2818 (1991).
7. E. A. Wagar and R. S. Stephens, *Infect. Immun.* **56**, 1678 (1988).
8. E. Perara and D. Ganem, in *Chlamydial Infections: Proceedings of the Seventh International Symposium on Human Chlamydial Infections*, W. R. Bowie *et al.*, Eds. (Cambridge Univ. Press, Cambridge, 1990), p. 44.
9. T. Hackstadt, *J. Bacteriol.* **173**, 7046 (1991).
10. S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1074 (1985).
11. M. Manafi, W. Kneifel, S. Bascomb, *Microbiol. Rev.* **55**, 335 (1991).
12. D. J. Arndt-Jovin and T. M. Jovin, *Methods Cell Biol.* **30**, 417 (1989).
13. B. Setlow *et al.*, *J. Bacteriol.* **173**, 6270 (1991).
14. J. W. Costerton, L. Poffenroth, J. C. Wilt, N. Kordova, *Can. J. Microbiol.* **22**, 16 (1976).
15. Plasmid DNA was isolated from K38(pGP1-2 pT763) by the boiling miniprep procedure of D. S. Holmes and M. Qigley [*Anal. Biochem.* **114**, 193 (1981)]. After centrifugation, lysates were precipitated with isopropanol and analyzed by PAGE and Western blotting.
16. O. G. Stonington and D. E. Pettijohn, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 6 (1971).
17. K. Sjåstad, P. Fadnes, P. G. Kruger, I. Lossius, K. Kleppe, *J. Gen. Microbiol.* **128**, 3037 (1982).
18. D. E. Pettijohn, *Cell* **30**, 667 (1982).
19. V. Deretic and W. M. Konyecsni, *J. Bacteriol.* **172**, 5544 (1990).
20. J. Kato, T. K. Misra, A. M. Chakrabarty, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2887 (1990).
21. V. Deretic, C. D. Mohr, D. W. Martin, *Mol. Microbiol.* **5**, 1577 (1991).
22. C. J. Dorman, N. N. Bhriain, C. F. Higgins, *Nature* **344**, 789 (1990).
23. M. Göransson *et al.*, *ibid.*, p. 682.
24. J. F. Miller, J. J. Mekalanos, S. Falkow, *Science* **243**, 916 (1989).
25. C. S. J. Hulton *et al.*, *Cell* **63**, 631 (1990).
26. S. S. Broyles and D. E. Pettijohn, *J. Mol. Biol.* **187**, 47 (1986).
27. K. Drlica and J. Rouviere-Yaniv, *Microbiol. Rev.* **51**, 301 (1987).
28. C. F. Higgins *et al.*, *Mol. Microbiol.* **4**, 2007 (1990).
29. C. F. Higgins *et al.*, *Cell* **52**, 569 (1988).
30. G. J. Pruss and K. Drlica, *ibid.* **56**, 521 (1989).
31. M. V. Solbrig, M. L. Wong, R. S. Stephens, *Mol. Microbiol.* **4**, 1535 (1990).
32. H. D. Caldwell, J. Kromhout, J. Schachter, *Infect. Immun.* **31**, 1161 (1981).
33. After heat induction, 20-μl samples were placed on slides, and 1 μl of 0.01% acridine orange in 150 mM sodium acetate, pH 4.0, was added. Samples were photographed with a Zeiss Photomicroscope III (Neofluar 100 oil-immersion lens, epi-fluorescence condenser III RS) onto Kodacolor P800/1600 color reversal film.
34. S. H. Pincus *et al.*, *J. Exp. Med.* **172**, 745 (1990).
35. L.-I. Larsson, *Immunocytochemistry: Theory and Practice* (CRC Press, Boca Raton, FL, 1988).
36. E. N. Robinson, Jr., *et al.*, *Infect. Immun.* **46**, 361 (1984).
37. The 25-ml cultures were prepared as described, except that at an $OD_{600\ nm}$ of 0.1, 10 μCi of [^{3}H]thymidine were added to each culture. After induction, the cells were lysed by the procedure of Sjåstad *et al.* (*17*), with the exception that hen egg-white lysozyme (500 μg/ml) was used in place of T4 lysozyme. Unincorporated label and soluble protein were removed by repeated centrifugation through 100 mM sucrose cushions as described. The pellet was resuspended in 500 μl of buffer A [20 mM tris, pH 8.1, 50 mM NaCl, 5 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol] and loaded onto a 0 to 60% (w/v) linear sucrose gradient in the same buffer. Samples were centrifuged at 1250*g* for 20 min, and 0.5-ml fractions were removed from the top and analyzed as described. For DNase I treatment, the pellet after three pelletings through 100 mM sucrose solutions was resuspended in 500 μl of buffer B (10 mM tris, pH 8.0, 0.5 mM $CaCl_2$, and 5 mM $MgCl_2$), 250 U of DNase I was added, and the suspension was incubated at 37°C for 15 min before loading onto a linear sucrose gradient as above.
38. We thank J. Sager for technical assistance; H. Caldwell, J. Swanson, S. Fischer, S. Hill, and T. Brickman for helpful comments; and S. Tabor for the T7 expression system.

12 December 1991; accepted 28 February 1992

Reciprocal Regulation of Adipogenesis by Myc and C/EBPα

Svend O. Freytag* and Tim J. Geddes

3T3-L1 adipoblasts that express large amounts of c-Myc cannot terminally differentiate, raising the possibility that Myc inhibits the expression of genes that promote adipogenesis. The CCAAT/enhancer binding protein (C/EBPα) is induced during 3T3-L1 adipogenesis when cells commit to the differentiation pathway. Transfection of 3T3-L1 adipoblasts with the gene that encodes C/EBPα caused overt expression of the adipocyte morphology. Expression of Myc prohibited the normal induction of C/EBPα and prevented adipogenesis. Enforced expression of C/EBPα overcame the Myc-induced block to differentiation. These results provide a molecular basis for the regulation of adipogenesis and implicate Myc and C/EBPα as pivotal controlling elements.

Proliferation and differentiation are often alternative and mutually exclusive pathways for living cells. Because specific genes control these pathways, the decision to either proliferate or differentiate may be governed by the ratio of gene products that promote each pathway. Inappropriate expression of genes that promote proliferation can favor proliferation over differentiation, which can result in neoplasia. Given that proliferation is generally incompatible with differentiation and vice versa, a gene that controls both pathways in a reciprocal manner might provide a molecular basis for this observation.

The decision to either proliferate or differentiate begins at the cell surface with cues received from the environment. These cues initiate events that cause changes in gene expression, a process often controlled at the level of transcription initiation. Because sequence-specific DNA binding proteins regulate transcription initiation (*1*), they are likely to participate in the regulation of cellular proliferation and differentiation.

The sequence-specific DNA binding protein c-Myc controls cellular proliferation and differentiation (*2*). Expression of c-Myc increases when quiescent cells are induced to proliferate (*3*) and decreases when actively growing cells enter either a quiescent or replicative senescent state (*4, 5*). Deregulated expression of c-Myc promotes cellular transformation and inhibits terminal differentiation both in vitro and in vivo (*2*). These and other observations suggest that c-Myc activates genes that promote proliferation (*2, 6*). However, it is also possible that Myc suppresses genes that restrict growth (*7*). One such candidate gene codes for the sequence-specific DNA binding protein C/EBPα (*8–11*), which promotes 3T3-L1 adipoblast differentiation (*11*). Expression of C/EBPα increases during adipogenesis (*9, 10*), and its premature expression in proliferating adipoblasts causes cessation of mitotic growth (*11*). That quiescent 3T3-L1 adipoblasts do not express C/EBPα suggests that C/EBPα is not a general growth suppressor. Enforced expression of c-Myc prevents 3T3-L1 adipogenesis by inhibiting the ability of cells to commit to the differentiation pathway (*12, 13*). Because C/EBPα has been implicated in the promotion of 3T3-L1 adipogenesis, we investigated whether expression of C/EBPα was sufficient for 3T3-L1 adipogenesis and whether Myc prevented adipogenesis by inhibiting induction of C/EBPα.

3T3-L1 adipoblasts were transfected (*14*) with pZip-NeoSV(X) (*15*) and a plasmid that contains the rat C/EBPα gene (pMSV-C/EBP) (*8*) under the control of the murine sarcoma virus (MSV) promoter. As controls, cells were transfected with a plasmid that contained the MSV promoter but lacked the C/EBPα gene (pEMSV) (*16*). After transfection, cells were selected in G418 (Boehringer Mannheim) for 2 to 3 weeks, and the number of G418-resistant colonies was recorded. Dishes of cells that received pMSV-C/EBP produced fewer colonies (~20% of the controls) than those that received pEMSV (Table 1). The yield of G418-resistant colonies, and the percentage of adipocyte colonies, was a func-

S. O. Freytag, Molecular Biology Research Program, Henry Ford Hospital, Detroit, MI 48202, and Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109.
T. J. Geddes, Molecular Biology Research Program, Henry Ford Hospital, Detroit, MI 48202.

*To whom correspondence should be addressed.

tion of the ratio of pMSV-C/EBP to pZIP-NeoSV(X) used in the transfections (*17*). These results suggest that C/EBPα expression inhibits 3T3-L1 adipoblast growth.

After 5 days, small colonies with adipocyte morphology developed in dishes that received pMSV-C/EBP (Fig. 1A). Even though cells were maintained in mitogen-rich medium and were never exposed to inducers of differentiation (*12*), a fraction of the colonies (~12%) became rounded and accumulated cytoplasmic lipid droplets, two characteristics of terminally differentiated adipocytes (*12*, *18*). No such colonies developed in control dishes (Fig. 1B) (Table 1). The adipocyte colonies expanded at a slower rate than did the control colonies and, after 2 weeks, had only 3 to 200 cells. Most of these colonies senesced within 2 to 3 weeks even though they were fed fresh media every 3 days. Few of them lost their adipocyte morphology and became established cell lines. By contrast, the control colonies expanded to several thousand cells over the same period and readily became established cell lines. These results indicate that expression of C/EBPα is sufficient for 3T3-L1 adipogenesis.

To confirm this possibility, we attempted to establish a correlation between development of the adipocyte morphology and expression of pMSV-C/EBP. Because most of the adipocyte colonies senesced and never became established cell lines, we used a reverse transcription–polymerase chain reaction (RT-PCR) assay to detect pMSV-C/EBP mRNA in colonies with fewer than 200 cells (*19*). Each assay included primers that detected the alpha 4 tubulin mRNA as an internal control and was performed with colonies from control and pMSV-C/EBP–transfected dishes. Of the 12 colonies examined that exhibited adipocyte morphology, 10 expressed the pMSV-C/EBP mRNA at a high concentration and 1 at a low concentration (Fig. 2). One adipocyte colony with only three cells failed to produce an RT-PCR product for pMSV-C/EBP and alpha 4 tubulin, which suggests that the colony size was below the sensitivity of the assay. Colonies that received pEMSV produced an RT-PCR product for tubulin but not for pMSV-C/EBP (Fig. 2). Colonies from pMSV-C/EBP–transfected dishes that did not exhibit adipocyte morphology did not produce an RT-PCR product for pMSV-C/EBP (Fig. 2). Thus, with one exception, there was a good correlation between development of adipocyte morphology and high expression of the pMSV-C/EBP mRNA.

We next examined the expression of the *aP2* gene, which increases (~100-fold) (*20*) during 3T3-L1 adipogenesis and is under the control of C/EBPα (*10*). The RT-PCR product specific for *aP2* mRNA was detected in colonies that did and did not exhibit the adipocyte morphology (Fig. 3). However, the amount of *aP2* product generated by the adipocyte colonies was much greater than that of control colonies, even though the adipocyte colonies contained fewer cells. These results demonstrate that all the colonies that expressed the pMSV-C/EBP mRNA and exhibited the adipocyte morphology also showed activation of a gene that is expressed in the differentiated adipocyte.

To investigate whether enforced expression of Myc inhibited the induction of C/EBPα during 3T3-L1 adipogenesis, we used two cell lines that constitutively expressed either a stably transfected human c-*myc* (*13*) or N-*myc* gene (*21*). Neither cell line can undergo adipogenesis (*13*, *17*). As previously demonstrated (*9*), antibodies to rat C/EBPα detected two polypeptides in the differentiated 3T3-L1 adipocyte (Fig. 4). One polypeptide corresponded to intact C/EBPα (43 kD) and the other was probably a degradation product (34 kD). The immunoblot demonstrated that quiescent 3T3-L1 adipoblasts did not express C/EBPα and that C/EBPα was induced in terminally differentiated adipocytes. In addition, C/EBPα was not induced in either the c-Myc (cM21) or the N-Myc (NM12) cell line after treatment with inducers of differentiation. Thus, expression of Myc in 3T3-

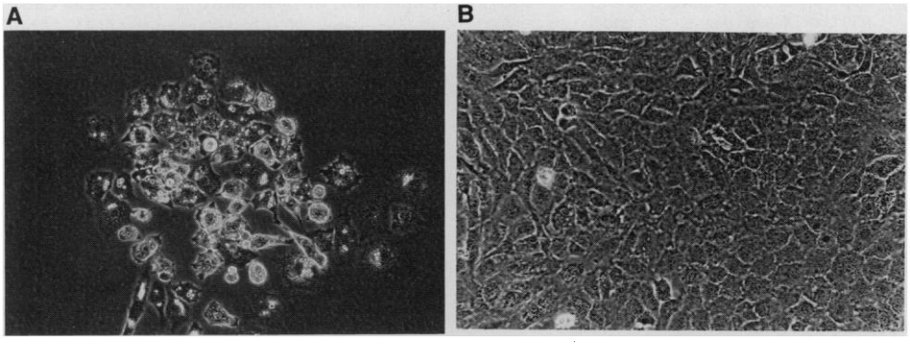

Fig. 1. Photomicrographs of 3T3-L1 colonies. (**A**) An adipocyte colony from a pMSV-C/EBP–transfected dish. The refractile bodies in the cytoplasm are lipid droplets. (**B**) A typical colony from a pEMSV-transfected dish.

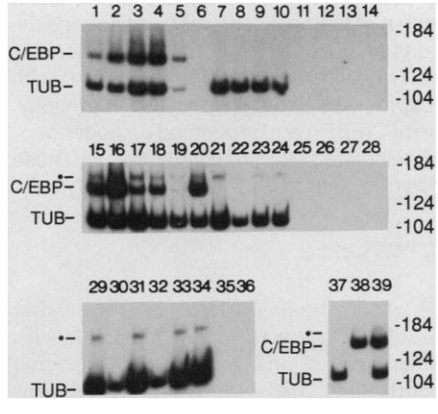

Fig. 2. RT-PCR analysis of pMSV-C/EBP and alpha 4 tubulin mRNA in transfected 3T3-L1 adipoblasts. The top two autoradiograms represent different transfection experiments. Lanes 1 to 6 and 15 to 20, 3T3-L1 colonies, transfected with pMSV-C/EBP, that exhibited the adipocyte morphology; lanes 7 to 10 and 21 to 24, 3T3-L1 colonies transfected with pEMSV; lanes 29 to 34, 3T3-L1 colonies, transfected with pMSV-C/EBP, that did not exhibit the adipocyte morphology; lanes 11, 25, and 35, no extract added; lanes 12, 26, and 36, same as lanes 1, 15, and 29, respectively, but without RT; lanes 13 and 27, same as lanes 1 and 15, respectively, but the extract was pretreated with deoxyribonuclease-free ribonuclease A; lanes 14 and 28, same as lanes 1 and 15, respectively, but the extract was pretreated with 0.1 N NaOH; lane 37, same as lane 15, but including only the tubulin primers; lane 38, PCR product obtained with linearized pMSV-C/EBP DNA; lane 39, same as lane 15. The RT-PCR products for pMSV-C/EBP (C/EBP, 168 bp) and tubulin (TUB, 110 bp) are indicated. The numbers on the right indicate DNA markers in base pairs. Asterisks indicate an RT-PCR product obtained unreproducibly with the tubulin primers (see lane 37).

Table 1. Number of G418-resistant and adipocyte (A) colonies in 3T3-L1 adipoblasts transfected with pMSV-C/EBP or pEMSV. The number of G418-resistant (G418^r) and adipocyte colonies was scored 12 to 14 days after commencement of selection. Colonies in which more than 50% of the cells contained cytoplasmic lipid droplets were scored as adipocyte colonies.

Experiment	pEMSV		pMSV-C/EBP		
	G418^r	A	G418^r	A	%
1	581	0	102	10	10
2	661	0	130	11	8
3	544	0	86	7	8
4	851	0	179	23	13
5	640	0	156	24	15
6	1163	0	214	32	15
Total	4440	0	867	107	12*

*Mean.

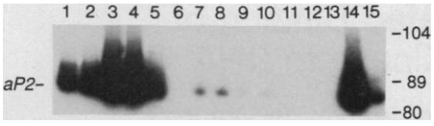

Fig. 3. RT-PCR analysis of *aP2* mRNA in transfected 3T3-L1 adipoblasts. Lanes 1 to 13, same as in Fig. 2; lanes 14, RNA (1 ng) from differentiated 3T3-L1 adipocytes; lane 15, RNA (1 ng) from undifferentiated 3T3-L1 adipoblasts. The *aP2* RT-PCR product (*aP2*, 84 bp) is indicated. The numbers on the right indicate DNA markers in base pairs.

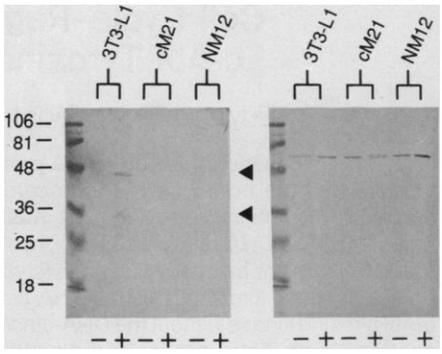

Fig. 4. Protein immunoblot of C/BPα in normal and Myc-transformed 3T3-L1 adipoblasts. Cells were harvested on days 0 (−) and 8 (+) after initiation of the differentiation program (*12*). Soluble nuclear protein (5 μg) was applied to duplicate SDS-polyacrylamide (15%) gels. The immunoblots were probed with either the alpha 14 polyclonal antisera to rat C/EBP (on the left) (*9*) or antisera to mouse β tubulin (on the right). The numbers on the left indicate protein molecular size markers in kilodaltons. Arrowheads indicate the positions of the C/EBPα polypeptides; 3T3-L1, untransfected 3T3-L1 cell line; cM21, 3T3-L1 cell line expressing a stably transfected human c-*myc* gene (pM21) (*13*); and NM12, 3T3-L1 cell line expressing a stably transfected human N-*myc* gene (pMP34.1^N) (*21*).

L1 adipoblasts prohibited induction of C/EBPα and prevented adipogenesis.

It is possible that the loss of C/EBPα induction in Myc-transformed cells is necessary for the Myc-induced block to differentiation. If this were true, C/EBPα expression in Myc-transformed cells should overcome the differentiation block and allow normal adipogenesis. To test this possibility, we transfected Myc-1 cells, which expressed a stably transfected pRSV*myc* gene (*12*), with either pMSV-C/EBP or pEMSV and examined them for the ability to differentiate into adipocytes. We chose Myc-1 cells for these experiments because the function of c-Myc in preventing 3T3-L1 adipogenesis is well-characterized (*12*). Similar to the results obtained with 3T3-L1 adipoblasts, a fraction (~5%) of the colonies in pMSV-C/EBP–transfected dishes developed the adipocyte morphology (Table 2). No such colonies developed in the control dishes. RT-PCR analysis confirmed that only the adipocyte colonies expressed the pMSV-C/EBP mRNA and that they continued to express the pRSV*myc* mRNA (*17*). Thus, development of the adipocyte morphology correlated well with expression of the pMSV-C/EBP mRNA and was not a result of suppression or loss of the stably integrated pRSV*myc* gene. These results demonstrate that expression of C/EBPα overcame the Myc-induced block to differentiation.

There were two notable differences between the results of the Myc-1 and 3T3-L1 cell transfections. First, the effect of pMSV-C/EBP on the yield of G418-resistant colonies was less dramatic with Myc-1 cells (~50% of pEMSV) than with 3T3-L1 adipoblasts (~20% of pEMSV). Second, the percentage of G418-resistant colonies that developed the adipocyte morphology was also less with Myc-1 cells (~5%) than with 3T3-L1 adipoblasts (~12%). Thus, the ability of C/EBPα to promote adipogenesis was compromised somewhat in Myc-1 cells. It is possible that the resulting phenotype is dependent on the relative amounts of the Myc and C/EBPα proteins.

The findings of this study provide a molecular basis for the observation that deregulated proliferation is generally incompatible with differentiation. Deregulated expression of Myc, which promotes proliferation, prohibits the expression of C/EBPα, which promotes terminal differentiation. When Myc levels are high, normal 3T3-L1 adipoblasts are locked in a proliferation-competent state, and normal differentiation cannot be activated. When Myc concentrations are low, resting 3T3-L1 adipoblasts can proceed along a pathway that leads to either proliferation or differentiation depending on cues from the environment. Thus, Myc functions at a pivotal control point in the proliferative and differentiation pathways. Although it is not known how Myc inhibits the induction of C/EBPα, the promoter of the mouse C/EBPα gene (*22*) contains the Myc binding site core sequence (CACGTG) (*23*). Thus, Myc may inhibit expression of C/EBPα directly, by binding to C/EBPα promoter sequences, or indirectly, either by activating genes that repress C/EBPα transcription or by preventing the function of proteins that activate the C/EBPα gene.

Expression of C/EBPα in proliferating 3T3-L1 adipoblasts causes growth arrest (*11*) (Table 1). This effect of C/EBPα was demonstrated when cells were in mitogen-rich media and at low density. Because quiescent 3T3-L1 adipoblasts do not express C/EBPα (Fig. 4), it is unlikely that C/EBPα mediates reversible growth arrest, or quiescence. Instead, these observations, together with the fact that C/EBPα is induced when cells commit to the differentiation pathway, argue that expression of C/EBPα promotes a state of irreversible growth arrest that is associated with terminal differentiation, or replicative senescence. Enforced expression of Myc does not inhibit the ability of cells to enter a quiescent state in G0/G1 (*12*) but rather precludes entry into a replicative senescent state. We show here that Myc prohibits the expression of a gene that induces a state of growth arrest resembling replicative senescence. Myc may immortalize primary rodent cells in vitro by means of a similar mechanism. Moreover, because the ability of Myc to inhibit terminal differentiation is closely linked to its transforming activity (*13*), deregulated expression of Myc in vivo may initiate tumorigenesis by preventing replicative senescence.

Table 2. Number of G418-resistant and adipocyte (A) colonies in Myc-1 cells transfected with pMSV-C/EBP or pEMSV. The number of G418-resistant (G418^r) and adipocyte colonies was scored 12 days after commencement of selection. Colonies in which more than 50% of the cells contained lipid droplets were scored as adipocyte colonies.

Experiment	pEMSV		pMSV-C/EBP		
	G418^r	A	G418^r	A	%
1	313	0	156	7	4
2	296	0	147	9	6
Total	609	0	303	16	5*

*Mean

REFERENCES AND NOTES

1. P. J. Mitchell and R. Tjian, *Science* **245**, 371 (1989); M. Ptashne, *Nature* **335**, 683 (1988).
2. B. Luscher and R. Eisenman, *Genes Dev.* **4**, 2025 (1990).
3. K. Kelly *et al.*, *Cell* **35**, 603 (1983).
4. J. Campisi, H. Gray, A. Pardee, M. Dean, G. Sonenshein, *ibid.* **36**, 241 (1984); M. Dean *et al.*, *J. Biol. Chem.* **261**, 9161 (1986); L. Westin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2490 (1982); P. H. Reitsma *et al.*, *Nature* **306**, 492 (1983); H. Lachman and A. Skoultchi, *ibid.* **310**, 592 (1984).
5. We are using the term replicative senescence to describe the irreversible withdrawal from the cell cycle, which is often required for cellular differentiation. Quiescence, on the other hand, is reversible growth arrest induced by serum deprivation or high cell density. The term replicative senescence is also used to describe the in vitro cessation of cell division of normal human fibroblasts (*24*). This process is thought to be a type of terminal differentiation (*24*). Use of the term replicative senescence in this study is not meant to imply that the phenomenon described here is identical to the process of replicative senescence described for human fibroblasts, or that these processes are controlled by the same genes.
6. E. M. Blackwood and R. N. Eisenman, *Science* **251**, 1211 (1991); G. Prendergast and E. Ziff, *Cell* **65**, 395 (1991).
7. G. Klein, *Science* **238**, 1539 (1987); R. Sager, *ibid.* **246**, 1406 (1989).
8. W. H. Landschulz, P. F. Johnson, S. L. McKnight, *ibid.* **240**, 1759 (1988); A. Friedman *et al.*, *Genes*

Dev. **3**, 1314 (1989); Z. Cao, R. Umek, S. McKnight, *ibid.* **5**, 1538 (1991).
9. E. Birkenmeier *et al.*, *Genes Dev.* **3**, 1146 (1989).
10. R. Christy *et al.*, *ibid.*, p. 1323.
11. R. M. Umek, A. D. Friedman, S. L. McKnight, *Science* **251**, 288 (1991).
12. S. Freytag, *Mol. Cell. Biol.* **8**, 1614 (1988).
13. _____, C. Dang, W. Lee, *Cell Growth Differen.* **1**, 339 (1990).
14. The 3T3-L1 adipoblasts were grown as previously described (*12*). Subconfluent monolayers (~5 × 10^5 cells, 100-mm-diameter dish) were transfected with 5 μg of pZip-NeoSV(X) and with either 50 μg of pMSV-C/EBP or pEMSV using the $CaPO_4$-precipitation method (*12*). After 5 hours, cells were shocked for 1 min with Dulbecco's modified Eagle's medium that contained 15% glycerol and then allowed to recover overnight in growth medium. Cells were subcultured in growth medium and subjected to selection in growth medium that contained G418 (400 μg/ml) for 2 to 3 weeks.
15. C. Cepko *et al.*, *Cell* **37**, 1053 (1984).
16. R. Davis *et al.*, *ibid.* **51**, 987 (1987).
17. S. Freytag, unpublished results.
18. H. Green and O. Kehinde, *Cell* **1**, 113 (1974); H. Green and M. Meuth, *ibid.* **3**, 127 (1974).
19. After G418 selection, individual colonies were isolated with cloning cylinders and lysed in 10 mM tris-HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40 (50 μl) at room temperature for 1 min. The RT assay (20 μl) contained cell extract (4 μl), 50 mM tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM each of deoxyguanosine 5′-triphosphate, deoxyadenosine 5′-triphosphate, deoxythymidine 5′-triphosphate, deoxycytidine 5′-triphosphate, random hexamers (5 μM), 1 mM dithiothreitol, and 200 U of Moloney murine leukemia virus RT. The PCR assay (100 μl) was performed with the addition of the following to the RT mixture: 10 × PCR buffer [100 mM tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, and 0.1% (w/v) gelatin (10 μl)], 5′ and 3′ PCR primers (5 pmol, 1 μl), ^{32}P-labeled 5′ PCR primer (10^6 cpm, 1 μl), and *Taq* DNA polymerase (2.5 U, 0.5 μl). Samples were amplified in a DNA Thermal Cycler (Perkin-Elmer Cetus) for 30 cycles with denaturation at 95°C for 1 min, reannealing at 65°C for 2 min, and extension at 72°C for 1 min. Aliquots (10 μl) from the RT-PCR reactions were applied to 10% nondenaturing polyacrylamide gels. Gels were dried and analyzed by autoradiography. The PCR primers were: pMSV-C/EBP, 5′-GCGCCAGTCTTCCGATAGACTGCG-3′ and 5′-AAAGCCAAAGGCGGGCGTTGCTGG-3′, corresponding to nucleotides 3 to 26 of the MSV RNA (*25*) and nucleotides 209 to 232 of the rat C/EBPα sequence (*8*), respectively; mouse alpha 4 tubulin, 5′-GGCACCGGCTCTGGCTTCACCTCTC-3′ and 5′-GCCGTGGACACTTGGGGGGCTGG-3′, corresponding to nucleotides 459 to 483 and 546 to 568, respectively, of the mRNA sequence (*25*); and *aP2*, 5′-GTGGGAACCTGGAAGCTTGTCTCC-3′ and 5′-CACTTTCCTTGTGGCAAAGCCCAC-3′, corresponding to nucleotides 83 to 106 and 143 to 166, respectively, of the mRNA sequence (*20*).
20. C. Hunt, J. Ro, D. Dobson, H. Min, B. Spiegelman, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3786 (1986).
21. M. Schwab and J. M. Bishop, *ibid.* **85**, 9585 (1988).
22. R. Christy *et al.*, *ibid.* **88**, 2593 (1991).
23. T. K. Blackwell *et al.*, *Science* **250**, 1149 (1990); G. C. Prendergast and E. B. Ziff, *ibid.* **251**, 186 (1991).
24. S. Goldstein, *ibid.* **249**, 1129 (1990).
25. R. Dhar *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3938 (1980); A. Villasante *et al.*, *Mol. Cell. Biol.* **6**, 2409 (1986).
26. We thank B. Villeponteau, M. Anderson, and B.-S. Yang for readings of the manuscript, the McKnight Laboratory for the C/EBP expression plasmids and antisera, and S. Pendelton for generating and characterizing the NM12 cell line. Supported by NIH grant CA51748.

14 November 1991; accepted 19 February 1992

Cell Cycle–Regulated Binding of c-Abl Tyrosine Kinase to DNA

Edward T. Kipreos* and Jean Y. J. Wang†

The proto-oncogene c-*abl* encodes a protein tyrosine kinase that is localized in the cytoplasm and the nucleus. The large carboxyl-terminal segment of c-Abl was found to contain a DNA-binding domain that was necessary for the association of c-Abl with chromatin. The DNA-binding activity of c-Abl was lost during mitosis when the carboxyl-terminal segment became phosphorylated. In vitro phosphorylation of the DNA-binding domain by cdc2 kinase abolished DNA binding. Homozygous mutant mice expressing a c-Abl tyrosine kinase without the DNA-binding domain have been reported to die of multiple defects at birth. Thus, binding of the c-Abl tyrosine kinase to DNA may be essential to its biological function.

The proto-oncogene c-*abl* was first isolated from the mouse genome as a gene with similarity to the v-*abl* oncogene of Abelson murine leukemia virus (*1*). The c-*abl* gene encodes a protein tyrosine kinase that shares several common features with other cytoplasmic tyrosine kinases, for example, c-Abl has the Src-homology domains 2 (SH2) and 3 (SH3) (*2*). Unique to the c-Abl tyrosine kinase, however, is a large COOH-terminal segment. Mutant mice homozygous for a 3′-deletion of c-*abl* and expressing an active c-Abl tyrosine kinase truncated at its COOH-terminus have multiple defects at birth (*3*). This observation indicates that the COOH-terminal region of c-Abl is essential for its biological function. The c-Abl protein undergoes a cell cycle–regulated Ser-Thr phosphorylation and all the mitosis-specific phosphorylation sites are localized in the COOH-terminal segment (*4*). The mitotic phosphorylation does not appear to alter the tyrosine kinase activity of c-Abl (*4*). This result suggested that Ser-Thr phosphorylation might regulate other functions associated with the COOH-terminal segment.

While attempting to purify the c-Abl protein, we found that it was preferentially retained on a calf thymus DNA-cellulose column. When NIH 3T3 cell lysates were applied to DNA-cellulose columns (1.2 mg of protein per milliliter of bed volume in 100 mM KCl), less than 20% of the total cellular protein was bound, whereas 60 to 80% of the total c-Abl protein was retained

Department of Biology and Center for Molecular Genetics, University of California San Diego, La Jolla, CA 92093–0116.

*Present address: Department of Biology, Johns Hopkins University, Baltimore, MD 21218.
†To whom correspondence should be addressed.

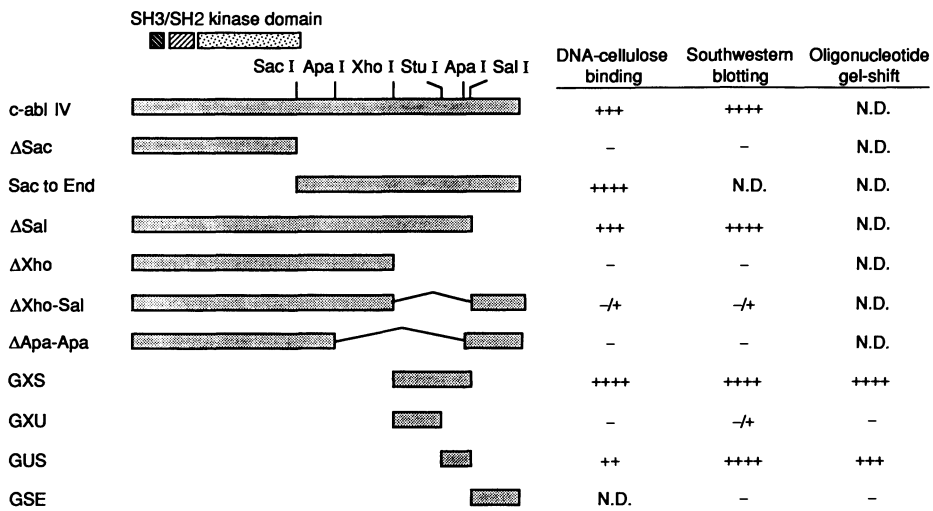

Fig. 1. Summary of constructs and results on DNA-binding assays. SH3, Src-homology domain 3; SH2, Src-homology domain 2; and N.D., not determined. The deletion and fusion proteins were prepared with the restriction enzymes indicated in the figure by standard recombinant DNA methods. Proteins were expressed in COS cells or in bacteria. DNA-cellulose binding (*5*): ++++ indicates 80 to 100% of the Abl protein in cell lysates was bound; +++ indicates 60 to 80% bound; ++ indicates 30 to 50% bound; −/+ indicates <5% bound; and − indicates no binding. Refer to Fig. 2 for Southwestern blotting and oligonucleotide gel shift results.