Proc. Natl. Acad. Sci. USA
Vol. 77, No. 2, pp. 1078–1082, February 1980
Genetics

# Functional expression in yeast of the *Escherichia coli* plasmid gene coding for chloramphenicol acetyltransferase

(chloramphenicol resistance/*Saccharomyces cerevisiae* transformation/heterologous gene expression)

JOE D. COHEN*, T. ROSS ECCLESHALL*, RICHARD B. NEEDLEMAN†, HOWARD FEDEROFF*, BARBARA A. BUCHFERER*, AND JULIUS MARMUR*

*Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York, 10461; and †Department of Biochemistry, Wayne State University, School of Medicine, 540 East Canfield Avenue, Detroit, Michigan 48201

*Communicated by Salome G. Waelsch, November 13, 1979*

**ABSTRACT** **The *Escherichia coli* R factor-derived chloramphenicol resistance ($cam^r$) gene is functionally expressed in the yeast *Saccharomyces cerevisiae*. The gene was introduced by transformation into yeast cells as part of a chimeric plasmid, pYT11-LEU2, constructed *in vitro*. The plasmid vector consists of the *E. coli* plasmid pBR325 (carrying the $cam^r$ gene), the yeast 2-μm DNA plasmid, and the yeast *LEU2* structural gene. Yeast cells harboring pYT11-LEU2 acquire resistance to chloramphenicol and cell-free extracts prepared from such cells contain chloramphenicol acetyltransferase (acetyl-CoA: chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28), the enzyme specified by the $cam^r$ gene in *E. coli*. Resistance to chloramphenicol and the presence of chloramphenicol acetyltransferase activity segregate with the yeast marker *LEU2*, carried by the transforming plasmid, during both mitotic growth and meiotic division.**

The eukaryotic microorganism *Saccharomyces cerevisiae* appears to occupy an intermediate position between prokaryotes and higher eukaryotes in the processing of nuclear transcripts. In prokaryotes, transcription and translation are closely coupled; in all eukaryotes, the nuclear transcripts are initially generated and primarily modified in the nucleus before being transported to the cytoplasm where they are subsequently translated. In *S. cerevisiae*, as in other eukaryotes, modification of mRNA involves posttranscriptional capping and poly(A) addition at their 5′ and 3′ ends, respectively; however, in contrast to higher eukaryotes, splicing of the mRNA has not yet been demonstrated. In the most extensively studied case in yeast there is a strict colinearity between the nucleotide and amino acid sequences of the iso-1-cytochrome *c* gene and the protein for which it codes (1).

The advent of recombinant DNA technology and the development of a procedure for the transformation of yeast (2) makes it possible to investigate the behavior and expression of genes in heterologous cellular environments. Although several laboratories have reported that eukaryotic genes can be transcribed (3, 4) and in some cases translated and expressed (5–8) in *Escherichia coli*, there are no published reports of prokaryotic genes being expressed in yeast. The present paper describes experiments which demonstrate that the bacterial plasmid gene $cam^r$, which specifies resistance to chloramphenicol in *E. coli* (9), is functionally expressed in yeast. The enzymatic activity coded for by the $cam^r$ gene in *E. coli*, chloramphenicol acetyltransferase (acetyl-CoA: chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) (10), is detectable in yeast cells harboring a plasmid carrying the gene. It is significant that the expression of the $cam^r$ gene in yeast results in the acquisition by transformed cells of an easily detectable phenotype—namely, resistance to chloramphenicol. The possible use of the latter observation for the investigation of the mechanisms of transcription, RNA processing, and translation in eukaryotes will be discussed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

## MATERIALS AND METHODS

**Bacterial and Yeast Strains.** The following strains were used in this work: *E. coli*, RR101 ($F^-$ *pro leu thi lac*Y $str^r$ $r_k^-$ $m_k^-$) harboring plasmid pBR325; and *S. cerevisiae* 21D (*α leu 2-3 leu 2-112 his 4 lys 1*), AH22 (*a leu 2-3 leu 2-112 his 4*), 11C (*a leu 2-3 leu 2-112*), and 2A (*α leu 2-3 leu 2-112*).

**Media.** L medium (11), supplemented with chloramphenicol at 20 μg/ml when required, was used for culturing bacterial cells. Solid or liquid media for the growth of yeast cells were: YEPD (1% yeast extract, 2% peptone, 2% glucose); YEPGE (1% yeast extract, 2% peptone, 3% glycerol, 2% ethanol) supplemented, when necessary, with chloramphenicol at the appropriate concentration as indicated in table and figure legends; and YNBD (0.7% yeast nitrogen base without amino acids, 2% glucose) supplemented with the required amino acids as described (12).

**Transformation of Yeast.** The procedure described by Hinnen *et al.* (2) was used.

**Assay of Chloramphenicol Acetyltransferase.** Cell-free extracts from *E. coli* strain RR 101 harboring pBR325 were prepared by suspending the cells in 100 mM Tris·HCl at pH 7.8 and sonicating the suspension three times for 15 sec each time. The supernatant resulting from centrifugation at 27,000 × *g* for 30 min was used for enzyme assays. Yeast cell-free extracts were prepared from 7-ml cultures grown in appropriately supplemented YNBD medium. The cells in early stationary phase (≈$10^8$ cells per ml) were harvested by centrifugation, washed with water, suspended in 0.4 ml of 100 mM Tris·HCl at pH 7.8, and homogenized in the presence of glass beads for 2 min in a Braun homogenizer. After centrifugation at 7800 × *g*, the supernatant was used for enzyme assays. The enzyme reaction mixture (total volume, 0.25 ml) contained 23 nmol of Tris·HCl (pH 7.8), 40 nmol of acetyl-CoA, 5–10 nmol of [$^{14}$C]chloramphenicol (43.8 mCi/mmol; 1 Ci = 3.7 × $10^{10}$ becquerels; New England Nuclear), and cell-free extract containing 0.2–1 mg of protein. Incubation of the reaction mixture was at room temperature for the yeast extracts and at 37°C for bacterial extracts. The reaction was terminated by adding 1 ml of ice-cold 2 M Tris base and extracting with ice-cold ethyl acetate when the activity was to be assayed by thin-layer chromatography or with ice-cold benzene when selective extraction of the acetylated derivatives of chloramphenicol was

desired (10). The organic extracts were evaporated to dryness in an air stream and dissolved in 100 μl of 95% ethanol. For determination of radioactivity, aliquots were added to 5 ml of Bray's scintillation fluid and assayed in a liquid scintillation counter. For identification of acetylated chloramphenicol, aliquots were chromatographed on silica gel sheets (Eastman chromatogram sheet, no. 6061) with a chloroform/methanol, 9:1 (vol/vol) solvent system. The dried thin-layer plates were autoradiographed at −70°C with Kodak No-Screen film.

**Yeast Genetic Techniques.** The techniques and media have been described (12).

## RESULTS

**Description of the Recombinant Plasmids Used in This Work.** Plasmid pYT11-LEU2 is a composite vector consisting of the bacterial plasmid pBR325 (13), the yeast 2-μm DNA plasmid (14), and a fragment of DNA containing the yeast *LEU2* structural gene derived from plasmid pYeleu10 (6) (Fig. 1). The entire 2-μm DNA was ligated to pBR325 at the *Pst* I restriction site within the ampicillin-resistance ($amp^r$) gene. An *Xho* I/*Sal* I restriction fragment of plasmid pYeleu10, containing the yeast *LEU2* gene, was ligated at the *Sal* I cleavage site of the pBR325-2 μm DNA chimera, within the tetracycline-resistance ($tet^r$) gene. The resulting composite vector has the genotype $cam^r$ $amp^s$ $tet^s$. Plasmid pYe13 was constructed and kindly made available to us by J. Hicks, J. Broach, J. Strathern, and A. Klar. It is a composite of pBR322 (15), the smaller *Eco*RI restriction fragment of the 2-μm DNA, and a DNA segment containing the yeast *LEU2* structural gene. It has the genotype $amp^r$ $tet^r$. Unlike pYT11-LEU2, pYe13 does not contain the $cam^r$ gene. Both plasmids transform yeast cells with high frequencies and both behave as nonchromosomal genetic elements in the yeast host (data not shown).

**Yeast Cells Harboring pYT11-LEU2 Acquire Resistance to Chloramphenicol.** A leucine-requiring yeast strain, 21D, carrying a double mutation in the *LEU2* gene, was transformed to leucine prototrophy with either pYT11-LEU2 or pYe13 DNA. Several independent leucine prototrophic (Leu$^+$) transformants were randomly selected and tested for their level of resistance to chloramphenicol. Resistance to the drug was determined by replica plating on solid medium containing the nonfermentable energy sources glycerol and ethanol (YEPGE), supplemented with chloramphenicol at 500 μg/ml. Cellular

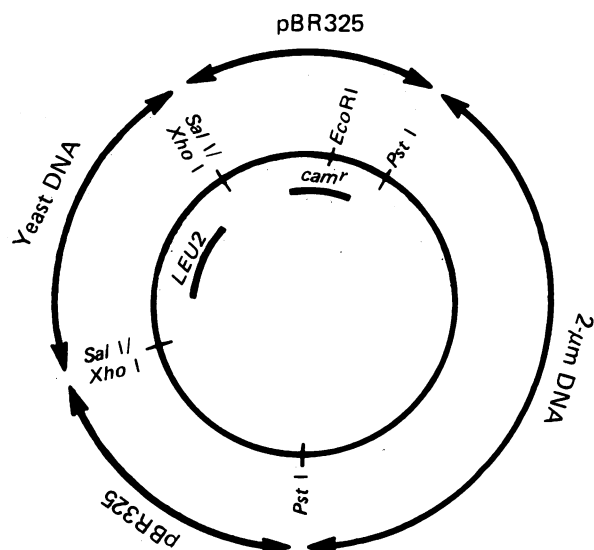



FIG. 1. Diagram of the components of plasmid pYT11-LEU2 (9.6 × 10$^6$ daltons).

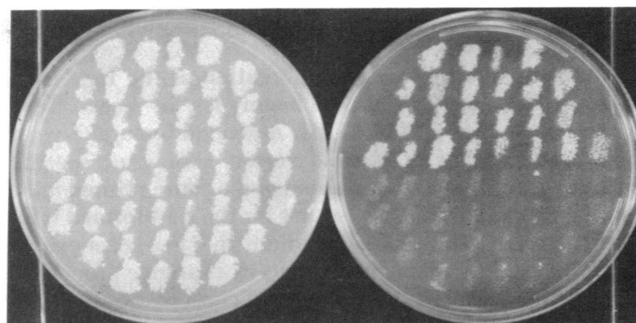


FIG. 2. Chloramphenicol resistance of Leu$^+$ transformants obtained in strain 21D with either pYT11-LEU2 DNA (top half of plates) or pYe13 DNA (bottom half of plates). (*Left*) YNBD supplemented with lysine and histidine but lacking leucine. (*Right*) YEPGE plus chloramphenicol at 500 μg/ml.

metabolism and growth on these carbon sources require functional mitochondria which, in yeast, is the cellular target of chloramphenicol; only cells that are resistant to the antibiotic will grow on YEPGE plus chloramphenicol. Invariably, all Leu$^+$ transformants generated with pYT11-LEU2 DNA expressed a higher level of resistance to chloramphenicol than did those obtained with pYe13 DNA (Fig. 2). Identical results were obtained when several different *leu2* mutant strains were used as recipients in similar transformation experiments (data not shown). In addition, the chloramphenicol-resistance level of pYe13 transformed cells was identical to that of untransformed cells of the same strain (data not shown). Growth experiments in liquid media confirmed the results obtained by the replica plating tests (Fig. 3). This initial observation can be simply explained by postulating that a plasmid-borne genetic determinant, very likely the $cam^r$ gene, is responsible for the resistance to chloramphenicol of yeast cells transformed with pYT11-LEU2. The results of the experiments described in the

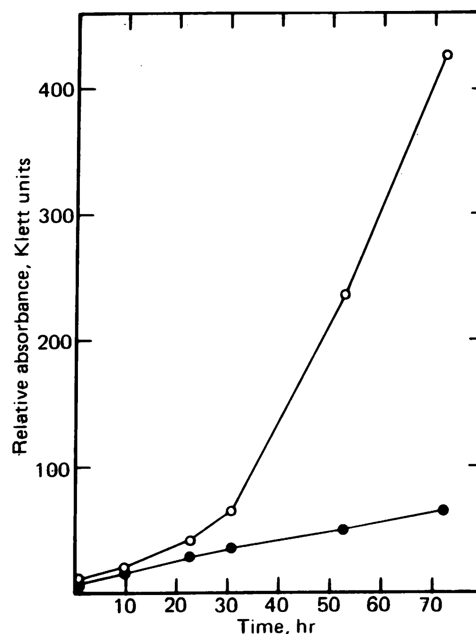



FIG. 3. Growth curves, in YEPGE plus chloramphenicol (200 μg/ml), of Leu$^+$ transformants of strain 21D. The cultures were grown in a water bath at 30°C with vigorous shaking. Turbidity was measured by using a Klett colorimeter fitted with a red filter. ○, Transformants obtained with plasmid pYT11-LEU2; ●, transformants obtained with plasmid pYe13.

following sections demonstrate that this hypothesis is indeed correct.

**Mitotic Instability and Cosegregation of the Leu$^+$ and Chloramphenicol-Resistance Phenotypes in Yeast Cells Transformed with pYT11-LEU2.** A general feature of yeast cells transformed with plasmid vectors containing specific segments of the 2-$\mu$m DNA plasmid or the entire plasmid is the mitotic instability that they exhibit for the transformed trait. Growth in rich (nonselective) medium and, to a lesser extent, in synthetic selective medium results in a variable proportion of segregants losing the transformed phenotype (16–18). Cells transformed with pYT11-LEU2 are no exception to this general rule. This characteristic provides a simple means for testing the hypothesis advanced in the preceding section. Thus, the concurrent loss or retention of the plasmid-specified Leu$^+$ phenotype and of resistance to chloramphenicol would provide strong evidence that the latter is also plasmid determined. The following experiment was therefore performed.

A Leu$^+$ transformant of strain 21D, obtained with pYT11-LEU2 DNA, was grown under the conditions described in the legend to Table 1. The phenotype of several individual segregants, derived from each of the growth media used, was then tested by replica plating on the appropriate solid media. There was an absolute correlation between the retention of the Leu$^+$ and of the chloramphenicol-resistance phenotypes, or their loss, among mitotic segregants of cells transformed with pYT11-LEU2 DNA (Table 1). Furthermore, growth of these transformants on rich medium (YEPGE) containing chloramphenicol was as efficient as growth in synthetic medium lacking leucine, in exerting selective pressure for the retention of the plasmid-determined phenotype Leu$^+$. These results confirm that resistance to chloramphenicol is itself plasmid determined.

**Meiotic Segregation of the Leu$^+$ and Chloramphenicol-Resistance Phenotypes.** Further evidence of the physical linkage between the genetic determinants for Leu$^+$ and chloramphenicol resistance is provided by the segregation pattern of both traits during meiotic division. Table 2 presents the results of such a meiotic analysis. The tetrads analyzed here were derived from a cross between a Leu$^+$CAM$^R$ transformant generated in strain AH22 with pYT11-LEU2 DNA and a leu$^-$cam$^s$ strain, 2A. As expected, a non-Mendelian segregation pattern was obtained for both traits. But more importantly, the type of segregation obtained indicates strong genetic linkage

Table 1. Cosegregation of Leu$^+$ and CAM$^R$ phenotypes among mitotic segregants of transformants obtained with plasmid pYT11-LEU2

| Growth medium | Clones scored, no. | Phenotypes among segregants*: Leu$^+$CAM$^R$ | leu$^-$cam$^s$ |
|---|---|---|---|
| YEPGE | 248 | 179 (72%) | 69 (28%) |
| YNBD + histidine + lysine | 167 | 143 (86%) | 24 (14%) |
| YEPGE + chloramphenicol (200 $\mu$g/ml) | 250 | 242 (97%) | 8 ( 3%) |

A Leu$^+$ transformant of strain 21D, obtained with plasmid pYT11-LEU2, was grown for 10 generations in the media listed. The cells from each culture medium were then diluted, plated on YEPD, and subsequently replica-plated on YNBD plus histidine plus lysine or on YEPGE plus chloramphenicol (500 $\mu$g/ml). The phenotype of individual clones was determined after incubation of the plates at 30°C for 4–6 days

* CAM$^R$, resistant to chloramphenicol; cam$^s$, sensitive to chloramphenicol.

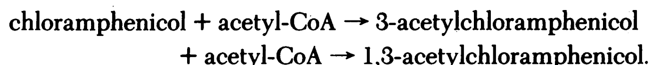
Table 2. Meiotic segregation of the Leu$^+$ and CAM$^R$ phenotypes

| Phenotype of spore clones and pattern of segregation in complete tetrads: Leu$^+$CAM$^R$ | leu$^-$cam$^s$ | No. of tetrads in each segregational class |
|---|---|---|
| 4 | 0 | 11 |
| 3 | 1 | 2 |
| 2 | 2 | 1 |
| 1 | 3 | 1 |
| 0 | 4 | 11 |

A Leu$^+$ transformant of strain AH22, obtained with plasmid pYT11-LEU2, was crossed to strain 2A. Both untransformed AH22 and 2A are resistant to up to 2 mg of chloramphenicol per ml, but both are sensitive on medium containing 3 mg/ml. The Leu$^+$ transformants of AH22 used in this cross are resistant to 3 mg/ml chloramphenicol. Spore clones from complete tetrads were tested by replica plating on YNBD plus histidine and on YEPGE plus chloramphenicol at 3 mg/ml. The plates were scored after 4–6 days' incubation at 30°C.

between the determinants for the Leu$^+$ and CAM$^R$ phenotypes.

**Yeast Cells Transformed with Plasmid pYT11-LEU2 Contain Chloramphenicol Acetyltransferase Activity.** In *E. coli*, the plasmid *cam$^r$* gene codes for a detoxification enzyme, chloramphenicol acetyltransferase (10). The enzyme inactivates the antibiotic via the following reactions:



chloramphenicol + acetyl-CoA → 3-acetylchloramphenicol
+ acetyl-CoA → 1,3-acetylchloramphenicol.

Both the mono- and diacetylated derivatives of the drug are inactive and can be readily identified by thin-layer chromatography (10). Cell-free extracts of strain 21D transformed with pYT11-LEU2 were assayed for chloramphenicol acetyltransferase activity, along with appropriate controls. The presence of monoacetylated chloramphenicol among the reaction products (Fig. 4) demonstrates that the extracts contained chloramphenicol acetyltransferase activity. In contrast, extracts from cells transformed with plasmid pYe13 did not exhibit this enzymatic activity. The activity was thermolabile and dependent on acetyl-CoA (data not shown). The kinetics of accumulation of acetylated chloramphenicol was linear for up to 40 min (Fig. 5).

To further verify that monoacetylchloramphenicol was a reaction product [*acetyl*-$^3$H]acetyl-CoA (1.5 nmol; 1.3 Ci/mmol; New England Nuclear) was added in addition to nonlabeled acetyl-CoA in the reaction mixture (see *Materials and Methods*). The reaction products were analyzed by thin-layer chromatography, and the spot corresponding to monoacetylchloramphenicol was cut out and assayed for radioactivity. From the ratio of $^3$H to $^{14}$C, it was determined that the molar ratio of acetyl groups to chloramphenicol was 0.97:1. A similar value (0.95:1) was obtained when the *E. coli* extract was substituted in the reaction. It was concluded that the yeast extract catalyzes the formation of monoacetylated chloramphenicol, as does the bacterial extract, by the transfer of the acetyl group from acetyl-CoA to chloramphenicol. Fig. 6 illustrates the fact that, in the meiotic progeny of appropriate crosses, chloramphenicol acetyltransferase activity cosegregated with the Leu$^+$ CAM$^R$ phenotype.

## DISCUSSION

The data presented in this paper demonstrate that the *E. coli* plasmid gene *cam$^r$* is functionally expressed in yeast cells. Yeast cells harboring plasmid pYT11-LEU2, carrying the *cam$^r$* gene, display the following characteristics: (*i*) resistance to chloramphenicol at a level sufficiently higher than that of control

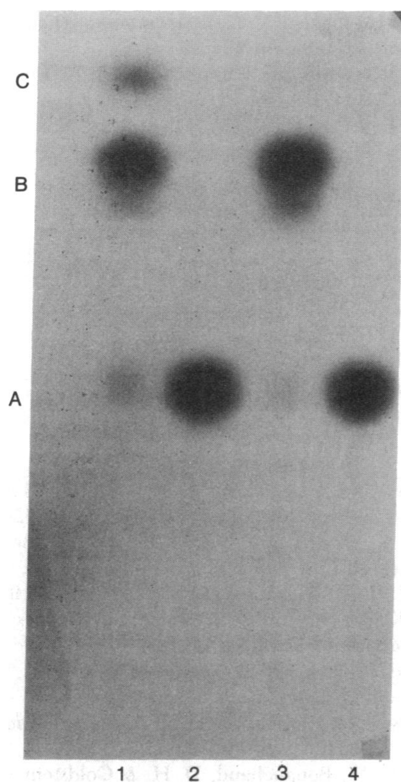



FIG. 4. Conversion of chloramphenicol to its acetylated derivatives by crude extracts of yeast cells harboring plasmid pYT11-LEU2. Extracts were from: *E coli* RR101 harboring pBR325 (lane 1); yeast strain 21D harboring pYe13 (lane 2); 21D harboring pYT11-LEU2 (lane 3). For reference, [$^{14}$C]chloramphenicol was run in lane 4. Bands: A, chloramphenicol; B, monoacetylated chloramphenicol; C, diacetylated chloramphenicol.

cells so as to be easily detectable on either solid or liquid media; (*ii*) linkage of the genetic determinant for chloramphenicol resistance to the plasmid-borne *LEU2* marker; and (*iii*) presence, in cell-free extracts, of chloramphenicol acetyltransferase,

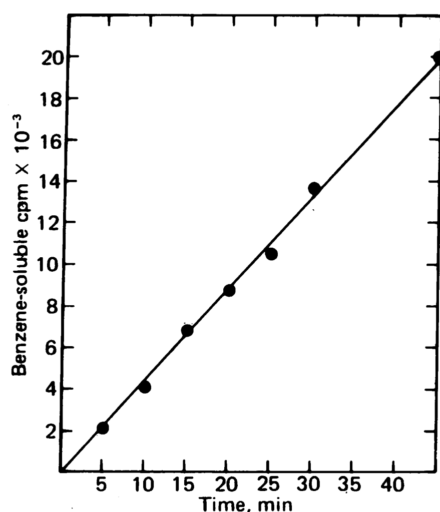



FIG. 5. Kinetics of formation of acetyl chloramphenicol by an extract of a yeast transformant of strain 21D harboring plasmid pYT11-LEU2. Benzene extraction at alkaline pH allows the selective recovery of the acetylated derivatives of the drug in the organic phase; the unmodified chloramphenicol remains in the aqueous phase (10). This was verified by chromatographing aliquots of the benzene extracts (data not shown here).

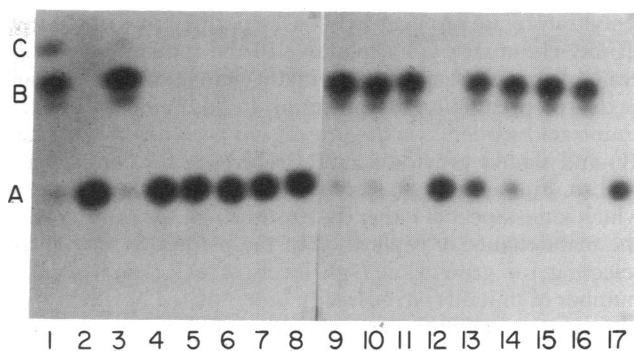



FIG. 6. Meiotic segregation of chloramphenicol acetyltransferase activity. Extracts of spore clones of three complete tetrads derived from a cross between strain 21D harboring pYT11-LEU2 and strain 11C were tested for their ability to convert chloramphenicol to the acetylated derivative. Extracts were from *E. coli* RR101 harboring pBR325 (lane 1), strain 11C (lane 2), strain 21D harboring pYT11-LEU2 (lane 3), strain 21D harboring pYe13 (lane 4); spore clones of a tetrad that gave 0:4 segregation for Leu$^+$CAM$^R$:leu$^-$cam$^s$ (lanes 5–8); spore clones of a tetrad that gave 3:1 segregation for Leu$^+$-CAM$^R$:leu$^-$cam$^s$ (lanes 9–12); spore clones of a tetrad that gave 4:0 segregation for Leu$^+$CAM$^R$:leu$^-$cam$^s$ (lanes 13–16). For reference, [$^{14}$C]chloramphenicol was run in lane 17. Bands: A, chloramphenicol; B, monoacetylated chloramphenicol; C, diacetylated chloramphenicol.

the enzymatic activity specified by the *cam$^r$* gene in *E. coli.* None of these characteristics is displayed by cells of the same strain harboring a related plasmid, pYe13, which does not carry the *cam$^r$* gene. An interesting question raised by this observation concerns the way by which the *cam$^r$* gene is transcribed and translated in yeast cells. Expression is probably not the result of plasmid rearrangement because, invariably, all Leu$^+$ transformants obtained are also resistant to chloramphenicol. However, this remains to be demonstrated directly.

If it is assumed that plasmid rearrangement has not occurred, the expression of the *cam$^r$* gene could then arise in one of the following ways.

(*i*) The legitimate bacterial control sequences are utilized throughout transcription and translation of the gene.

(*ii*) Bacterial DNA sequences adjacent to but other than the legitimate bacterial control sequences are similar enough to the yeast control signals so as to be recognized and utilized by the yeast transcriptional and translational apparatuses.

(*iii*) Transcription is initiated via a promoter region located somewhere in the yeast DNA portion of the plasmid (given the direction of transcription of the *cam$^r$* gene, a 2-$\mu$m DNA promoter would be the most likely candidate).

(*iv*) Some combination of two or more of the preceding possibilities.

In all four cases the primary transcript may or may not be posttranscriptionally modified (polyadenylylated at the 3′ terminus or capped at the 5′ end) before translation. In case *i*, one might expect that the physical and enzymatic characteristics of the protein synthesized in yeast would be similar, if not identical, to those of the protein made in *E. coli.* In all other cases, differences in the primary transcript could possibly result in differences between the characteristics of the protein synthesized in yeast and bacteria. Fortunately, the *cam$^r$*-acetyltransferase gene–protein system should lend itself rather well to the resolution of these possibilities. For example, a cloned DNA sequence corresponding to the *cam$^r$* gene can be used as a specific hybridization probe for the characterization of mRNA transcripts. The sequences of the coding region of the *cam$^r$* gene and of the flanking regions have now been determined (19) and will be useful in this respect. Also, the chloramphenicol

acetyltransferase protein can be easily purified by a single-step affinity-chromatography column (10) and is therefore readily available for physical and enzymatic characterization as well as for the production of antiserum. In addition, most of the amino acid sequence of the protein has been determined (20, 21) and should provide a useful reference for comparative studies. Finally, it is possible to obtain, in yeast, mutants in which some aspect of either the expression of the $cam^r$ gene or the maintenance or replication of the plasmid is altered. By selecting for growth on high levels of chloramphenicol, a number of mutants have already been isolated in which acetyltransferase activity is increased 20- to 40-fold over the levels observed in the primary "wild type" transformants. The investigation of the mutational event(s) leading to such variants might prove useful in increasing our understanding of the mechanisms of transcription, mRNA processing, and translation in eukaryotes.

This work was supported by National Institutes of Health Grants 1R01 CA 12410 (to J.M.) and 2T32 GM 07001-04 (to J.D.C.). Support for T.R.E. was from Grant 1T32AG 00052-01. Partial salary support for J.D.C. and T.R.E. from the David Opinsky–Henry Segal Memorial Fund is gratefully acknowledged. Partial support for J.M. was obtained from Grant 2P5DGM 19100. R.B.N. was the recipient of a Research Development Award from Wayne State University.

1. Sherman, F. & Stewart, J. W. (1978) in *Biochemistry and Genetics of Yeast*, eds. Bacila, M., Horecker, B. L. & Stoppani, A. D. M. (Academic, NY), pp. 273–316.
2. Hinnen, A., Hicks, J. B. & Fink, G. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1929–1933.
3. Chang, A. C. Y., Lansman, R. A., Clayton, D. A. & Cohen, S. N. (1975) *Cell* **6**, 231–244.
4. Kedes, L. H., Chang, A. C. Y., Houseman, D. & Cohen, S. N. (1975) *Nature (London)* **255**, 533–538.
5. Struhl, L., Cameron, J. R. & Davis, R. W. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1471–1475.
6. Ratzkin, B. & Carbon, J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 487–491.
7. Vapnek, D., Hautala, J. A., Jacobson, J. W., Giles, N. H. & Kushner, S. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3508–3512.
8. Dickson, R. C. & Markin, J. S. (1978) *Cell* **15**, 123–130.
9. Shaw, W. V. (1967) *J. Biol. Chem.* **242**, 687–693.
10. Shaw, W. V. (1975) *Methods Enzymol.* **43**, 737–755.
11. Miller, J. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
12. Sherman, F., Fink, G. R. & Lawrence, C. W. (1974) *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
13. Bolivar, F. (1978) *Gene* **4**, 121–136.
14. Hollenberg, C. P., Degelman, A., Kustermann-Kuhn, B. & Royer, H. D. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2072–2076.
15. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L. & Boyer, H. W. (1977) *Gene* **2**, 95–113.
16. Beggs, J. D. (1978) *Nature (London)* **275**, 104–109.
17. Struhl, K., Stinchcomb, D. T., Scherer, S. & Davis, R. W. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1035–1039.
18. Gerband, C., Fournier, P., Blanc, H., Aigle, M., Heslot, H. & Guerineau, M. (1979) *Gene* **5**, 233–253.
19. Alton, N. K. & Vapnek, D. (1979) *Nature (London)* **282**, 864–869.
20. Shaw, W. V., Bouanchaud, D. H. & Goldstein, F. W. (1978) *Antimicrob. Agents Chemother.* **13**, 326–330.
21. Zaidenzaig, Y. & Shaw, W. V. (1978) *Eur. J. Biochem.* **83**, 553–562.