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Reply from John A. Baross* and Jody W. Deming†

* School of Oceanography, Oregon State University, Corvallis, Oregon 97331, USA
† Chesapeake Bay Institute and Department of Biology, The Johns Hopkins University, Shady Side, Maryland 20764, USA

The conclusion of Trent *et al.* that no bacterial growth occurred in our experiments[1] is not tenable because of crucial differences between the results of our work and theirs, and because of additional data not addressed by these authors. If the scores of morphologically diverse bacteria we observed in thin sections of a 250 °C sample were contaminants, they would have appeared in the 300 °C sample also reported[1], since both samples were processed simultaneously using the same reagents. Such was not the case. Recent amino acid analyses[2] from our original experiments[1] indicate that at 250 °C the total amino acid content of particulate protein increased from 1.6 to 145 $\mu g\ ml^{-1}$ in 5.4 h, a doubling time of approximately 1 h. The same doubling time was calculated for concentrations of each of the 15 amino acids (including serine and threonine) detected by the standard methods we used[1,2]. We continue to work with the original culture of black smoker bacteria, as well as the viable culture recovered from the titanium syringe upon termination of the 250 °C experiment, and one of us will dive with the French in March 1984 to search for similar bacteria in unexplored smokers at 13° N.

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Role of an upstream regulatory element in leucine repression of the *Saccharomyces cerevisiae leu2* gene

Alfonso Martinez-Arias*‡, H. Joseph Yost† & Malcolm J. Casadaban*†

* Department of Biophysics and Theoretical Biology, and
† Committee on Genetics, University of Chicago, Chicago, Illinois 60637, USA

The expression of a number of eukaryotic genes has been shown to involve at least two sequences located upstream of the actual transcription unit[1–7]: one of these sequences, centred on a widely conserved TATAAT sequence, is thought to be involved in determining the precise site of initiation of transcription[1,8]; the other has a gene-specific sequence, can function at a variable distance upstream of the initiation site[3,9], and is involved in the regulation of transcription[3,4,7,10]. By constructing β-galactosidase gene fusions, to facilitate measuring gene expression *in vivo*, we have now defined a *cis*-acting regulatory element of the *Saccharomyces cerevisiae leu2* gene. This element is located within a 280 base pair (bp) fragment which occurs 125 bp upstream of the *leu2* translation initiation codon and which contains a short G+C-rich palindromic sequence. A fragment of the *Escherichia coli* transposable element Tn*9* which contains a similar palindromic sequence can functionally replace the natural *leu2* regulatory element. Our results are contrary to previous speculations that the *leu2* gene is regulated by an attenuation mechanism[11,12].

Biochemical studies of the *leu2* locus of *S. cerevisiae*, which encodes β-isopropylmalate dehydrogenase, an enzyme of the leucine biosynthetic pathway, have indicated that enzymatic activity is repressed when cells are grown in the presence of leucine and threonine[13]. DNA sequence analysis of the region upstream of *leu2*[12] has revealed the complex arrangement (Fig. 1) of a Ty*1* transposable element, a leucyl tRNA gene and an open reading frame with the potential for encoding a 24 amino acid polypeptide containing 6 leucine residues. This led to speculation about the regulatory mechanisms that may be operating at this locus[11,12]. To investigate further the nature of this regulation an *Xho*I–*Bst*EII fragment, which extends from the Ty*1* δ element to the 13th *leu2* codon, was fused *in vitro* to the *E. coli* β-galactosidase gene on the vector pMC1790[14,15], as described in Fig. 1. Comparable fusions to the β-galactosidase gene have been used to study the regulated expression of several other genes in yeast[16–18]. The resulting fusion plasmid pMC1876 expresses a level of β-galactosidase in transformed[19] yeast cells that is repressed fivefold when the cells are grown in the presence of 1 mM leucine and 1 mM threonine (Table 1).

The *leu2* control region in the fusion plasmid was dissected as illustrated in Fig. 2 and assayed as summarized in Tables 1 and 2. A deletion from the upstream side extending to the middle of the $tRNA_3^{leu}$ gene sequence (Δ1) results in a small increase in β-galactosidase expression but no qualitative changes in the pattern of regulation. Total removal of $tRNA_3^{leu}$ gene sequences in this region (Δ8) leads to a further increase in β-galactosidase expression. This suggests that the tRNA gene is not directly involved in the regulation of this locus, although its presence does exert a small effect on *leu2* expression, perhaps due to steric interactions between factors bound to these two closely linked genes[20]. Removal of sequences up to the *Hin*cII site located 125 bp upstream of the *leu2* translation initiation codon (Δ2) results in a 100-fold decrease in β-galactosidase activity and the loss of leucine-specific regulation. This indicates that regions important for regulation exist between the tRNA gene and the *Hin*cII site. Moreover, as the Δ2 deletion does not remove the major transcription initiation site (A. Andreadis and P. Schimmel, personal communication) the regulation of this locus presumably takes place primarily at the level of transcription.

To explore this further, we exchanged the DNA fragment between the *Hin*cII site and the *Bst*EII site in Δ1 with a 250 bp piece of DNA containing the transcription and translation initiation regions from the *cyc1* gene of *S. cerevisiae* fused to the β-galactosidase gene[17,21]. When this fusion, which has a hybrid promoter (HYΔ1), was assayed in yeast, β-galactosidase activity was regulated by leucine and threonine (Table 2). This fusion does not respond to other amino acids in the culture medium nor to catabolite repression, which is the normal regulatory pattern of the *cyc1* gene. This indicates that the *leu2* regulatory element is located upstream of the *Hin*cII site and well before the *leu2* coding region. It is interesting, however, that the levels of β-galactosidase expressed by HYΔ1 are lower than those of the wild-type *leu2* promoter, and that the ratio of the levels of expression in repressing and non-repressing conditions is larger. This might reflect differences in translation or stability of the hybrid proteins from the fusions, or it might reflect a requirement for a precise interaction between the upstream regulator and the initiation site-proximal sequences, which is altered in the hybrid promoter.

In the DNA sequence[12] between the Δ8 and the Δ2 end points, we noted the presence of a short palindromic sequence

‡ Present address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

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