performed without (-) or with (+) CAT enzyme. CAT activity was estimated by densitometric scans of the autoradiogram.

The integration pattern of the pmycCAT-NEO construct was analysed in seven single cell clones (Sk-1 to Sk-7). Southern blot analysis revealed integration of about 2-4 copies of pmycCAT-NEO in all cell lines. Six out of seven cell lines (except cell line Sk-4) showed intact integration of c-myc/CAT sequences revealed by a 4.4 kb HindIII-BamHI fragment (Figure 1a).

Cells were starved for 36h in 0.5% serum and subsequently stimulated by addition of 20% serum. The proliferative activity of the cells was measured by incorporation of 5-bromodeoxyuridine and visualized by indirect immunofluorescence with a monoclonal antibody against BUDR (22). About 30-40% of the cells of a normally growing population incorporated BUDR, whereas only 2% of the cells were in S phase after serum deprivation for 36h (data not shown).

The CAT activity of pmycCAT-NEO transfectants was measured in proliferating cells at 5% serum (P), after 36h serum starvation at 0.5% serum (R), and 2 and 4h after addition of 20% serum to serum-deprived cells (Figure 2). CAT activity did neither decrease

significantly in pmycCAT-NEO cells upon serum reduction nor increase when serum-deprived cells were stimulated with 20% serum. Cell lines Sk-4 and Sk-7 revealed an about 10 fold higher CAT activity compared to the other cell clones tested. The high CAT activity is reflected by an elevated amount of myc/CAT specific RNA in Sk-7 cells (Figure 4). The high CAT activity in Sk-4 cells, however, does not correlate with the low amount of myc/CAT specific RNA. Furthermore, CAT activity still increased after serum deprivation. It is unclear, whether the integration of truncated copies of pmycCAT-NEO in Sk-4 cells is responsible for this phenomenon.

We next tested the responsiveness of the endogenous c-myc gene of NIH3T3 cells to serum. Total RNA was isolated from serum deprived and serum stimulated cells and studied by Northern blotting. Densitometric evaluation of the Northern blots revealed an about 16 fold (2h) and 8 fold (4h) increase in the steady-state level of RNA transcribed from the endogenous mouse c-myc gene upon serum stimulation (Figure 3).

Hybrid RNAs derived from the exogenously introduced pmycCAT-NEO construct were monitored directly by S1-analysis (Figure 4a). S1 analysis revealed bona fide usage of the human dual c-myc promoter in a ratio P1<P2. The amount of RNA transcribed from the construct upon serum stimulation was measured in Sk-7 cells and did not change significantly in agreement with the results of the CAT analysis (Figure 4b).

We thus conclude that the pmycCAT-NEO construct is uncapable to confer responsiveness to serum in NIH3T3 cells. There are several possibilities which may account for the lack of responsiveness of pmycCAT-NEO in our system. First, the upstream sequences of the human c-myc gene may contain serum responsive elements which operate in human but not in mouse cells. This assumption is conceivable, however, the sequence of the serum response element

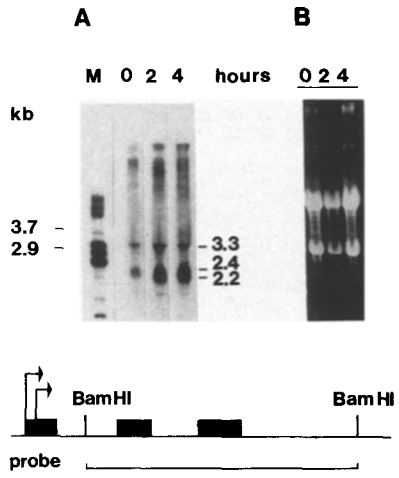


Figure 3. Northern blot analysis of the endogenous mouse c-myc RNA after serum stimulation.
RNA was extracted from cells deprived of serum for 36h (0) and 2 and 4h after addition of 20% serum. The RNA was fractionated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose. (A) The filter was hybridized with a [$^{32}$P]-multi-prime labeled DNA of a 5 kb BamHI-BamHI fragment containing exon 2 and exon 3 of the mouse c-myc gene. The 2.2 and 2.4 kb RNAs correspond to $P_1$ and $P_2$ derived transcripts, the 3.3 kb RNA is presumably derived from the 5' located $P_0$ promoter. Lane M contains a molecular size marker. (B) The ethidium bromide stained gel before transfer.

(SRE) of the fos gene in mouse as well as its binding protein are highly conserved in evolution and can interact with the corresponding elements of the gene for cytoskeletal actin in Xenopus (18). Second, the sequences responsible for transcriptional activation of the c-myc gene may not be present on the pmycCAT-NEO construct. This notion is supported by the fact that a fos gene related SRE 5' of the c-myc gene was not found in a computer search. Third, transcriptional regulation of the pmycCAT-NEO construct may require the chromatin configuration of the endogenous c-myc gene which may not be established after transfection. In this context it has recently been demonstrated that transiently and stably transfected promoters response differently to TPA (phorbol 12-myristate 13-acetate) (23), indicating that the transcriptional activity and regulation of an integrated construct is influenced by chromatin and position effects. The construct pmycCAT-NEO described here directs specific

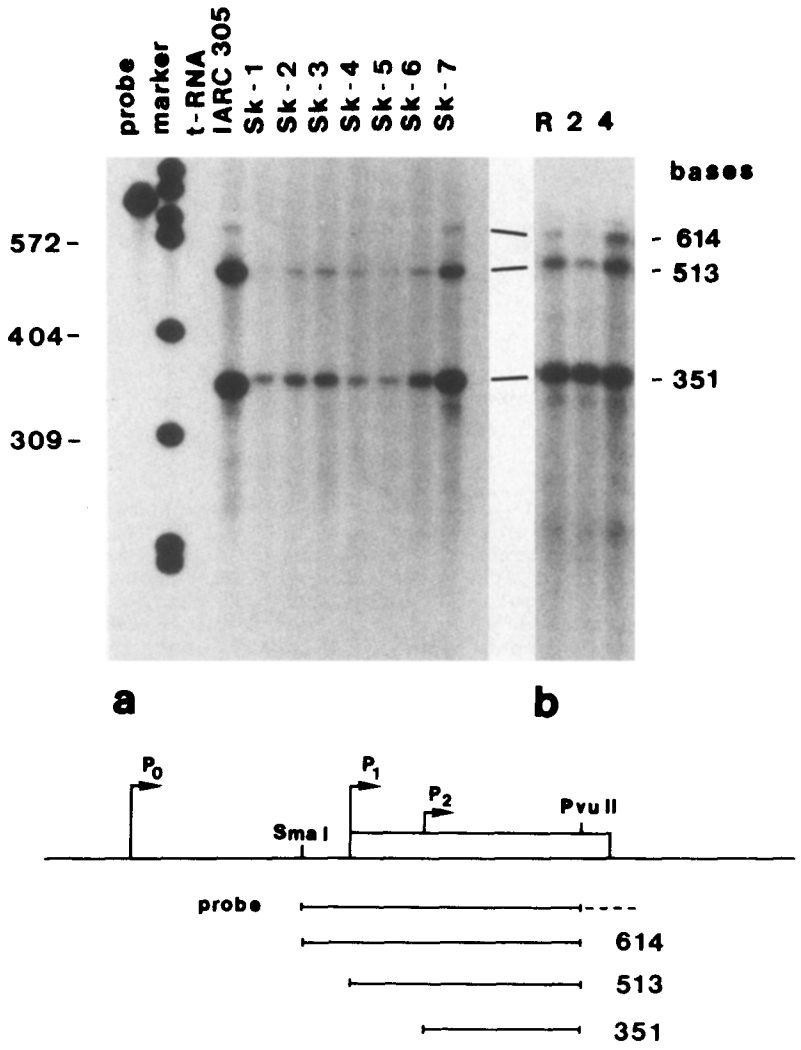


Figure 4. S1 analysis of c-myc/CAT hybrid RNA.
(a) RNA was extracted from proliferating pmycCAT-NEO-transfected cells (Sk-1 to Sk-7). RNA of the lymphoblastoid cell line IARC305 served as control for normal expression of the human c-myc gene. The RNA was hybridized with an uniformly [$^{32}$P]-labeled probe, specific for the human c-myc exon 1. The probe protected fragments of 614 b ($P_0$), 513 b ($P_1$) and 351 b ($P_2$).(b) RNA was extracted from Sk-7 cells deprived of serum for 36h (R) and after stimulation with 20% serum for 2 and 4h.

promoter-dependent transcription of c-myc. Proper regulation of c-myc by serum in a position-independent manner, however, may require additional sequences 5'- and 3' of the first exon, as demonstrated for the $\beta$-globin gene (24).

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REFERENCES

1. Yang, J.Q., Remmers, E.F., and Marcu, K.B. (1986) EMBO J. 5, 3553-3562.
2. Remmers, E.F., Yang, J.Q., and Marcu, K.B. (1986) EMBO J. 5, 899-904.
3. Chung, J.E., Sinn, R.R., and Leder, P. (1986) Proc. Natl. Acad. Sci. USA 83, 7918-7922.
4. Lipp, M., Schilling, R., Wiest, S., Laux, G., and Bornkamm, G.W. (1987) Mol. Cell. Biol. 7, 1393-1400.
5. Hay, N., Bishop, M., and Levens, D. (1987) Genes & Development 1, 659-671.
6. Leder, P., Battey, J., Lenoir, G.M., Moulding, C., Murphy, W., Potter, H., Stewart, T., and Taub, R. (1983) Science 222, 765-770.
7. Siebenlist, U., Henninghausen, L., Battey, J., and Leder, P. (1984) Cell 37, 381-391.
8. Fahrlander, P.D., Piechaczyk, M., and Marcu, K.B. (1985) EMBO J. 4, 3195-3202.
9. Dyson, P.J., Littlewood, T.D., Forster, A., and Rabbitts, T.H. (1985) EMBO J. 4, 2885-2891.
10. Eick, D., and Bornkamm, G.W. (1986) Nucleic Acids Res. 14, 8331-8346.
11. Eick, D., Berger, R., Polack, A., and Bornkamm, G.W. (1987) Oncogene 2, 61-65.
12. Bentley, D.L., and Groudine, M. (1986) Nature 321, 702-706.
13. Nepveu, A., and Marcu, K.B. (1986) EMBO J. 5, 2859-2865.
14. Mechti, N., Piechaczyk, M., Blanchard, J.M., Marty, L., Bonnieu, A., Jeanteur, P., and Lebleu, B. (1986) Nucleic Acids Res. 14, 9653-9666.
15. Kelly, K., Cochran, B.H., Stiles, C.D., and Leder, P. (1983) Cell 35, 603-610.
16. Greenberg, M.E., and Ziff, E.B. (1984) Nature 311, 433-437.
17. Nepveu, A., Levine, R.A., Campisi, J., Greenberg, M.E., Ziff, E.B., and Marcu, K.B. (1987) Oncogene 1, 243-250.
18. Treisman, R. (1987) EMBO J, 6, 2711-2717
19. Gilman, M.Z., Wilson, R.N., and Weinberg, R.A. (1986) Mol. Cell. Biol. 6, 4305-4316.
20. Mohun, T., Garrett, N., and Treisman, R. (1987) EMBO J. 6, 667-673.
21. Croce, C.M., Erikson, J., ar-Rushdi, A., Aden, D., and Nishikura, K. (1984) Proc. Natl. Acad. Sci. USA 81, 3170-3179.
22. Gratzner, H.G. (1982) Science 218, 474-475.
23. Lebkowski, J.S., McNally, M.A., Okarma, T.B., and Lerch, L.B. (1987) Nucleic Acids Res. 15, 9043-9055.
24. Grosveld, F., Blom van Assendelft, G., Greaves, D.R., and Kollias, G. (1987) Cell 51, 975-985.
25. Graham, F.L., and van der Eb, A.J. (1973) Virology 52, 456-467.

26. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, published by Cold Spring Harbor Laboratory Press, NY.
27. Auffray, C., and Rougeon, F. (1980) Eur. J. Biochem. 107, 303-314.
28. Eick, D., Piechaczyk, M., Henglein, B., Blanchard, J.M., Traub, B., Kofler, E., Wiest, S., Lenoir, G.M., and Bornkamm, EMBO J. 4, 3717-3725.
29. Berk, A.J., and Sharp, A.P. (1977) Cell 12, 721-732.
30. Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044-1051.
31. Feinberg, A.P., and Vogelstein, B. (1983) Biochemistry 132, 6-13.