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Role of heme in mitochondrial biogenesis: Transcriptional and post-transcriptional regulation of the expression of Iso-l-cytochrome C gene during glucose repression-derepression in cells of *Saccharomyces cerevisiae*

K. S. K. BALAJI, G. GOPALAN and C. RAJAMANICKAM*

Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

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Abstract. Exogenous addition of hemin to glucose-repressed cells of *Saccharomyces cerevisiae* restores the level of Iso-1-cytochrome C messengers to that observed in derepressed cells. *In vitro* transcription in isolated nuclei has shown a 4-fold stimulation in the synthesis of Iso-1-cytochrome C messengers in repressed but hemin-treated and derepressed cells compared to the repressed cells. Studies on *in vitro* transport of RNA from isolated nuclei have revealed that there is a 50% drop in the transport of total RNA from nuclei isolated from repressed but hemin-treated and derepressed cells when compared with the nuclei from repressed cells. However, under these conditions, there is an enhanced transport of translatable RNA. Hybridization analysis of the transported RNA using Iso-1-cytochrome C gene-specific probe has shown that there is preferential transport of Iso-1-cytochrome C messengers in repressed but hemin treated and derepressed cells.

Keywords. Glucose repression-derepression; transcription; RNA transport; heme; Iso-1-cytochrome C gene.

Introduction

The phenomenon of glucose-repression of mitochondrial functions in *Saccharomyces cerevisiae* is well established (Perlman and Mahler, 1974; Polakis and Bartley, 1965; Tzagoloff, 1969) and extensively used for studying mitochondrial biogenesis (Falcone *et al.,* 1983; Szykely and Montgomery, 1984). In recent years, the regulatory mechanisms involved in the coordinated expression of nucleo-cytoplasmic and mitochondrial genetic systems have become the focal point of several investigations.

We have earlier shown that cAMP relieves the cells from the effect of glucose-repression by increasing the endogenous levels of heme (Gopalan and Rajamanickam, 1985). Furthermore, exogeneous addition of hemin to glucose-repressed cells alleviates glucose-repression of mitochondrial functions (Gopalan *et al.,* 1984). Our attempts to understand the molecular mechanisms responsible for the reversal of glucose repressed mitochondrial functions by exogenous addition of hemin have revealed that hemin enhances the levels of poly (A) containing RNA in the cytosol. *In vitro* translation and subsequent immunoprecipitation studies have revealed that hemin preferentially increases the messages for hemoproteins (Gopalan and Rajamanickam, 1986). Iso-1-cytochrome C (CYC1) gene and other aerobic genes have been shown to be induced by heme and the influence of heme on

*To whom all the correspondence should be addressed.

Abbreviations used: CYC1, Iso-1-cytochrome C; SDS, sodium dodecyl sulphate; DTT, dithiothreitol.

the expression of these genes has been mediated by the binding of regulatory proteins (Lowry and Lieber, 1986; Pfeifer *et al.,* 1987, 1989). In an attempt to provide direct evidence and to delineate the molecular mechanism by which hemin influences the synthesis of hemoproteins, we have chosen to study the expression of CYC1 gene, a nuclear gene coding for the mitochondrial hemoprotein, cytochrome C1, during glucose repression-derepression and the role of heme in this process.

Mehtods

Chemicals

Most of the chemicals used in this study were of analytical grade and obtained from BDH, Bombay and all fine chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. [^{32}P] phosphate (carrier free) and [^{3}H] UTP were purchased from Bhabha Automic Research Centre, Bombay and [^{32}P] dCTP (3000 Ci/m mol) was purchased from Radiochemical Centre, Amersham, England.

Strain and growth conditions

A haploid strain of *S. cerevisiae* D273-10B, was used in this study. The maintenance and growth conditions were as described by Jayaraman *et al.* (1966). Cells grown overnight in YPD medium (1 % glucose, 2% peptone and 1% yeast extract) were transferred into repression medium (1% glucose, 0·4% yeast extract, 0·9% KH_2PO_4 0·06% $(NH_4)_2$ SO_4, 0·05% $MgSO_4$ and 0·04% $CaCl_2$) at a concentration of 4mg wet weight/ml. Under these conditions, mitochondrial functions are repressed for the initial 2·5 h. Once glucose in the medium gets exhausted (at 2·5 h), the cells enter the derepression phase (Jayaraman *et al.,* 1966). A second addition of glucose to a final concentration of 1% at 2·5 h extends the repression phase (Dharmalingam and Jayaraman, 1971).

Plasmid

Plasmid pAB68 is a derivative of the shuttle vector, YEpl3 which bears CYC1 gene insert (5·2 kb *Hind*III fragment). The plasmid was a kind gift from Professor Fred Sherman, Rochester, New York. For preparing the hybridization probe, the plasmid was digested with *Sal*I and *Hind*III and the 1·6 kb *Sal*I-*Hind*III fragment containing the upstream sequence, the coding sequence and the 5′ and 3′ untranslated regions of CYC1 gene was electroeluted as described by Maniatis *et al.* (1982).

Preparation of repressed, repressed but hemin treated, derepressed and levulinic acid treated cells

Cells grown overnight in YPD medium were transferred into repression medium at a concentration of 4 mg wet weight/ml. At 2·5 h the culture was divided into 4 portions. To one portion, glucose was added to a final concentration of 1%. This is refered to as glucose-repressed cells. To another portion, hemin (4 μM) was added along with glucose (1 %) to give rise to repressed but hemin-treated cells. The cells

to which no addition was made were taken as derepressed cells. As a negative control, levulinic acid (1·6 mg/ml), an inhibitor of heme biosynthesis, was added to derepressed cells. The cells were grown under these conditions for 30 min.

Preparation of nuclei

For studies involving *in vitro* transcription, *in vitro* transport and for the preparation of nuclear RNA, nuclei were prepared from protoplasts and purified by density-gradient centrifugation as described by Duffus (1979). Briefly, the cells were harvested and washed twice with 1 M sorbital and resuspended in 10 ml of 1 M sorbitol. To this 150μ1 of zymolase (10mg/ml) was added and shaken at 30°C for about 30 min. The protoplasts were pelleted by centrifugation at 2000 *g* and washed with 1 M sorbitol. The protoplasts were lysed at 4°C by suspending 1 g of protoplasts in 10 ml of polymer solution (15–18% w/v ficoll in 0·02 M phosphate buffer, pH 6·5) and homogenised. The homogenate was centrifuged at 3000 *g* for 15 min at 4°C and the supernatant was removed and layered onto a solution of 30% ficoll in 0·02M K H_2 PO_4 (pH 6·5) and the resulting step gradient was centrifuged at 1,00,000 *g* for 45 min at 4°C. The pellet represents the purified nuclei and was suspended in appropriate buffer.

Dot blot analysis of cytosolic, polysomal and nuclear RNA

Cells grown under different conditions for 30 min were harvested, washed with saline and cell-free extracts were prepared by homogenising the cells with an equal volume of glass beads (0·45–0·5 mm) in a Braun homogeniser. The paste was extracted with a buffer containing 0·1 M Tris-HCl, pH 7·5, 0·25 M sucrose and 2 mM EDTA. The lysate was cleared by centrifugation. From the post-mitochondrial supernatant, total RNA was extracted following the method described by Shapiro *et al.* (1974).

Polysomal RNA was extracted following the method of Longacre and Rutter (1977), Briefly, the cells were homogenized in a buffer containing 10 mM Tris-HCl, pH 7·4, 10 mM NaCl, 1·5 mM $MgCl_2$ and cycloheximide (0·1 mg/ml). The nuclei and mitochondria were removed by centrifugation at 16000g for 20 min. The supernatant was layered over 4 ml of 36% sucrose cushion in 10 mM Tris-HCl, pH 7·4, 10 mM NaCl and 1·5 mM $MgCl_2$ and centrifuged at 90,000 *g* for 3·5 h. The pellet was suspended gently in 5 ml of extraction medium [100 mM Tris-HCl, pH 9, 100 mM NaCl, 1 mM EDTA and 1% sodium dodecyl sulphate (SDS)] by homogenising and RNA was isolated by phenol/chloroform extraction. RNA from purified nuclei was extracted following the method described by Penman (1969).

Nick-translation of the CYC1 gene was performed following the method of Rigby *et al.* (1977). For dot blot analysis, 20 μg of denatured RNA (both cytosolic total as well as polysomal) was spotted onto nitrocellulose membrane and hybridized to nick-translated CYC1 probe following the method described by Thomas (1983). Hybridization signals obtained in this study are in the linear range.

Quantitation of the hybrid

The spots on the nitrocellulose filter corresponding to the hybridization signals

were cut out and counted in a scintillation based counter. Based on these counts, the fold differences were calculated.

Preparation and hybridization of labelled in vitro transcribed RNA

In vitro transcription in isolated nuclei was performed following the modified method of Jerome and Jaehning (1986). The purified nuclei were suspended in a buffer containing 50 mM Tris-HCl, pH 7·5, 0·6 M sucrose, 1 mM $MgCl_2$, 1 mM NaCl, 1 mM dithiothreitol (DTT) and 35% glycerol. The transcription assay was performed in a buffer containing 0·5 mM GTP, 0·5 mM CTP, 0·5 mM ATP, 0·02 mM UTP, 0·5 mM Tris-HCl, pH 7·5, 0·5 mM $MgCl_2$, 25 μl of nuclear suspension and 50μCi of [^{3}H] UTP in a final volume of 100 μl. The reaction mixture was incubated at 30°C for 15 min and the reaction was stopped by adding 100 μl of a 0·5 % SDS, 10 mM EDTA solution. Labelled nuclear RNA was extracted by hot phenol/SDS extraction and following ethanol precipitation, RNA was purified by 2 M LiCl washing at 4°C. Following mild alkalai cleavage (in 0·1 M NaOH at 0°C for 10 min) and neutralization by 2 M Hepes, 10^5 cpm in 1·5 ml of 5 × SSC was used, for each hybridization. RNA hybridization to the DNA matrix was carried out at 65°C for 36 h. Filters were washed and digested with RNase (Salditt-Georgieff *et al.*, 1980). A control dot without DNA was carried out and RNase treated to monitor the background. In order to quantify the extent of transcription under different conditions, the corresponding dots were cut out and the radioactivity associated with the dots were measured in a scintillation based counter.

DNA dots were prepared as previously described (Kafatos and Estradiadis, 1979). Each DNA dot represents 1 μg of DNA (1·6 Kb *Sal*I-*Hind*III fragment) denatured in 0·2 M NaOH at 100°C for 5 min, neutralized with 2 M Hepes and applied to the membrane in 5 × SSC.

In vitro transport of RNA from isolated nuclei

The cells after growth in repression medium for 2·5 h, were subjected to different conditions (*i.e.*, repressed, derepressed and repressed but heme treated) along with the supplement of [^{32}P] phosphoric acid (20 μCi/ml) for 30 min. The nuclei were isolated as described previously. *In vitro* transport of RNA from isolated labelled nuclei was performed following the method described by Schumm and Webb (1972) with minor modifications. The incubations were carried out in a medium containing labelled nuclei (4·5 × 10^6 ml), 50 mM Tris-HCl, pH 7·5, 25 mM KCl, 2·5 mM MgCl2, 0·5 mM $CaCl_2$, 2·5 mM Na_2 HPO_4, 150 mM DTT, 2 mM ATP, 5 mM creatine phosphate, 35 units of creatine Phosphokinase and 500 μg yeast RNA/ml. Actinomycin D at a concentration of 15 μg/ml was present throughout the incubation period to prevent further incorporation during incubation. After incubation at 25°C for 30 min, the nuclei were pelleted by centrifugation at 5000 *g* for 10 min and RNA was extracted from the supernatant. RNA was suspended in Tris-HCl and subjected to DNase (RNase free) digestion and again precipitated with ethanol. The percentage of RNA transported was calculated based on the total radioactivity in the RNA released into the supernatant and the total radioactivity associated with RNA extracted from the nuclei before incubation.

*In vitro transport of CYC*1 *RNA from isolated nuclei*

In vitro transport of RNA from cold nuclei isolated from cells subjected to different conditions (*i.e.*, repressed, derepressed, repressed but heme treated) was performed and the transported RNA was extracted as above. Twenty μg of denatured RNA, transported under each condition was spotted onto a nitrocellulose membrane and hybridized to nick translated CYC1 probe following the method described by Thomas (1983). In order to quantify the level of transport of CYC1 RNA the radioactivity associated with the spots were measured in a scintillation based counter.

In vitro translation of in vitro transported RNA

In vitro translation of *in vitro* transported RNA from cold nuclei isolated from the cells grown under different conditions, was carried out in reticulocyte lysate following the method described by Pelham and Jackson (1976). A reaction mixture in a total volume of 25 μl contained 10 mM creatine phosphate, 10 μM GTP, 1 mM ATP, 300 μM spermidine, 50 μM pottassium acetate, 20 mM Hepes, pH 7·4, 2 mM DTT , 25 μg creatine Phosphokinase, 10 mM magnesium acetate, 10 μl of the rabbit reticulocyte lysate, 20 μg of RNA and 20 μCi of [^{35}S] methionine (800Ci/m mol) per assay. The mixture was incubated at 37°C for 1 h. The translation products were precipitated with trichloroacetic acid and processed for counting (Beattie, 1979).

Measurement of the half-life of CYC1 mRNA in the cytosol

The cells, after growth in repression medium for 3 h (allowed the cells to derepress for 30 min), were treated with actinomycin D (15 μg/ml) and cordycepin (100 μg/ml) to block fresh transcription and transport respectively during the chase and divided into 3 portions. These were grown under repressed, repressed but heme-treated and derepressed conditions. At different time intervals, the cells were harvested and post-mitochondrial supernatant prepared. RNA was extracted and hybridized to nick-translated CYC1 gene. The counts associated with the hybrid were checked.

Results

As a first step towards understanding the role of heme in the expression of CYC1 gene in yeast cells undergoing glucose repression-derepression, the steady state levels of CYC1 RNA in the cytosol was checked. For this purpose, cytosolic RNA from cells grown under repressed, repressed but heme-treated, derepressed and levulinic acid treated conditions were extracted and hybridized to a cloned CYC1 gene. Figure 1 shows that the level of CYC1 RNA in repressed cells is 6–8-fold lower as compared to derepressed cells. However, exogenous addition of hemin to repressed cells restores the level to that observed in derepressed cells. Involvement of heme is further confirmed by the decreased level seen in levulinic acid treated cells.

To look into the functional significance of this observation, the amount of CYC1

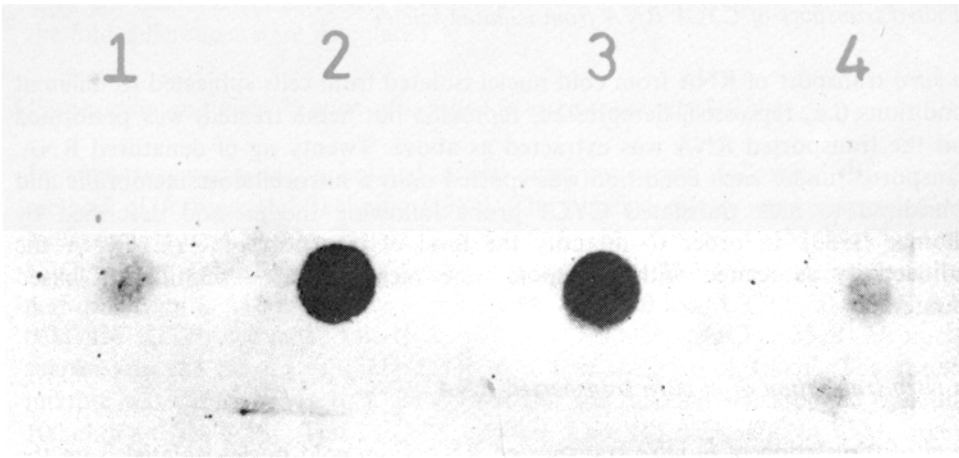

Figure 1. Dot blot analysis of CYC1 RNA in the cytosol.

The cells after growth in repression medium for 2·5 h were divided into 4 portions and grown under repressed (1), repressed but heme-treated (2), derepressed (3) and levulinic acid-treated (4) conditions for another 30 min. The cells were then harvested and post-mitochondrial supernatant prepared. Total RNA was extracted from the post-mitochondrial supernatant. RNA (20 μg) was heat denatured by incubating at 65°C for 15 min and hybridized to nick-translated CYC1 DNA. The counts associated with the hybrid were calculated as given under 'methods'.

RNA associated with the polysomes was checked by extracting RNA from the polysomes prepared from cells grown under the above conditions and hybridizing to CYC1 gene. Figure 2 shows a pattern similar to the one seen with cytosolic RNA.

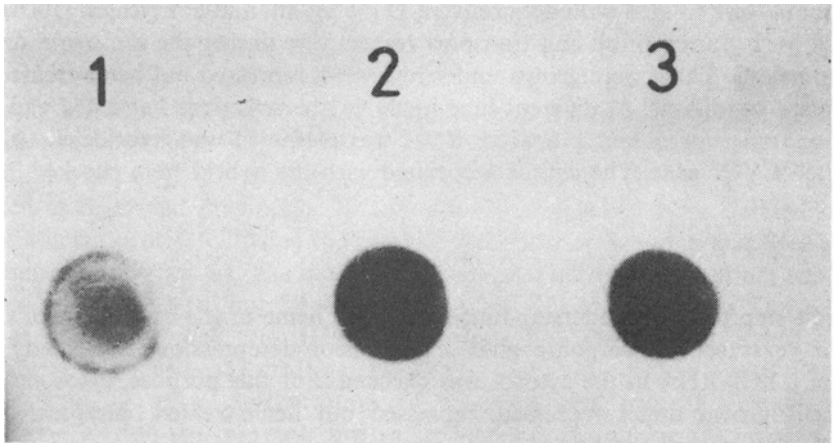

Figure 2. Relative levels of CYC1 RNA associated with the polysomes.

The cells after growth in repression medium for 2·5 h were subjected to different conditions [repressed (1), repressed but heme-treated (2) and derepressed (3)] for 30 min. The cells were then harvested and polysomes were prepared. RNA was extracted from the polysomes and 20 μg of RNA was heat denatured and hybridized to nick-translated CYC1 DNA. The counts associated with the hybrid were calculated as mentioned under 'methods'.

In order to account for the increased levels of CYC1 RNA in the cytosol, the possible role of heme in triggering the transcription of CYC1 gene was investigated. Towards this, studies on *in vitro* transcription in isolated nuclei prepared from repressed, repressed but heme-treated and derepressed cells were carried out using [^{3}H] UTP. The *in vitro* transcripts were extracted and hybridized to unlabelled CYC1 probe immobilized on a nitrocellulose membrane. The counts associated with the hybrid was checked. Table 1 shows that there is a 4-fold stimulation in the transcription of CYC 1 gene in repressed but heme-treated and derepressed nuclei over the repressed nuclei. *In vitro* addition of hemin to repressed nuclei, however, does not restore the level to that seen in derepressed nuclei, thus excluding the possibility of direct interaction between hemin and the transcriptional machinery. In order to see whether heme plays any role in post-transcriptional regulation of CYC 1 gene expression, the transport of RNA from isolated nuclei was studied. Table 2 shows, contrary to our expectations, that the amount of RNA transported from repressed but heme-treated and derepressed nuclei is only around 50% of that observed from repressed nuclei. However, *in vitro* translation of the *in vitro* transported RNA in a heterologous cell free system revealed (table 3) a 3–4-fold stimulation in the incorporation of labelled amino acid into polypeptides with the total RNA transported from derepressed and repressed but heme-treated nuclei rather than from the repressed nuclei. These results indicate that though there is a decrease in the transport of total RNA, the amount of translatable RNA transported upon hemin treatment is greater than that observed from repressed nuclei. Hybridization of the transported RNA with CYC1 probe has lent further evidence for the preferential transport of mRNA (figure 3).

Table 1. *In vitro* transcription of CYC1 gene in isolated nuclei.

	cpm hybridized			
Source of nuclei	Exp. 1	Exp. 2	Exp. 3	Stimulation (%)
Repressed	30	42	33	100
Repressed + hemin (*in vivo*)	136	199	142	452·47 ± 17·77
Derepressed	126	181	140	425·07 ± 4·53
Derepressed + levulinic acid	20	27	20	63·87 ± 2·51
Repressed + hemin (*in vitro*)	35	52	37	117·83 ± 4·78

Equal numbers ($4{\cdot}5 \times 10^6$) of nuclei isolated from cells grown under different conditions mentioned in the table were incubated with transcription assay buffer containing [^{3}H] UTP (50 μCi/assay). After terminating transcription following 25 min of incubation, RNA was extracted. Labelled RNA (10^5 cpm) transcribed *in vitro* was hybridized to CYC1 DNA immobilized on (nitrocellulose) filter and the radioactivity of the hybridized RNA was measured. The results presented here represent values from 3 sets of experiments. Percentage stimulation is given as the mean ± SD of the values obtained from the 3 experiments taking repressed value as 100%.

It has been shown by Dharmalingam and Jayaraman (1971) that there is an induction of hydrolytic enzymes during glucose-repression. Hence, the alterations in the level of CYC1 RNA in the cytosol could also be due to the differential stability of CYC1 mRNA under different conditions. To check this possibility, the half-life of CYC1 RNA was measured. Figure 4 shows that there is no significant variations in the half-life of CYC 1 RNA in cells grown under different conditions.

Table 2. *In vitro* transport of RNA from isolated nuclei.

Source of nuclei	Exp. No.	Total radio-activity in the nuclei (cpm)	Transported radioactivity (cpm)	Transport (% of total)	Decrease in transport (% of repressed)
Repressed	1	263190	21555	8·19	
	2	140148	10063	7·18	
	3	134566	9823	7·30	
Repressed + hemin	1	232090	8843	3·81	
	2	256310	10483	4·09	50·21 ± 6·21
	3	187247	6272	3·35	
Derepressed	1	324100	11408	3·52	
	2	183687	6172	3·36	53·23 ± 3·78
	3	152832	5640	3·69	

After labelling the cells with [^{32}P] phosphoric acid (20 μCi/ml) under different conditions for 30 min, nuclei were isolated and purified. Equal numbers of nuclei ($4{\cdot}5 \times 10^6$/ml) were incubated with transport assay buffer. After 30 min of incubation, nuclei were pelleted and RNA was extracted from the supernatant and checked for radioactivity. Percentage transport was calculated from the radioactivity associated with released RNA and the radioactivity associated with RNA extracted from the nuclei before incubation. The results presented here represent values from 3 sets of experiments. The mean ± SD of the percentage decrease in transport under repressed but heme treated and derepressed conditions were calculated taking the percentage transport in repressed condition as 100%.

Table 3. *In vitro* translation of *in vitro* transported RNA.

Nuclei-source of transported RNA	Radioactivity incorporated (cpm) Exp. 1	Exp. 2	Exp. 3	Stimulation (%)
Repressed	28075	32167	23522	100
Repressed + hemin	130425	146731	105734	456·74 ± 7·54
Derepressed	87750	48928	73629	311·04 ± 3·04

RNA (20 μg) transported under *in vitro* conditions from nuclei isolated from cells grown under different conditions mentioned in the table was translated in a reticulocyte lysate suplemented with 25μCi of [^{35}S] methionine (see 'methods'). The radioactivity incorporated into the translated products were measured. The results presented here represent values from 3 sets of experiments. Percentage stimulation is given as the mean ± SD of the values obtained from the 3 experiments taking repressed values as 100%.

Discussion

In this report we have shown that hemin increases the levels of CYC1 mRNA in the cytosol. This increase has been shown not due to the differential stability of CYC1 mRNA under different conditions employed. The fact that there is an increased association of CYC1 RNA with the polysomes upon hemin treatment, not only reflects the increased availability of CYC1 RNA in the cytosol but also the increased availability of translatable RNA. Studies on *in vitro* transcription clearly demonstrated an increased transcription of CYC 1 RNA. However, the unexpected results of the in *vitro* transport studies definitely warrant further discussion. The transport of RNA has been shown to be regulated in a variety of cells by their metabolic status (Hill, 1975; Johnson *et al.,* 1974; Meenakshi *et al.,* 1983; Shaerer, 1974). The increased transport of RNA under repressed condition can be mostly

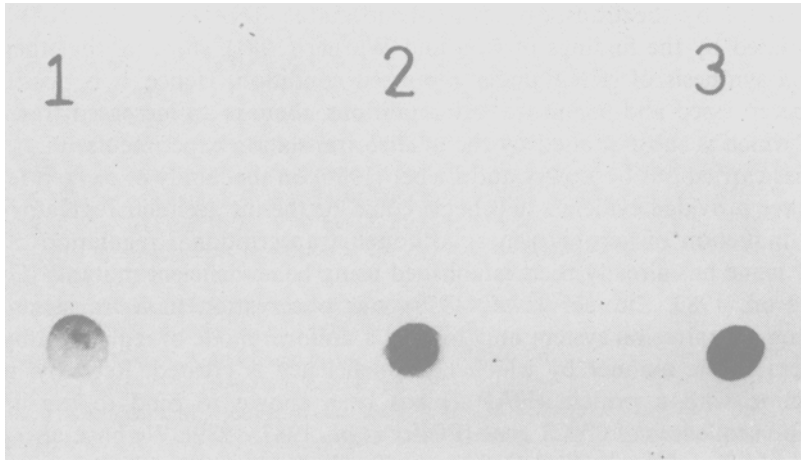

Figure 3. *In vitro* transport of CYC1 RNA from isolated nuclei.
The cells after growth in repression medium for 2·5 h were subjected to different conditions [repressed (1), repressed but heme-treated (2) and derepressed (3)] for 30 min. The cells were then harvested and nuclei isolated. Equal numbers of nuclei ($4{\cdot}5 \times 10^6$/ml) from these cells were incubated with transport assay medium at 25°C. After 30 min of incubation, the nuclei were pelleted and RNA was extracted from the supernatant. RNA (20 μg) was hybridized to nick translated CYC1 DNA.

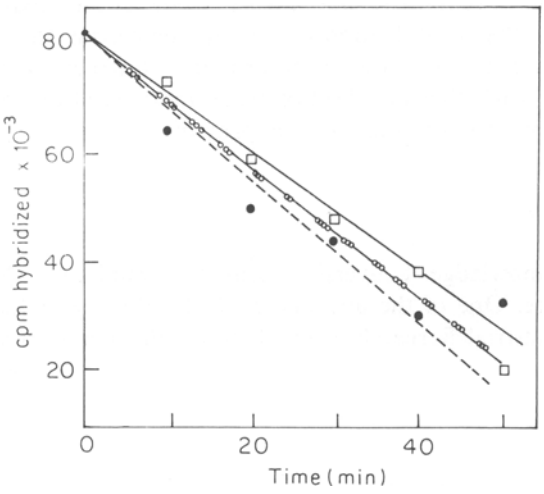

Figure 4. Half-life of CYC1 RNA.
The cells after growth in repression medium for 3 h (the cells were allowed to derepress for 30 min) were treated with actinomycin D (15 μg/ml) and cordycepin (100 μg/ml) and divided into 3 portions. These were grown under repressed (●) repressed but heme-treated (_) and derepressed conditions (O). At indicated time intervals the cells were harvested and post-mitochondrial supernatant prepared. Total RNA was extracted from the post-mitochondrial supernatant and 20 μg of it was hybridized to nick-translated CYC1 DNA. The radioactivity associated with the hybrid were determined by cuting out the spots corresponding to the hybridization signal and counting in a liquid scintillation counter.

accounted for by the transport of non-translatable RNA (mainly rRNA). This is substantiated by the findings of Kief and Warner (1981), showing that there is an increased synthesis of rRNA under repressed condition. Hence, it is possible that under derepressed and hemin-treated conditions, there is an increased transport of mRNA which is substantiated by the *in vitro* translation experiments.

Studies carried out by Lowry and Lieber (1986) on the family of oxygen regulated genes have provided evidence that heme could be the intracellular regulatory signal for the induction of aerobic genes. Although transcriptional regulation of CYC1 gene by heme has already been established using heme-deficient mutants (Guarente and Mason, 1983; Zitomer *et al.,* 1979), our observation in a transient glucose repression-derepression system only implies a uniform mode of regulation by hemin regardless of the manner by which heme-deficiency is created. Recently, heme in conjunction with a protein (HAP 1) has been shown to bind to the upstream ctivation sequences of CYC1 gene (Pfeifer *et al.,* 1987, 1989). We have also purified a protein (different from HAP1) that specifically binds to the CYC1 gene and that the level of this protein varies depending upon the physiological status of the cell (unpublished results). Such binding has been shown to promote transcription. Similar observation has also been reported in the case of catalase T gene (Spevak *et al.,* 1986). These evidences strongly suggest that heme, in addition to being the prosthetic group of the hemoproteins, is also involved in the synthesis of the apoproteins.

In conclusion, heme has been shown to play an important role in the expression of CYC1 gene in cells undergoing glucose repression-derepression. A predominant effect has been shown to be exerted at the level of transcription and transport of CYC1 RNA. This strengthens our earlier observations that heme specifically stimulates the synthesis of hemoproteins by increasing the availability of the translatable messengers for the hemoproteins. Further work is required to demonstrate clearly whether the effect of heme observed at the level of transcription and post-transcription is specific for hemoproteins.

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