

Chapter 2

Adaptation to Environment

2.1 Growth and Multiplication

2.1.1 Introduction

Of the many carbon sources, yeast prefers glucose. In the presence of glucose, enzymes of the galactose metabolic pathway are not expressed. Even a disaccharide containing glucose moiety such as sucrose, is not utilized until all the available free glucose is completely consumed. Yeast maintains a strict hierarchy in terms of sugar utilization and glucose is at the top. Does it offer any advantage to yeast despite the free energy content between say galactose and glucose is the same? Growth is a resultant of all the biological activities of a cell. Quantitative analysis of growth provides insights into the metabolic strategies adapted by different organisms or same organisms under different experimental or physiological conditions. In this section, I shall briefly discuss the basic aspects of cell growth with a focus on what a cell or a living organism considers important for its evolutionary success.

2.1.2 Growth Kinetics

Growth rate is determined by measuring cell number or biomass as a function of time. Cell number is measured by monitoring the optical density of the cell culture, counting the cell number using a haemocytometer, or determining the viable cell count. Biomass is determined by measuring the dry weight of the cells.

An increase in cell number obviously reflects an increase in biomass. However, an increase in biomass need not necessarily be due to an increase in cell number. This can occur due to an accumulation of cell material without a concomitant increase in cell number (Fig. 2.1.1a). If the cells present at the start of the experiment are at the same stage of cell cycle, that is they are synchronized, then the increase in cell number occurs in discrete steps (Fig. 2.1.1b). For most of the growth studies, it is not required to monitor the growth of a synchronously growing population.

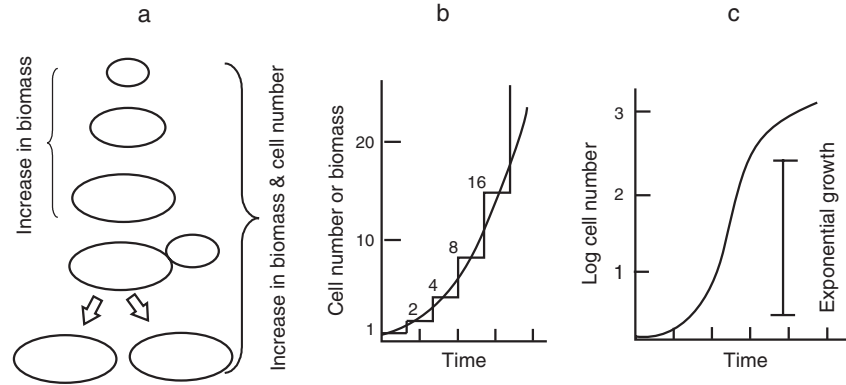


Fig. 2.1.1 Kinetics of growth: **a** The relationship between cell growth and cell multiplication. **b** The increase in cell number in a step-wise manner starting from a synchronized cell population. A curve with continuously increasing slope is obtained if cell numbers of an unsynchronized population is plotted against time. The curve also indicates the increase in biomass of a synchronized or unsynchronized cell population. **c** The growth of an unsynchronized cell population on a log scale

Unless the cell cycle is synchronized using special techniques, the initial cell population is heterogeneous with respect to the growth cycle. Because of this, cell division is a continuum, i.e., in a population of cells, at any given instant of time, cells would be at different stages of cell cycle. Therefore, if the cell number is plotted as a function of time or number of generations lapsed (Fig. 2.1.1) on arithmetic coordinates, a curve with constantly increasing slope is obtained (Fig. 2.1.1b). A similar pattern is also obtained if the biomass is plotted instead of cell number. That is, the rate of increase escalates as a function of growth. Cell multiplication of a synchronized cell population would show step-wise growth (Fig. 2.1.1b).

A typical growth profile of yeast in a batch culture is represented in Fig. 2.1.1c. The initial cell population in the inoculums is heterogeneous with respect to the physiological state and therefore different cells multiply at different rates. This initial phase of the growth profile is the lag phase, which is characterized by a slow growth rate. Once the cells adapt to the new environment, all the cells start multiplying at the same rate and the cell density increases exponentially and eventually reaches a stationary phase beyond which the cells do not multiply. Attainment of a stationary phase is due to a number of factors, such as diminishing nutrients and accumulation of metabolites. Under typical experimental conditions, yeast can grow up to a cell density of 10^8 to 10^9 cells/ml.

A fundamental parameter that describes growth is its rate. The time period required to double the cell density is called “doubling time” (t_d), which is fixed for a given organism under a given set of experimental conditions. Total cell (N_t) number at any time point during growth is proportional to the number of generations and can be calculated using the following equation, provided we know the initial cell number (N_0) and the number of generations n (n = total time/doubling time).

$$N_t = N_0 \cdot 2^n$$

How do we calculate the doubling time? The number of cells can be plotted either as a function of time or as function of generations. This representation of growth kinetics is inconvenient and needs to be transformed into a more suitable form. In the above case, the number of cells increase by geometric progression, but the parameter on the X-axis is in arithmetic progression. To convert the geometric increase in cell number to a linear form, one needs to express the above equation in a logarithmic form, which is

$$\log N_t = \log N_0 + n \log 2$$

From the above equation one can calculate n by experimentally determining N_0 and N_t

$$n = (\log N_t - \log N_0) / \log 2$$

If we want to calculate the number of generations per unit of time, then

$$n/t = (\log N_t - \log N_0) / t \log 2$$

n/t is called the growth rate constant k . The inverse of the growth rate constant is t_d .

$$t_d = t/n = 1/k$$

Therefore, $N_t = N_0 2^{kt}$

The increase in cell number occurs by a factor of k . The unit of k is t^{-1} . Here, the increase is considered to occur in a discrete step-wise manner, in other words, k is an average value for the population over a finite period of time, but we know growth occurs in a continuous manner. For this purpose, we need to have an instantaneous growth rate constant. This is because growth occurs even without cell division and we need to account for this as well. For this purpose, we need to consider a small time interval to get instantaneous increase in growth. That is, for a small increase in say cell mass per small increase in time.

dx/dt —cell mass existing at that instant of time.

$$dx/dt = \mu X$$

μ is the proportionality constant designated as specific growth rate constant with t^{-1} as unit

$$\mu = \text{rate of growth/amount of biomass}$$

To calculate the increase in cell mass that occurs between any two time points, cell growth occurring during small time periods will have to be added up. Mathematically, this is achieved by integrating the equation $dx/dt = \mu X$.

Thus we get $X_t = X_0 \cdot e^{\mu t}$.

This is same as the equation that we got previously and is transformed into logarithmic form

$$\ln X_t = \ln X_0 + \mu t$$

From the above equation we can calculate

$$\mu = (\ln X_t - \ln X_0) / t$$

The actual increase in biomass per unit time becomes greater at each instant during exponential growth and the growth rate remains constant. Thus, a value of $\mu = 0.1 \text{ h}^{-1}$ is equivalent to a 10% increase per hour. This does not mean that the doubling of cell density would occur in 10h, but would occur in 6.93 h. This is because the increase occurs in a continuous fashion, similar to an increase in compound interest.

μ and k are related as shown

$$2^n = (e^{0.693})^{kt}$$

$$e^{0.693kt} = e^{\mu t}$$

$$kt \cdot 0.693 = \mu t,$$

$$\mu = k \cdot 0.693$$

$$t_d = 0.693 / \mu$$

2.1.3 Effect of Nutrients on Growth

Nutrient concentration affects the growth rate and the total biomass. The nutrient that limits the growth in this way is called the “limiting nutrient”. For example, if yeast is grown in a medium containing a different amount of glucose keeping other nutrients unlimited, μ would keep increasing and reach a maximum (Fig. 2.1.2, panel A). The relationship between the specific growth rate and the nutrient concentration is hyperbolic (Fig. 2.1.2b). The concentration of limiting nutrient at which the μ is maximum is called as μ_{\max} . Nutrient concentration at $1/2 \mu_{\max}$ is K_s . The relationship between μ and the substrate concentration is given by an empirical formula $\mu = [S] \mu_{\max} / K_s + [S]$, given by Monod.

A linear relationship is obtained between the net biomass and the concentration of limiting nutrient over a wide concentration range. The mass of cells produced per unit of nutrient is called the “growth yield coefficient” or “yield constant”, defined as $Y_s = (X - X_0) / S$; X is the dry weight of cell (mg/liter) at the beginning of stationary

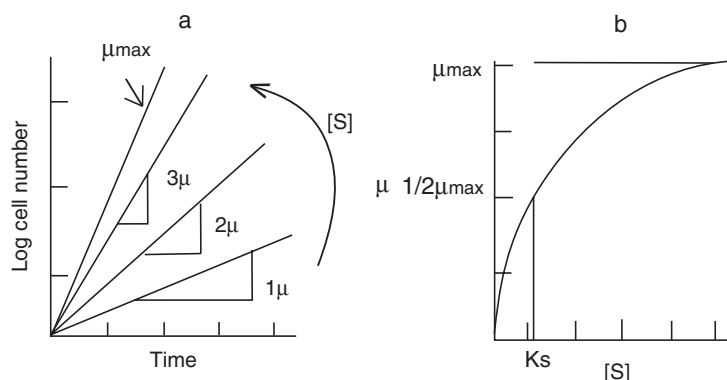


Fig. 2.1.2 Relationship between the concentration of the limiting nutrient and specific growth rate. *Panel a* indicates the growth as a function of time at different concentration of limiting nutrient. The relationship between the growth rate constant and the concentration of limiting nutrient is given in *panel b*

Table 2.1.1 Growth properties of anaerobic cultivation of *Saccharomyces kluyveri* and *S. cerevisiae* (data obtained with permission from Moller and Pisker 2001)

Growth parameter	<i>S. kluyveri</i>	<i>S. cerevisiae</i>
Growth rate (μ_{\max} , h^{-1})	0.24	0.41
Biomass (Y_{sx} [g/g]) ^a	0.089	0.092
Ethanol (Y_{se} [g/g])	0.350	0.376
Carbon dioxide (Y_{sc} [g/g])	0.389	0.397
Glycerol (Y_{sgly} [g/g])	0.109	0.107

^aYield coefficient (Y) is expressed as grams of biomass, ethanol, glycerol, carbon dioxide per gram of glucose consumed

phase, X_0 is the dry weight of the inoculum and S is the concentration of limiting nutrient (in mg/L). Growth yield can also be expressed as the dry weight in grams of biomass formed per mole of substrate (Table 2.1.1). The parameters such as μ , Y_s or K_s allow us to compare the growth performance of different strains or same strains under different growth conditions. For example, μ_{\max} h^{-1} of *Candida tropicalis* and *Saccharomyces cerevisiae* is 0.74 and 0.47, while Y_{molar} (gm dry weight/mole glucose) of *Zymomonas mobilis* and *Saccharomyces cerevisiae* is 9 and 29, respectively. These comparisons provide insights into how organisms have optimized these parameters to remain competitive in a constantly changing environment.

2.1.4 Metabolic Strategy

As mentioned before, yeast prefers to obtain energy by fermenting glucose and not by oxidation, despite the fact that oxidation provides more energy per glucose as compared to fermentation. In fact, during fermentation on glucose, mitochondrial

oxidation machinery is severely suppressed. Not only that, a significant fraction of cells in the population sporadically lose mitochondria during growth on glucose. After exhaustion of glucose, subsequent growth occurs due to mitochondrial oxidation of ethanol, which is accumulated during the fermentative stage. Cells that have spontaneously lost mitochondria during growth on glucose form small colonies on solid medium and are referred to as petites. The small colony size of petites is because of their inability to use ethanol after glucose is exhausted from the medium. This is referred to as “petite-positive phenotype” (Box 2.1.1). This phenotype is not exhibited when cells grow on other fermentative carbon sources such as galactose, nor is it exhibited by other species of *Saccharomyces*. For example, *Saccharomyces kluyveri*, a close relative of *Saccharomyces cerevisiae*, can grow anaerobically, but cannot survive if mitochondria are lost, and therefore is referred to as “petite negative yeast”.

What is the teleological reason for the sporadic loss of mitochondria when yeast grows on glucose? *S. kluyveri*, and *S. cerevisiae*, when grown on glucose under anaerobic condition, display similar growth parameters except the μ_{\max} (Table 2.1.1). This suggests that in *S. cerevisiae*, the metabolic energy derived from fermentation of glucose is diverted for cell multiplication rather than for maintaining mitochondria, at least in a fraction of cell population. This could account for the overall higher μ_{\max} of *S. cerevisiae* as compared to *S. kluyveri*. In evolutionary terms, it may mean that the petites, which have a disadvantage once glucose exhausts, seem to “sacrifice” their growth on ethanol for achieving higher growth rate for the common good.

Fermentation of glucose by yeast reveals a near-perfect metabolic design to remain competitive. First, because fermentation is an energy-inefficient process compared to oxidation, it has to consume more glucose per cell division. In this context, high growth rate would result in faster depletion of glucose from the

Box 2.1.1 *Petite-positive phenotype*

During growth on glucose as the carbon source, cells of *Saccharomyces cerevisiae* constantly produce mutants characterized by reduced colony size and referred to as petites. Petite mutants, a special class of respiratory-deficient mutants, either lack a part or whole mitochondrial genome. Yeast groups such as *S. cerevisiae* that give rise to petite mutants without any apparent selective pressure are said to exhibit petite-positive phenotype while those that cannot generate petites are said to exhibit a petite-negative phenotype.

Saccharomyces kluyveri can grow anaerobically but is not petite-positive. That is, it cannot lose mitochondria. On the other hand, *Kluyveromyces lactis*, a close relative of *Saccharomyces*, ferments glucose to ethanol, but neither exhibits petite-positive phenotype nor can grow anaerobically. In the latter two cases, mitochondrial function is absolutely essential. It is suggested that the petite-positive phenotype of *Saccharomyces cerevisiae* evolved due to the reorientation of metabolism as a consequence of genome duplication followed by rearrangement of genes.

Table 2.1.2 Doubling time of normal and petite strains of *Saccharomyces cerevisiae* on hexoses (data obtained with permission from Deken 1966)

Carbon	Wild-type		Petite	
	Doubling time (in min)	Fermentation ($\mu\text{l CO}_2/10 \text{ min}/10^7 \text{ cells}$)	Doubling time (in min)	Fermentation ($\mu\text{l CO}_2/10 \text{ min}/10^7 \text{ cells}$)
Glucose	53	78.0	70	72.4
fructose	53	69.0	70	70
Mannose	63	46.0	96	52.0
Galactose	72	15.3	139	30.0

medium, making it unavailable for the competing organism. It has been estimated that the rate of glucose uptake is 2×10^7 molecules per second per cell. Remember, that during fermentation, 1/3 carbon of glucose is preserved as ethanol for future use. Unlike yeast, ethanol is toxic to many microorganisms and cannot use ethanol as a carbon source. However, once glucose is exhausted, mitochondrial activity is derepressed, and yeast switches over to oxidative mode to consume ethanol as the source of energy and carbon. Thus, *Saccharomyces cerevisiae* owes its competitiveness to a combination of several features that evolved over millions of years.

Unlike growth on glucose, growth on galactose is an expensive affair as it necessitates synthesis of Leloir enzymes, which constitute approximately 5% of total cellular proteins when cells grow on galactose as the sole carbon source (Box 2.2.2). This overwhelming energy demand probably cannot be met by fermentation alone, which yields just two ATP/galactose consumed. This is consistent with the observation that mitochondria-less yeast grow at a rate half that of

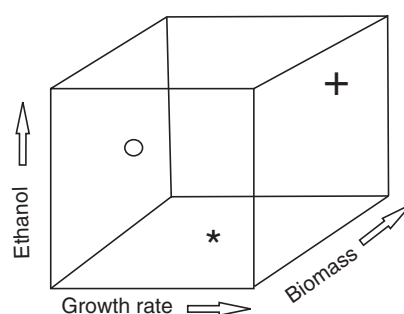


Fig. 2.1.3 Schematic illustration of metabolic space. Hypothetical metabolic space bounded by growth rate, ethanol production and biomass production. Organism represented by “O” has optimized high ethanol production and biomass production but low growth rate, “*” has optimized high growth rate but low biomass and ethanol production while “+” has optimized high ethanol and biomass and growth rate. Parameters are optimized as dictated by the evolutionary trajectory taken by the organisms. For example, humans can be considered to have optimized complex functions at the expense of growth rate

Box 2.1.2 *Fermentation*

Fermentation has been loosely used to indicate large-scale cultivation of microorganisms for industrial purposes. It has also been used as a synonym for respiration in the absence of oxygen. In fermentation, no external electron acceptor is required and redox reactions are balanced internally. Moreover, carbon is not completely oxidized. In fact, some industrial processes are aerobic and involve complete oxidation of the carbon source. Respiration in the absence of oxygen, that is, anaerobic respiration, differs from fermentation in that an external electron acceptor is used. For example, certain organisms use NO_3^- as an electron acceptor and reduce it to NH_3 or SO_4^{2-} is reduced to H_2S .

Box 2.1.3 *Biosynthetic rate*

There is an inverse correlation between rates of metabolism and the size of organisms. If we take the rate of metabolism of humans as say 1, then elephant has 0.2, mouse has 10, and yeast has 100. This difference is essentially due to the large surface area/volume or surface area/weight. This enables the microorganisms to exchange matter and energy very efficiently. For example, a 200-lb. pound man has a surface area of $24,000 \text{ cm}^2/10,000 \text{ g}=2.4 \text{ cm}^2/\text{g}$. A bacterium $1 \times 10^{-7} \text{ cm}^2/2 \times 10^{-12} \text{ g}=50,000 \text{ cm}^2/\text{g}$.

The rate of protein synthesis is an index of biosynthetic activity. The protein biosynthetic rate in aged, adult, young adult, and infants is 1.9, 3.0, 6.9, and 17.4 g/kg/day, respectively. Assuming 50% dry weight is protein and 12% is the total dry weight, 60 g is protein/kg of cells. A bacterial cell doubles itself every 30 min.

That is 1 kg of bacterial cell becomes 2 kg and 4 kg. At the end of 1 h, 3 kg of biomass is produced, which is equivalent to 180 gm/kg/h.

the strains containing mitochondria (Table 2.1.2). It is intriguing to know that while glucose and galactose have the same free energy content, the design of their metabolism is vastly different.

Unlike yeast, humans depend on mitochondrial oxidation for energy demands. However, red blood cells (RBC) derive energy exclusively by fermentation of glucose to lactate. This adaptation ensures that RBC does not oxidize glucose using oxygen which is meant to be supplied to other tissues. This is an example of metabolic differentiation to ensure efficient transport of oxygen from lungs to tissues. Skeletal muscles also ferment glucose to lactate when mitochondrial oxidation is unable to keep pace with the influx of glucose under conditions of vigorous muscular activity. This is an example of physiological adaptation. What is the metabolic fate of lactate?

Lactate finds its way into the liver through blood circulation where it gets converted to glucose, which is released back into the blood circulation. This is similar to the metabolic strategy adapted by yeasts during fermentation.

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2.2 Enzyme Adaptation

2.2.1 Introduction

Current understanding of the concept of differential regulation of gene expression, which is fundamental for the understanding of a whole range of biological processes such as development, differentiation emerged from a detailed analysis of the nature of enzyme adaptation. Enzyme adaptation was initially observed in microbial systems as a phenomenon of switching from one metabolic state to another in response to the presence of specific substrates. In this section, regulation of galactose metabolism in yeast is discussed in the context of brief historical perspective of enzyme adaptation. This gives a glimpse of intellectual and experimental efforts directed at understanding the phenomenon of enzyme adaptation. This paradigm continues to provide insights into the working of not just transcriptional regulation but also helps us understand many rapidly evolving concepts of modern biology.

2.2.2 Adaptation to Nutrients

As early as the 1890s, Frederic Dierckx discovered that yeast pre-grown on glucose starts utilizing galactose with a delay, but yeast pre-grown on galactose starts using glucose or galactose without delay. Further, if yeast grows on a mixture of glucose

and galactose, it first ferments glucose to ethanol and temporarily ceases growth before it starts fermenting galactose to ethanol. This effect was called “the glucose effect”. During the course of these investigations, he also identified yeast strain unable to use galactose. By the turn of the 20th century, similar observations were rediscovered in bacteria. Henning Karstrome invoked the idea of enzyme adaptation to explain the delay in acclimatization when microbes start utilizing alternate carbon sources. He referred to enzymes existing in a living cell regardless of the nature of the nutrients present in the medium as “constitutive” while those formed only in the presence of their pathway substrate such as galactose as “adaptive”.

In 1938, Yarkin proposed a conceptual basis for enzyme adaptation and suggested that enzymes exist in equilibrium between active and inactive form. The equilibrium is in favor of inactive form for adaptive enzymes while it is the opposite for the constitutive enzymes. He further suggested that when the adaptive enzyme which exists in inactive form comes in contact with the substrate, the equilibrium shifts towards the active form. This theory was referred to as the mass-action theory of enzyme adaptation. The view that the substrate somehow influences the protein to change its activity was also used to explain the diversity of antibodies. Sol Spiegelman (who was to later spend considerable effort in understanding the “long-term adaptation” phenotype in yeast, see below) proposed the “plasmagene” hypothesis to explain adaptation. According to this hypothesis, the substrate would induce duplication of the relevant genes to increase the enzymes. This idea did not stand the test of scientific scrutiny and was quickly abandoned.

In the 1940s, Jacques Monod observed that in certain mixtures of carbon sources, *E. coli* showed single growth cycle while in others it showed two cycles of growth separated by temporary cessation of growth. He termed this phenomenon “diauxie” (Fig. 2.2.1). The adaptation of an enzyme system required to catabolise galactose occurred in the absence of cell division was first observed in yeast as early as 1900. Later, a similar observation was also made in *E. coli*. This indicated

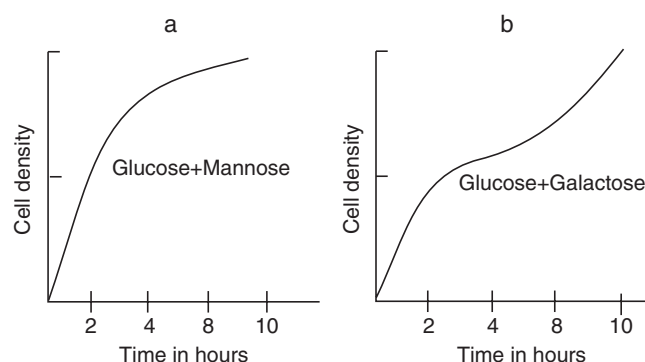


Fig. 2.2.1 Schematic illustration of growth profiles of *E. coli* in glucose medium either with mannose **a** or with galactose **b**. Note that in the presence of mannose and glucose there is only one exponential phase while in the presence of glucose and galactose the exponential phase is separated by a lag phase

that enzyme adaptation is not due to an alteration in the genetic structure, since the latter occurs only during cell multiplication. Second, the phenomenon of adaptation was sensitive to the presence of energy uncouplers, indicating that the expenditure of energy is a prerequisite for adaptation. These results convinced Monod that enzyme adaptation is due to the delay in the synthesis of enzymes rather than a delay in their transformation in the presence of the substrate. The challenge, however, was to relate the role of the substrate and the gene to account for the fresh synthesis of enzyme molecules. By 1960 Monod provided a molecular basis of enzyme induction, the cornerstone for our present understanding of regulation of gene expression. However, enzyme adaptation, which Dienert observed with respect to galactose utilization in yeast, took a curious turn.

2.2.3 Long-Term Adaptation

After the initial discovery of enzyme adaptation by Dienert, yeast played a key role as an experimental organism in the elucidation of glycolysis, but its use in genetic studies was a suspect for long time due to the non-Mendelian segregation pattern in genetic crosses. In the 1930s, Ojvind Winge began research in yeast genetics and showed that the yeast life cycle involves an alternation between haploid and diploid phase (discussed in the previous chapter). While investigating the ability of yeast strains to ferment sugars, Winge and Roberts encountered an unusual yeast, which took as many as 3–4 days to adapt to galactose as compared to few hours for a normal strain. This was referred to as “long-term adaptation” (Fig. 2.2.2).

An unusual feature of this phenotype was that galactose-adapted cells on subsequent exposure to galactose do not show long-term adaptation. However, if

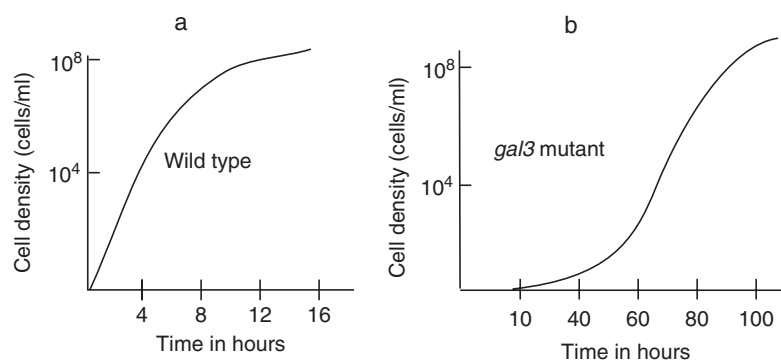


Fig. 2.2.2 Schematic illustration of growth kinetics of wild-type and *gal3* mutant. Growth of a wild-type strain **a** and *gal3* mutant **b** in galactose. A wild-type strain pre-grown on glucose starts growing on galactose without a significant lag. A *gal3* mutant pre-grown on glucose takes at least 48 h before it starts growing on galactose

galactose-adapted cells are cultivated in the absence of galactose for few generations, they lose the ability to rapidly adapt to the subsequent exposure to galactose. That is, during a few generations of growth on carbon sources other than galactose, the mutant strain loses the ability to rapidly adapt to galactose. Therefore, these cells are not only defective in responding quickly to galactose, but are unable to retain the property of rapid adaptation acquired during growth on galactose (Fig. 2.2.2). Preliminary genetic analysis indicated that it is a recessive defect at a genetic locus designated as *GAL3*. Following this discovery, Spiegelman and co-workers conducted a detailed analysis of long-term adaptation.

2.2.4 Single-Cell Analysis of Long-Term Adaptation

This phenotype provided a convenient experimental system for analyzing the phenomenon of enzyme adaptation. As yeast divides by budding, it is possible to monitor whether the mother and the successive daughters (daughters produced from the same mother) retain rapid induction phenotype when exposed to glucose. For this purpose, a *gal3* cell adapted to galactose is maintained in a glucose medium and buds are removed as and when they are formed. The ability of these buds and the mother cell to respond to galactose is independently assessed by transferring to galactose medium (Fig. 2.2.3).

It appeared that factors acquired by *gal3* cells during adaptation to galactose were reduced during the subsequent growth in the absence of galactose. A positive

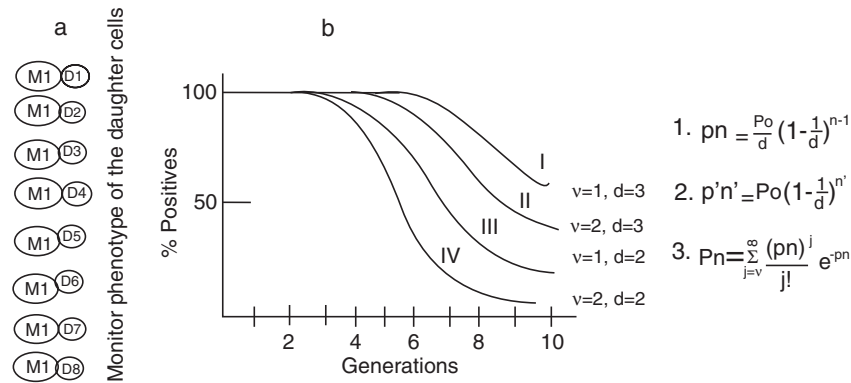


Fig. 2.2.3 Single-cell analysis of LTA. **a** The experimental strategy for determining the de-induction using single-cell analysis. **b** Theoretical curves generated based on statistical analysis. The average number of elements remaining in an n th generation daughter cell (Eq. 1), the average number of elements remaining in a mother cell after it has produced n daughter cell (Eq. 2), and the expected proportion of positive cells among the n th generation buds (Eq. 3) can be calculated. Po is the number of elements initially present, $1/d$ is the fraction of the parental elements that pass into the daughter cell and v is the minimal number of elements required for the cell to be positive (adapted with permission from Spiegelman et al. 1950)

mother cell itself becomes negative after six to seven generations. In some cases, the mother cell receives more while in other cases the daughter cell receives more. Overall, the data suggest that after a certain number of divisions have occurred, the number of elements available for distribution is such that the two cells produced as a result of division cannot both be positive. It is observed that the rapid induction and long-term adaptation phenotype of the daughter cells can alternate in successive generations. For example, in pedigree 2 (see pedigree 2, Table 2.2.1), the sixth and eighth buds show long-term adaptation while the seventh bud shows rapid induction. Table 2.2.2 gives the consolidated pedigree data.

This data is amenable for quantitative analysis and the proportions of the positives found in each generation among the mother and daughter cell can be determined. With a constant number of inducing elements present in a galactose adapted *gal3* cells, the proportion of positives to be expected at any given generation depends upon the following parameters. P_0 , the number of elements initially present; $1/d$ the fraction of elements that pass on to the daughter cell and v the minimum number of elements required to yield the positive phenotype. From the equation shown in Fig. 2.2.3, theoretical curves can be generated for different proportions of the positives as a function of generation for different values of $1/d$ and v . It turned out that the experimental data fits with the III curve. Of the three parameters, variation in P_0 would only alter the number of generations before the appearance of negative cells and this will not change the shape of the descending part of

Table 2.2.1 Single-cell analysis of four individual pedigrees (data obtained with permission from Spiegelman et al. 1950)

Pedigree	Generations									Mother cell
	1	2	3	4	5	6	7	8	9	
1	+	+	0	+	+	+	—	—	0 ^b	— ^a
2	+	+	+	+	+	—	+	—	—	—
3	+	+	+	+	+	+	—			+ ^a
4	+	+	+	+	+	+	+			

^a“+” and “—” signs indicate that the clone derived from the single cell exhibits rapid or slow induction, respectively

^b“0” indicates that the clone did not survive and a blank space indicates that bud isolation was not continued

Table 2.2.2 Summary of the pedigree analysis (data obtained with permission from Spiegelman et al. 1950)

Generations	Positives	Negatives	Total	Positives (%)
2	34	0	34	100
3	37	0	37	100
4	27	0	27	100
5	27	1	28	96.5
6	26	6	32	81.5
7	22	12	34	65.0
8	9	18	27	33.3
9	3	10	13	23.0

the curve. On the other hand, $1/d$ and v influence the descending part of the curve in opposite direction. Therefore, the value of $1/d=2$ and $v=1$ is not the only value that would fit the experimental data, other combinations of $1/d$ and v value would also fit the data. However, a more detailed analysis suggested that the value of $v=1$ and $1/d=2$ and P_0 was calculated to be 200.

Above analysis demonstrated that in *gal3* cells, galactose eventually induces a factor required for induction. If galactose is withdrawn, it gets diluted below a threshold required for rapid induction within six to seven generations. It was also observed that in glucose grown population of *gal3* mutants, one out of approximately 1,000 cells is a true galactose fermentor. Accordingly, it is the time required for the multiplication of this small fraction of cells that causes the delayed growth and not because of the slow induction of the factor. Based on these results, Spiegelman invoked the concept of heterogeneity in cell population as a mechanism of adaptation to galactose. Recent analysis, in fact, supports the view that LTA is due to the cellular heterogeneity (see section 8.2.3).

2.2.5 Galactose Metabolism

The galactose metabolic pathway is commonly referred to as the Leloir pathway after Luis Federico Leloir, who discovered that the intracellular galactose is converted to glucose through four distinct enzymatic reactions. Galactokinase catalyses the conversion of intracellular α D-galactose to galactose-1-phosphate. Galactose-1-phosphate is converted to glucose-1-phosphate by uridyl transferase. UDPglucose needed for this reaction is replenished by the conversion of UDPgalactose to UDP glucose by the epimerase. Glucose -1-phosphate is then converted to glucose-6-phosphate by phosphoglucomutase. As phosphoglucomutase is also involved in converting glucose-6-phosphate to glucose-1-phosphate during growth on glucose, it is not generally considered as a member of Leloir pathway. The need for three enzymes for epimerising galactose to glucose is quite unique in biochemistry.

Yeast utilizes melibiose, a disaccharide consisting of glucose and galactose linked through α -glycosidic linkage. α -galactosidase cleaves melibiose into glucose and galactose, which is taken up by yeast as carbon sources. *Saccharomyces cerevisiae* strains normally do not code for α -galactosidase, but *Saccharomyces cerevisiae* strains containing α -galactosidase have been derived by interspecies crossing with *Saccharomyces carlsbergensis*. Unlike the Leloir enzymes, α -galactosidase is an extracellular enzyme, and its expression is controlled by the same mechanisms as Leloir genes.

Free galactose exists as an equilibrium mixture of α and β forms and it is the α form that is the substrate for galactokinase. Aldose 1-epimerase (EC 5.1.1.3) or mutarotase interconverts these two forms. While in *E. coli* and humans, this enzyme is encoded by a distinct gene, in yeast, mutarotase is a part of the epimerase polypeptide.

The genetic basis of galactose metabolism was first demonstrated by Lindegren and Lindegren by conducting genetic analysis of haploid strains defective in galactose fermentation. It was believed that the sequential induction of enzyme activity

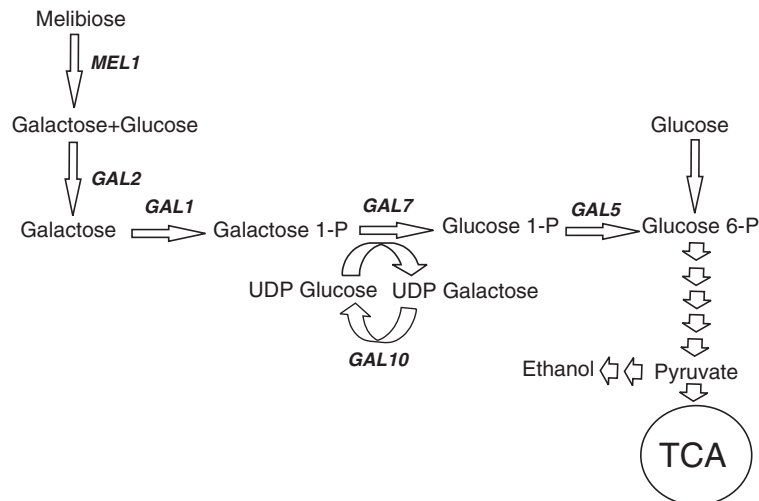


Fig. 2.2.4 Galactose metabolism in yeast. *GAL1*: Galactokinase (EC 2.7.1.6); *GAL7*: Galactose-1-phosphate uridyl transferase (EC 2.7.7.12); *GAL10*: Uridine diphosphoglucose 4-epimerase (EC 5.1.3.2) *GAL5*: (*PGM2*) Phosphoglucomutase (EC 2.7.5.1) ; *MEL1*: α -galactosidase (EC 3.2.1.22,) *GAL2*: galactose permease

occurs in response to the formation of a product which in turn acts as an inducer for the subsequent enzyme. Contrary to this expectation, galactose induced the activity of uridyl transferase and epimerase in a galactokinase-less mutant yeast strain. This study indicated that free galactose induces not just galactokinase but also the activities of all the three Leloir enzymes. Leloir enzymes were purified from cell-free extracts obtained from galactose adapted cells. Antibodies raised against these proteins were used as probes to determine the mechanism of galactose activation, which is discussed in the next chapter.

Box 2.2.1 Determination of enzyme activity

Leloir enzymes were purified using conventional protein-purification techniques. Purification of enzymes from a complex mixture of proteins requires an assay method to monitor the presence of the enzyme in fractions obtained during the purification. As an example, different methods for detecting galactokinase activity are discussed.

1. *Colorimetric method.* This method takes advantage of the fact that free galactose concentration decreases as the reaction proceeds. Free galactose concentration present in the reaction mixture after a specified time point is monitored by allowing it to react with 3,5, dinitrosalicylic acid. This oxidizes the free reducing sugar (R-CHO) to the corresponding acid

(continued)

Box 2.2.1 (continued)

(R-COOH) and in the process is reduced to 3 amino, 5 nitrate salicylate. The concentration of this can be determined from the molar extinction coefficient by recording absorption at 575 nm.

2. *Coupled assay.* ADP formed during the reaction is coupled to the conversion of phosphoenolpyruvate to pyruvate in the presence of pyruvate kinase. The pyruvate formed is then coupled to the formation of lactate from pyruvate in the presence of lactate dehydrogenase. NADH oxidation due the conversion of pyruvate to lactate is monitored by recording a decrease in absorbance at 340 nm. The decrease is proportional to pyruvate formed, which in turn is proportional to the ADP formed in the galactokinase reaction.
3. *Radioactive assay.* This assay takes advantage of the fact that galactose-1-phosphate formed can be separated from free galactose by adsorbing to a charged surface such as DEAE filter paper. For this purpose, ^{14}C labeled galactose, instead of normal galactose is used. The ^{14}C galactose-1-phosphate present in the reaction is separated by loading the reaction mixture onto DEAE paper strips followed by washing with excess water. During this step, radioactive galactose-1-phosphate retained on to the paper as it is charged while uncharged galactose is washed off. The filter paper is counted for the radioactivity. Other charged molecules such as ADP and unreacted ATP would also be retained as they are charged, but this will not interfere as the presence of only radioactivity is monitored.

Box 2.2.2 *Energetics of galactokinase synthesis*

A yeast cell yields $\sim 6 \times 10^{-9}$ mg of protein. One milligram of total protein extracted from galactose-adapted yeast contains sufficient galactokinase to catalyze $45 \mu\text{M}$ of galactose to galactose-1-phosphate in an hour. The turnover number of yeast galactokinase is 57/s per enzyme molecule. Based on this, the concentration of galactokinase in a yeast cell is determined to be in the nanomolar range. Its amino-acid sequence has also been deduced from its gene sequence. One molecule of galactokinase has 1,815 carbon atoms. If yeast grows on galactose as the sole source of carbon, this is equivalent to 302 galactose molecules. Consider the use of galactose as a source of carbon and energy to make a molecule of galactokinase. A total of 302 equivalent of galactose molecules are required to supply just the carbon alone. Assuming that four ATPs are required for one peptide bond formation, 2,108 ATPs are required for the synthesis of one molecule of galactokinase starting from amino acids. Here, the number of ATP required for the synthesis of amino acids is not considered. These calculations give us a glimpse of the energetics of galactose utilization. Remember, yeast also has to divert the carbon and the energy derived from galactose to other cellular activities when it grows on galactose as the sole source of carbon.

Box 2.2.3 *Galactose metabolic pathway is evolutionarily conserved*

In humans, the Leloir pathway of galactose metabolism is especially important during early childhood since galactose is one of the major sources of energy. In milk, galactose exists as a component of disaccharide lactose. This is absorbed as glucose and galactose after its hydrolysis by β -galactosidase, an enzyme present in the intestine. An individual bearing defect in galactokinase suffers from juvenile cataracts due to the accumulation of galactitol derived from un-metabolized galactose. Withdrawal of galactose from the diet of such individuals alleviates the symptoms considerably. Lack of transferase shows severe physiological disturbance due to the accumulation of galactose-1-phosphate. This leads to physiological disturbance such as ovarian dysfunction, learning disabilities, and liver enlargement. Due to the endogenous synthesis of galactose, this defect cannot be alleviated even upon withdrawing galactose from the diet. Individuals bearing the above defects occur in the population at a frequency of 1 in 30,000. Individuals lacking epimerase are very rare, indicating that its function might be essential.

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2.3 Induction of Leloir Enzymes

2.3.1 Introduction

We learned that Leloir enzyme activities are present only in yeast cells adapted to galactose but not in other carbon sources. The mechanism of how galactose increases the activities of these enzymes was not understood. The increase in enzyme activity could be due to either the activation of pre-existing enzyme by mechanisms such as posttranslational modification or to an increase in the absolute number of enzyme molecules per cell. An increase in the number of enzyme molecules could be a consequence of many factors, such as increased rate of transcription followed by translation, increase in mRNA stability, decreased rate of degradation of mRNA or protein or a combination of the above possibilities (Fig. 2.3.1, Box 2.3.1). In this section, experiments that demonstrated that galactose activates the synthesis of Leloir enzymes by increasing the steady-state concentration of transcripts of the corresponding genes are discussed.

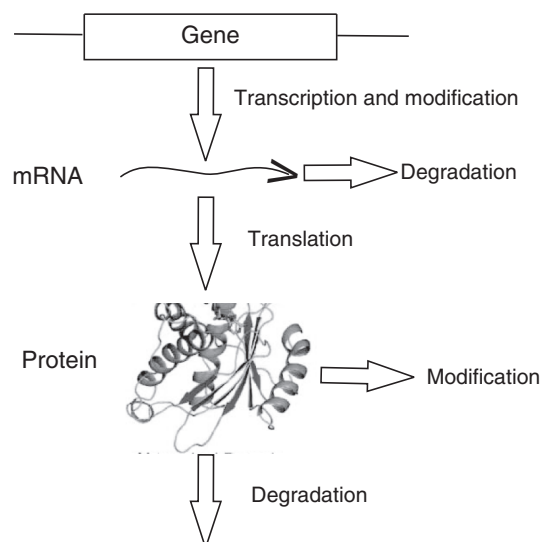


Fig. 2.3.1 Schematic representation of the regulation of gene expression. Any one or all of the above steps are potential targets for regulating the gene expression

Box 2.3.1 *Post-transcriptional and translational modifications*

Unlike prokaryotes, in eucaryotes, the primary product of mRNA, referred to as hnRNA, is processed to a mature functional form. hnRNA contains stretches of sequences called “introns”, which need to be removed to give rise to functional mRNA products. In yeast, only a few protein-coding genes contain introns, while in humans, almost every protein coding gene has an intron. The number of introns vary from gene to gene. The introns are removed by a complex enzymatic process called “splicing”, which occurs within the nucleus. Alternative splicing of the same hnRNA can give rise to different mature mRNA there by increasing the variation in the protein products formed per genetic unit. Another post-transcriptional modification of mRNA is processing of 5' and 3' ends. Generally, mRNA are capped at the 5' end by 7 methyl guanosine and polyadenylated at the 3' end. The above modifications are collectively called “posttranscriptional modifications”. Similarly, the protein product can also undergo many chemical modifications. For example, phosphorylation, ADP ribosylation, methylation, proteolysis, ubiquitinylation, etc. Almost all of these processes are regulated, thus increasing the stringency of biological regulation.

2.3.2 *Galactose Induces the Synthesis of Leloir Enzymes*

Hopper and his group investigated whether galactose induces the synthesis of Leloir enzymes by monitoring the incorporation of radiolabeled leucine into uridyl transferase. Yeast cells were grown in galactose (inducing carbon source) or acetate (non-inducing and non-repressing carbon source) as carbon source in presence of radioactively labeled leucine as a tracer. Cell-free extract prepared from yeast cells was immunoprecipitated using antibodies raised against pure uridyl transferase and separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE, see Box 2.3.2) followed by autoradiography (Fig. 2.3.2). They observed a band corresponding to the expected molecular mass of uridyl transferase only from extracts made from galactose but not acetate grown cells (Fig. 2.3.2) suggesting that galactose induces synthesis of uridyl transferase protein.

2.3.3 *Galactose Activates the Transcription of GAL Genes*

The increase in uridyl transferase synthesis in response to galactose could be due to a combination of reasons, such as increased transcription followed by translation or an increase in translation of the pre-existing mRNA. To distinguish between these possibilities, mRNA levels encoding uridyl transferase and galactokinase were monitored from a total mRNA population isolated from cells grown in acetate

Box 2.3.2 *In vitro translation*

This technique was developed to detect the presence of a specific mRNA. Wheat germ cell extract contains components necessary for translation of mRNA obtained from different sources. Wheat germ cell extract is processed to remove the endogenous amino acids and mRNA and supplemented with energy generating system required for protein synthesis. If exogenous mRNA is added to this lysate in the presence of labeled amino acids such as methionine or leucine supplemented with other cold amino acids, then synthesis of radioactively labeled proteins occur.

Radioactively labeled protein synthesis directed by the exogenously added mRNA present in the reaction mixture can be separated and analyzed by electrophoresis followed by detection. Before the advent of Northern blot analysis, this was the only technique available to detect a particular species of mRNA present in a heterogeneous population. It is now possible to monitor the expression of all the mRNAs simultaneously using microarray techniques, which will be discussed later.

Electrophoresis

This technique is one of the most widely used techniques for separating charged molecules such as proteins or nucleic acids. A sample containing a mixture of proteins or nucleic acids is separated through polyacrylamide or agarose gel under the influence of an electric field. Each molecular species migrates to a different extent depending upon its physicochemical properties. For example, nucleic acid is uniformly charged. That is, the charge-by-mass ratio remains the same regardless of the size, and therefore the separation is a function of the molecular weight. In proteins, it is possible that under native conditions, two proteins differing in molecular weight may have the same charge-by-mass ratio and would migrate to the same extent and separation would not occur. On the other hand, two proteins having the same molecular weight can differ in charge-by-mass ratio and could move to a different extent. By treating with sodium dodecyl sulphate (SDS), a denaturing agent, proteins can be conferred uniform charge-by-mass ratio. Therefore, SDS-treated proteins migrate through electrophoresis based on their molecular weight. Depending upon the experimental need, proteins can be separated either under native or under denaturing conditions. After separation, the samples are detected either by autoradiography or by staining, or both.

Staining and Autoradiography

After separation through electrophoresis, the gel is treated with a dye that imparts a specific color by interacting with nucleic acid or protein, which can be seen with the naked eye. For example, after separation through electrophoresis

(continued)

Box 2.3.2 (continued)

proteins are detected by staining with Coomassie blue, while nucleic acids are stained using ethidium bromides. For example, ethidium bromide intercalates with nucleic acid, which upon illumination at 280 nm, imparts characteristic fluorescence. The presence of radioactively labeled molecules can be detected by exposure to X-ray sensitive film. Here, the sample is kept in close contact with the film. An image corresponding to the position of the radioactive bands is imprinted on the film, which is detected after developing and fixing.

and galactose. The technique of detecting specific mRNA species in a mixture of heterogeneous population relies on the ability of wheat germ cell extract to support the translation of exogenous mRNA into proteins, in this case mRNA isolated from yeast cells. Total mRNA isolated from yeast cells grown in galactose and glucose were separately translated in wheat germ cell-free translation system in the presence of amino acids of which methionine is radioactively labeled. Immunoprecipitate obtained after treating the above reaction mixture with antibodies raised against galactokinase and uridyl transferase was subjected to SDS electrophoresis followed by autoradiography. Total mRNA isolated from galactose but not acetate grown cells directed the synthesis of a radiolabeled protein corresponding to the molecular weight of uridyl transferase (Fig. 2.3.3a, lanes 1 and 2) and galactokinase (Fig. 2.3.3b, lanes 1 and 2). This result indicated that mRNA directing the synthesis of galactokinase and uridyl transferase were present only in cells grown in galactose but not acetate.

To determine whether the same mRNA molecule encodes both galactokinase and transferase, total mRNAs isolated from galactose-grown cells were fractionated by sucrose density gradient centrifugation. During this procedure, mRNA gets

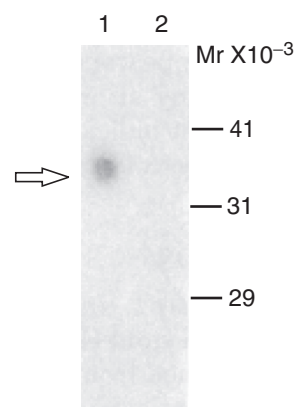


Fig. 2.3.2 Induction of synthesis of radioactively labeled uridyl transferase by galactose. Cell extract obtained from cells grown in presence of galactose (1) or acetate (2) with radioactively labeled leucine was immunoprecipitated by antibodies raised against uridyl transferase. Immunoprecipitate was separated on SDS-PAGE followed by autoradiography (reproduced with permission from Hopper et al. 1978). *Arrow* indicates radioactively labeled galactose-1-phosphate uridyl transferase

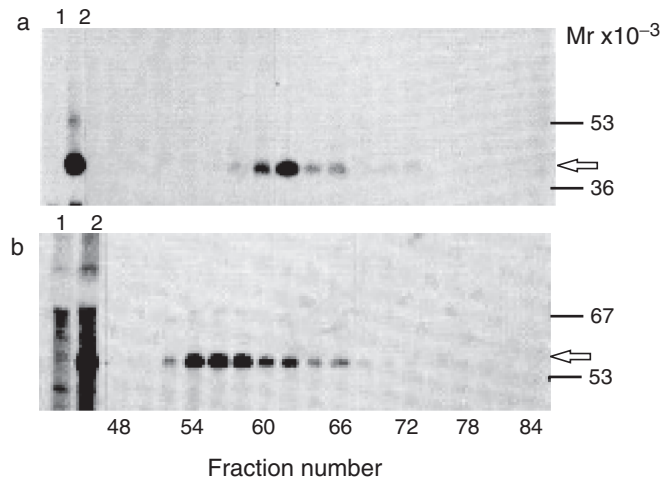


Fig. 2.3.3 Autoradiographic analysis of immunoprecipitated radiolabeled proteins obtained after in vitro translation. Total mRNA isolated from yeast cells grown in acetate (*lane 1*) and galactose (*lane 2*) were translated in vitro, immunoprecipitated by antibodies against uridyl transferase **a** or galactokinase **b**, and separated on SDS PAGE followed by autoradiography. In vitro translated products of mRNA obtained from alternate fractions collected after density gradient centrifugation (as indicated in the figure) are immunoprecipitated by antibodies against uridyl transferase **a** or galactokinase **b** and analyzed as before (adapted with permission from Hopper and Rowe 1978). Arrow indicates uridyl transferase **a** and galactokinase **b**

sedimented along the gradient depending upon the molecular weight. Fractions obtained after density gradient centrifugation were separately in vitro translated and immunoprecipitated and analyzed as before. Galactokinase and uridyl transferase synthesis were directed by mRNA present in different fractions obtained from density gradient centrifugation. If a single polycistronic mRNA were to code for both galactokinase and uridyl transferase, then they would have been translated from mRNA obtained from the same fraction, which was not what is observed. This indicated that mRNAs for galactokinase and uridyl transferase were transcribed from separate genetic units.

2.3.4 Galactose Activates a Genetic Program

Above results demonstrated that galactose activates the transcription of Leloir genes. We know that galactose is unable to do so if glucose is also included in the medium. How does galactose activate the transcription of these genes in the absence of glucose? How does glucose prevent galactose from activating the transcription of Leloir genes? The phenomenon wherein gene transcription is turned ON or OFF depending upon a specific stimulus is referred to as the “regulation of gene expression”, which is one of the predominant mechanisms by which cells regulate their

genetic potential for specific biological purposes. For example, a fertilized egg endowed with the complete repertoire of genetic program responds to different intra- and/or extracellular cues in a temporally and spatially regulated manner. A dried seed in response to water initiates a developmental program, which eventually leads to the formation of a full-fledged plant. Deciphering the molecular mechanisms of turning “ON” and “OFF” of genetic program starting from the primary step all the way through the manifestation of the phenotype is crucial for understanding the life process.

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