

SHORT REPORTS

Mutational analysis of the transcriptional activation domains of v-Myb

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A minimal transcription activation domain of the v-Myb oncoprotein was initially mapped to a central cluster of charged residues using GAL4-Myb fusion proteins. This region has been proposed to interact directly with the CBP co-activator in animal cells. Regions flanking this central domain of v-Myb are required for transcriptional activation by the native, unfused protein in both mammalian cells and in budding yeast. To identify the critical residues for transcriptional activation, we have now subjected the minimal activation domain and flanking regions including the heptad leucine repeat to random PCR-mediated mutagenesis. We found that the entire region examined can endure extensive substitutions without affecting transcriptional activation by v-Myb in budding yeast. The few mutations that did affect transcriptional activation altered acidic residues within the minimal activation domain or the heptad leucine repeat region, rather than leucine residues. Remarkably, there was a strong concordance between transcriptional activation in animal cells and in budding yeast, even though budding yeast have no known homologue of CBP or related co-activators. In contrast, there was not a strong correlation between transcriptional activation and oncogenic transformation.

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Introduction

We have previously demonstrated that both the v-Myb and c-Myb proteins can activate transcription in budding yeast (Chen and Lipsick, 1993). Analysis of a series of deletion mutants suggested a good correlation between transcriptional activation in animal cells and yeast. However, the functional importance of individual residues within the transcriptional activation domains remains unknown. Because of the relative ease of genetic manipulation in budding yeast, we have therefore performed random PCR-mediated mutagenesis on the region of v-Myb that encodes the transcriptional activation domains (Weston and

Bishop, 1989; Ibanez and Lipsick, 1990) (Figure 1a). For this purpose we cotransformed a gapped duplex 2 μ yeast shuttle plasmid that expresses v-Myb together with a pool of unligated PCR products generated under conditions favoring nucleotide misincorporation (Leung *et al.*, 1989). We then relied on the high frequency of homologous recombination in budding yeast to repair the gap with the mutagenized PCR products (Rothstein, 1991). The host strain contained a single integrated copy of a Myb-responsive reporter gene consisting of five tandem copies of a strong Myb-binding site from the chicken *mim-1* promoter, the minimal *CYCI* promoter and the *E. coli lacZ* reporter (Chen and Lipsick, 1993; Ness *et al.*, 1989). We used a standard blue-white colony lift assay to screen for either: (i) retention of transcriptional activation under highly mutagenic conditions; or (ii) loss of transcriptional activation under weakly mutagenic conditions (Scheda *et al.*, 1989).

In the first screen, colonies were identified that retained β -galactosidase activity (blue) on plates in which ~50% of the colonies had lost this activity (white). Plasmid DNA was isolated from individual replica-plated yeast colonies, cloned by transformation into *E. coli*, then amplified and retransformed into yeast to verify the phenotype. DNA sequencing of the region of v-Myb between the original PCR primers was then used to compile a list of amino acid substitutions in the region that are compatible with strong transcriptional activation (Figure 1b). Because of the high frequency of mutagenesis, each clone encoded more than one substitution. It should be noted that the nature of our PCR mutagenesis under low concentrations of dATP favored A to G or T to C transitions, consistent with the DNA substitutions that we observed. Nevertheless, we found that at least 80% of the residues in the region can be substituted without loss of function. Of the 27 remaining residues, 19 were in the group of codons that are refractory to this PCR mutagenesis method. A plot of the substitutions found in functional proteins revealed a clustering of non-mutable residues in the acidic and heptad leucine repeat regions (Figure 1c). A single threonine (Thr259) residue lying between these two regions also displayed no substitutions. However, site-directed mutagenesis of this threonine and an adjacent threonine to alanine residues (TT259/260AA) had no significant effect on transcriptional activation (data not shown). Remarkably, glycine and proline substitutions, both expect to serve as 'helix breakers', were well-tolerated at a

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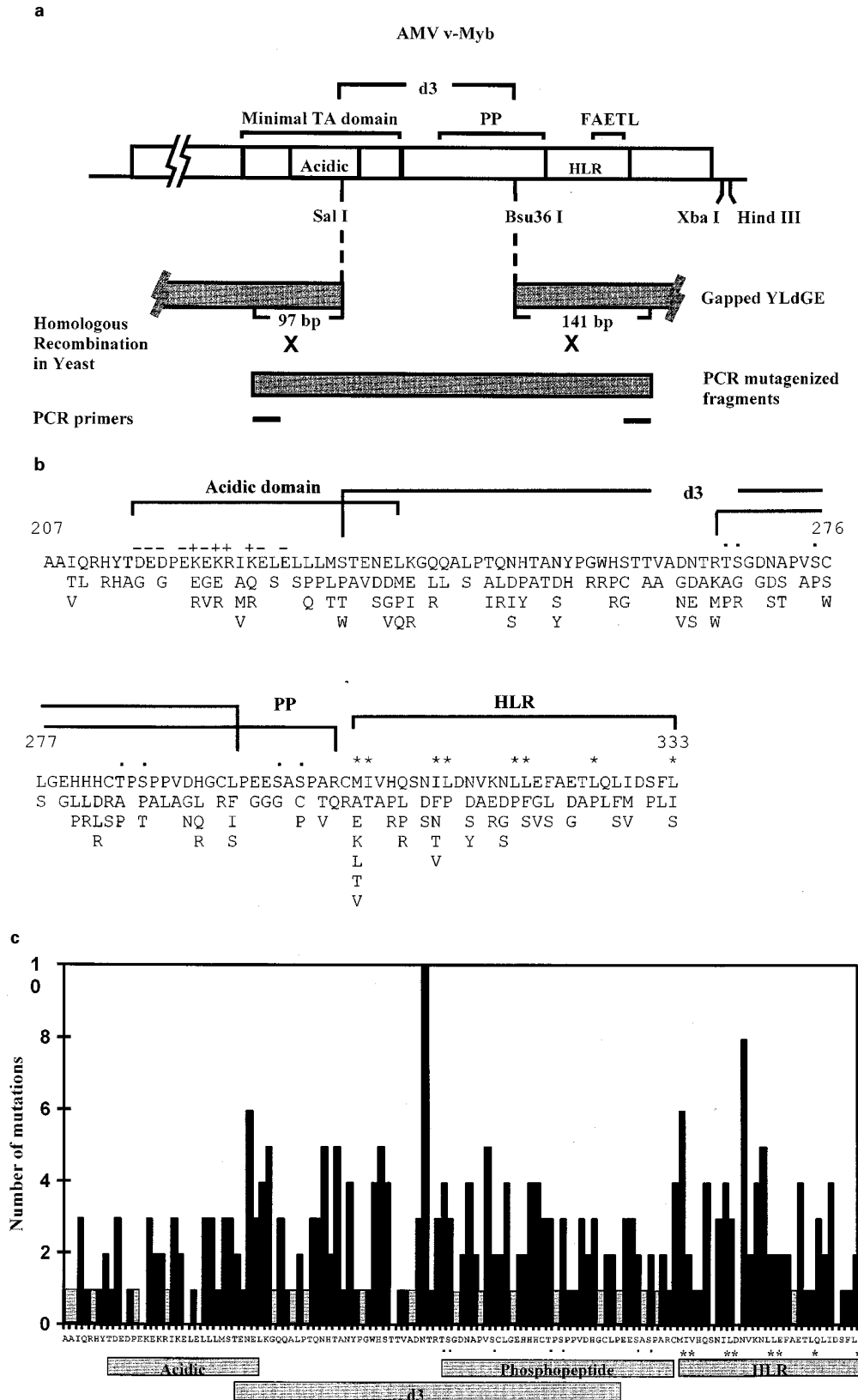


Figure 1 The amino acid substitutions of v-Myb mutants recovered from the retention of activity screen. (a) The top bar shows the various domains located in the C-terminal half of AMV v-Myb. These domains are termed minimal transactivation domain (TA), d3, phosphopeptide (PP) and heptad leucine repeat (HLR). The restriction enzyme sites used in this study are indicated below the bar. In the lower part of the figure, yeast vector (YLdGE) digested with *SalI* and *Bsu36I* to produce a gapped v-Myb plasmid was cotransformed with mutagenized PCR fragments into the yeast reporter strain, YSW5(+). The small black bars indicate the

variety of residues within the heptad leucine repeat that has been proposed to function as an amphipathic helix.

In a complementary analysis, we used less mutagenic conditions to isolate 170 non-functional mutant colonies (white) from plates on which most of the colonies (~90%) had wild type v-Myb function (blue). We then used immunoblotting to eliminate those non-functional mutants that did not encode full-length protein, most likely due to frameshift or nonsense mutations. The remaining eight mutants were then analysed by DNA sequencing along with two additional mutants from the first screen that consistently produced light blue colonies (Figure 2). Substitutions occurred sporadically throughout the analysed region. However, only five residues were mutated more than once, and four of these were clustered within the heptad leucine repeat. These four residues were acidic (aspartic or glutamic acid); none were leucines. Finally, when the two screens were compared, two residues stood out (Asp314 and Asp330). In particular, no substitutions of these two residues were ever recovered from blue colonies, but substitutions of one of these two residues occurred in five out of eight white colonies

in the loss of function screen. We therefore decided to focus further analysis on these residues.

To rule out the effects of substitutions outside the heptad leucine repeat region, we first reconstructed a set of the five white mutants with substitutions in Asp314 or Asp330 in which only the region 3' of the d3 deletion shown in Figures 1 and 2 was substituted into a wild type plasmid by swapping of a *Bsu36I/HindIII*-resistant DNA fragment. A quantitative β -galactosidase assay revealed that each of these mutants was severely compromised for transcriptional activation in yeast in a manner similar to the more amino-terminal d3 deletion mutant (Figure 3a). In addition, site-directed mutagenesis was used to show that alanine substitution of pairs of acidic residues or of all four acidic residues within the heptad leucine repeat region similarly reduced transcriptional activation in yeast (Figure 3a). The same v-Myb mutants were then expressed in quail QT6 fibroblasts and assayed for transcriptional activation using a Myb-responsive luciferase reporter gene (Figure 3b,c). Similar to the results obtained in yeast, these same mutations severely compromised transcriptional activation in animal cells.

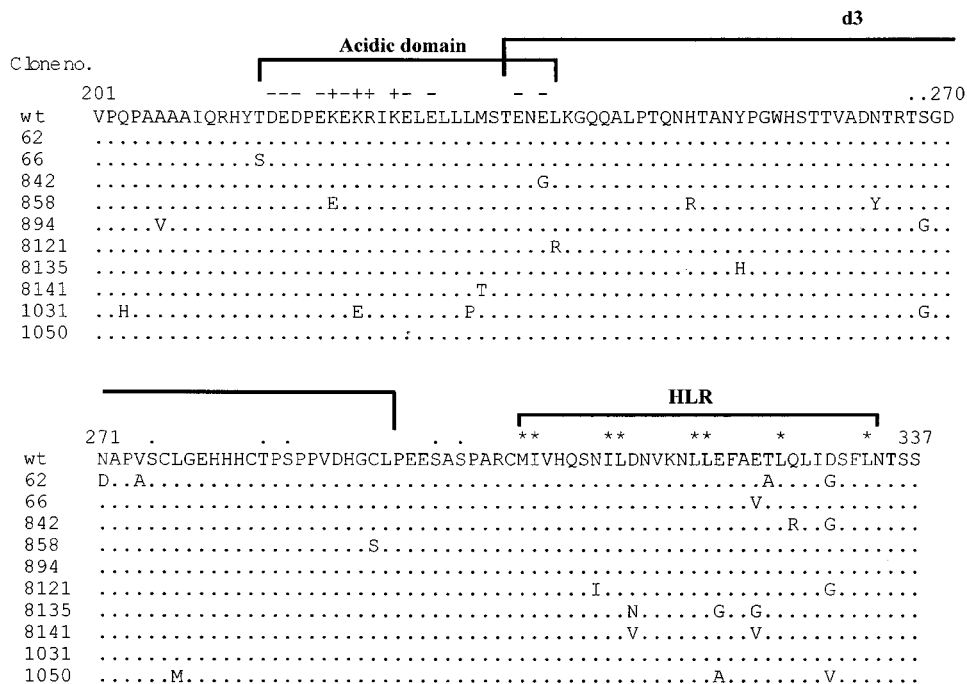


Figure 2 The amino acid substitutions of v-Myb mutants recovered from the loss of activity screen. The substitutions shown here are from the full-length v-Myb mutants with weak (#62, 66) or no detectable transcriptional activity in YSW5(+) cells in β -galactosidase filter assays. The dots represent the unchanged residues. All of the deduced amino acid substitutions present in each clone are indicated. The displayed sequence is the region subject to PCR mutagenesis. Sites of charged residues in the TA domain, potential phosphorylation sites, and residues that define the HLR are indicated with +/− signs, dots, and asterisks, respectively

PCR primer set used for the mutagenesis. (b) The compiled sequencing results shown here are from 47 transcriptionally-active v-Myb mutants analysed in the yeast reporter strain, YSW5(+). All the deduced amino acid substitutions are listed under the wild type v-Myb protein sequence. Only the region subjected to PCR mutagenesis is displayed (residues 207 to 333 of v-Myb). Sites of charged residues in the TA domain, potential phosphorylation sites in the major tryptic phosphopeptide, and residues that define the HLR are indicated with +/− signs, dots, and asterisks, respectively. (c) The total number of the substitutions found for individual residues is plotted against the v-Myb sequence. The gray bars indicate the unmutated residues (Ala, Gly, Pro and Arg) that are refractory to the PCR mutagenesis

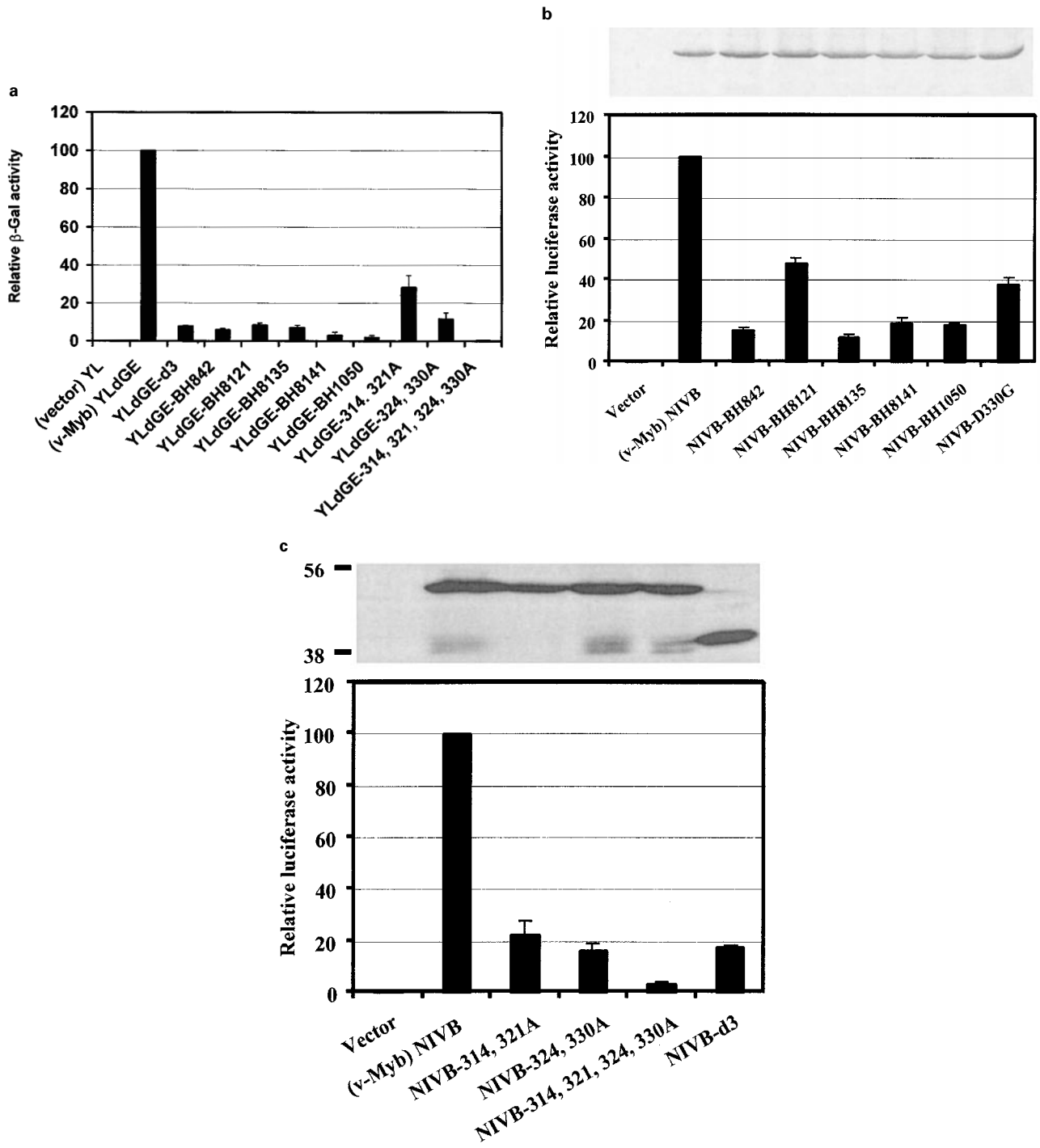


Figure 3 Transcriptional activation v-Myb mutants. (a) CPRG liquid β -galactosidase assays were performed on wild type v-Myb (YLdGE) and the indicated mutants in the yeast reporter strain, YSW5(+). YL is the empty vector control. Values were normalized using wild type v-Myb as 100. The error bars represent average deviations derived from at least three independent assays. (b and c) Luciferase assays were performed on wild type v-Myb (NIVB) and the indicated mutants in quail QT6 cells cotransfected with the EW5-E1b-luc reporter plasmid. After correcting for variations in transfection efficiency using an internal CMV- β -galactosidase control, values were normalized using wild type v-Myb as 100. The error bars represent average deviations derived from at least three independent assays. Immunoblots of representative transfections using an anti-Myb monoclonal antibody are shown above each graph

In addition, we showed that substitution of a single acidic residue found in two of the white mutants (D330G) was sufficient to significantly inhibit tran-

scriptional activation in animal cells. The steady-state levels of protein were unaffected by these mutations as shown by immunoblotting. These results demonstrate

Table 1 Transformation of primary chicken yolk sac cells by the v-Myb mutants

Sequence	Mutant	Trans activ in yeast	Trans activ in QT6	Oncogenic transformation
<div style="display: flex; justify-content: space-around; margin-bottom: 5px;"> 314 321 324 330 </div> <div style="display: flex; justify-content: space-around; margin-bottom: 5px;"> — — — </div> MIVHQSNILDNVKNLLEFAETLQLIDSFL	wild type	100	100	100
.....R..G...	BH-842	6	16	1
.....I.....G...	BH-8121	9	48	1
.....N.....G..G.....	BH-8135	7	12	16
.....V.....V.....	BH-8141	3	19	0
.....A.....V.....	BH-1050	2	18	0
.....A.....A.....	314, 321A	28	22	6
.....A.....A.....A.....	324, 330A	12	16	0
.....A.....A..A.....A...	314, 321, 324, 330A	1	3	0

The transforming activity of v-Myb mutants was analysed on primary hematopoietic cells derived from embryonic day 11 and 12 chicken yolk sacs (Fu and Lipsick, 1996). The virus-infected primary hematopoietic cells were seeded at 10^5 cells per 5-cm diameter dish in 0.8% methyl cellulose. Colonies were counted after 2 weeks. Relative colony numbers were obtained by assigning the colony count of wild type v-Myb (NIVB) a value of 100 for each experiment then calculating the mean among four different experiments

that specific acidic residues, but not the leucine residues within the heptad leucine repeat region of v-Myb are essential for transcriptional activation in both yeast and in animal cells. Furthermore, there was a good correlation between transcriptional activation by v-Myb mutants in yeast and in animal cells, even though yeast do not have homologues of many transcriptional co-activators found in animal cells. In particular, the CBP co-activator that has been proposed to bind directly to Myb proteins (Dai *et al.*, 1996; Oelgeschlager *et al.*, 1996) and to be required for transcriptional activation is not found in yeast.

Finally, we wished to further probe the correlation between transcriptional activation and oncogenic transformation by v-Myb. Our initial studies of linker insertion mutants of v-Myb had suggested a strong correlation between these two activities (Lane *et al.*, 1990). However, subsequent analyses of small deletion mutants demonstrated a dissociation of these two activities (Chen *et al.*, 1995). Furthermore, the heptad leucine repeat region was found to be critical for oncogenic transformation (Bartunek *et al.*, 1997; Fu and Lipsick, 1996). Because the series of white mutants described in the present study was isolated solely on the basis of their inability to activate transcription, we

thought that an analysis of their oncogenic transforming activities might be particularly informative. We found that although some mutants in the heptad leucine repeat were severely compromised for both transcriptional activation and oncogenic transformation, two mutants did not fit this pattern (Table 1). In particular, the mutant with the greatest transcriptional activity in animal cells did not cause oncogenic transformation (BH-8121). Conversely, the single mutant which consistently caused oncogenic transformation was an extremely weak transcriptional activator (BH-8135). These results support models in which the oncogenic transformation by v-Myb results not from transcriptional activation, but rather from some other function such as transcriptional repression or non-transcriptional interference with normal cell cycle regulation (Ganter and Lipsick, 1999).

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