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(71) Demandeur/Applicant:
VALTION TEKNIILLINEN TUTKIMUSKESKUS, FI

(72) Inventeurs/Inventors:
PAKULA, TIINA, FI;
SALOHEIMO, MARKKU, FI;
UUSITALO, JAANA, FI;
HUUSKONEN, ANNE, FI;
WATSON, ADRIAN, GB;
JEENES, DAVID, GB;
ARCHER, DAVID, GB;
PENTTILAE, MERJA, FI

(74) Agent: OGILVY RENAULT

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(57) **Abrégé/Abstract:**

This invention relates to a promoter and to a fungal host for improved protein production. According to the invention the promoter has been modified in its response to the mechanisms mediating transcriptional down-regulation of secreted proteins under secretion stress. This invention relates also to methods for optimised protein production of secretable proteins in fungi.



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(74) Agent: **SEPPO LAINE OY**; Itämerenkatu 3 B,
FIN-00180 Helsinki (FI).

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(71) Applicant (*for all designated States except US*): **VAL-
TION TEKILLINEN TUTKIMUSKESKUS** [FI/FI];
Vuorimiehentie 5, FIN-02044 VTT (FI).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **PAKULA, Tiina**
[FI/FI]; Haahkatie 12 A 6, FIN-00200 Helsinki (FI).
SALOHEIMO, Markku [FI/FI]; Kyntäjäsentie 18 B,
FIN-00390 Helsinki (FI). **UUSITALO, Jaana** [FI/FI];
Kaitaankulma 6 B 4, FIN-02360 Espoo (FI). **HUUSKO-
NEN, Anne** [FI/FI]; Hopeatie 10 C 53, FIN-00440
Helsinki (FI). **WATSON, Adrian** [GB/GB]; Institute of
Food Research, Genetics and Microbiology Department,
Norwich Research Park, Colney, Norwich, Norfolk NR4
7UA (GB). **JEENES, David** [GB/GB]; Institute of Food
Research, Genetics and Microbiology Department, Nor-
wich Research Park, Colney, Norwich, Norfolk NR4
7UA (GB). **ARCHER, David** [GB/GB]; School of Life
and Environmental Sciences, University of Nottingham,
University Park, Nottingham, Nottinghamshire NG7 2RD
(GB). **PENTTILÄ, Merja** [FI/FI]; Vattuniemenkatu 2 A
22, FIN-00210 Helsinki (FI).

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(54) Title: IMPROVED METHOD FOR PRODUCTION OF SECRETED PROTEINS IN FUNGI

(57) Abstract: This invention relates to a promoter and to a fungal host for improved protein production. According to the invention the promoter has been modified in its response to the mechanisms mediating transcriptional down-regulation of secreted proteins under secretion stress. This invention relates also to methods for optimised protein production of secretable proteins in fungi.



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IMPROVED METHOD FOR PRODUCTION OF SECRETED PROTEINS IN FUNGI

This invention relates to an optimised method for the production of secreted proteins in
5 fungi. In particular, this invention relates to DNA sequences, promoters and fungal hosts
used in the method.

BACKGROUND OF THE INVENTION

10 Certain species of fungi, in particular *Trichoderma reesei* and *Aspergillus niger*, are
commonly used in biotechnological industry for protein production. The recombinant
proteins, either heterologous or homologous, are typically produced under the regulation of
promoters of abundantly expressed genes encoding secreted proteins in the fungi, e.g. the
promoter of *cbh1* of *T. reesei* and the promoter *gla* of *A. niger*. *T. reesei* and *A. niger*
15 produce homologous hydrolases very efficiently into the culture medium, but the yields of
heterologous proteins produced are typically much lower compared to those of
homologous proteins. Especially, the proteins originating from distant species e.g.
mammalian proteins are produced at a very low level (Archer and Peberdy, 1997, Penttilä,
1998). As reasons for the low yields have been suggested inefficient translation and
20 translocation of the polypeptide into the secretory pathway, hindrances in folding and
transport of the protein, and low transcript levels of the heterologous gene due to mRNA
instability (MacKenzie et al. 1993, Gouka et al. 1997).

The impairment of protein folding and further transport, likely to occur during production
25 of a heterologous protein, is known to induce stress responses in the cell. In recent years,
two feedback mechanisms have been reported that allow the cell to sense the state of the
lumen of the ER and respond to perturbations in the normal functioning of this specialised
environment for protein folding and processing. These mechanisms constitute the Un-
folded Protein Response (UPR), which increases the transcriptional activity of genes
30 encoding chaperones and folding catalysts in response to the presence of unfolded proteins
in the lumen of the ER (Shamu *et al.*, 1994) and phosphorylation of the eukaryotic
initiation factor 2 α which down-regulates translation activity in the cells (Harding *et al.*,
1999). The cellular response to unfolded proteins in the endoplasmic reticulum of yeast
and mammalian cells has been reviewed recently by Mori (2000).

However, very little is known about the transcriptional regulation of genes coding for endogenous secreted proteins in these stress conditions. Especially, no reported data is available on the feed-back regulation of genes encoding the secreted proteins in response to limitations in the capacity of the cell to fold and transport the proteins. In some cases it has been observed that concomitant to the expression of heterologous genes the transcript levels of genes encoding endogenous extracellular proteins are lower compared to the expression in the control strains (Margolles-Clark et al. 1996). The explanation given has been that the amount of transcription/regulatory factors needed for efficient expression of the genes could be limiting during expression of multiple copies of the heterologous gene.

Regulation of the genes encoding secreted proteins on different carbon and nitrogen sources has been studied in detail in filamentous fungi, the cellulase and hemicellulase expression in *T. reesei* being a good example of that. In *T. reesei* the cellulase and hemicellulase expression is readily adapted to the environmental requirements and the availability of nutrients. On complex plant material containing medium, the (hemi)cellulase genes are coordinately induced, but also specific induction mechanisms are known (Margolles-Clark et al. 1997). Cellulose and certain oligosaccharides, such as lactose or sophorose, are known to be efficient inducers of the genes. In the presence of glucose the expression of cellulases and hemicellulases are tightly repressed by the carbon catabolite repression mechanism. Several regulatory factors mediating the regulation of cellulase gene expression have been isolated, including the glucose repressor gene *cre1* and as well genes that have been postulated to function as cellulase gene activators (*ace1* and *ace2*) (Saloheimo et al. 2000).

Specifically, modified *Trichoderma* promoters which are inducible by sophorose and not repressed by the presence of glucose and which comprise a nucleotide sequence from *Trichoderma reesei cbh1* promoter upstream of the protein coding region is described in United States Patent No 6, 001, 595. The publication mentions the regions -184 to -1, -161 to -1, -140 to -1 and -161 to -133. Although the publication describes certain truncated regions of *cbh1*, it does not suggest their use in the production of secreted proteins under stress conditions. The promoters are designed for protein production in the presence of glucose or sophorose. Cellulase regulators *ace1* and *ace2* have been described in the International Patent Publication WO 98/23642, which describes their use as

activators of protein production and suggests improved hemi(cellulase) expression by overexpression of the factors. Modifications that result in glucose derepression are described in WO 94/04673.

5 SUMMARY OF THE INVENTION

This invention is based on the novel finding that the expression level of genes encoding secreted proteins in filamentous fungi is decreased in conditions, in which the protein synthesis, folding or transport is impaired. This regulation mechanism has been
10 demonstrated to function in cultures treated with chemical agents interfering with protein synthesis, folding or transport (DTT, Ca²⁺-ionophore A23187, BrefeldinA, respectively), or in strains with functionally incomplete protein folding system (strains expressing anti-sense transcript for the gene *pdiA*). In addition, strains producing heterologous proteins, such as tPA (tissue plasminogen activator) have been shown to display activated UPR as
15 well as lower expression levels of endogenous genes coding for secreted proteins. We have as first found that this type of feed-back regulation occurs in the production of secreted proteins in filamentous fungi.

This phenomenon called down-regulation or feed-back regulation of genes encoding
20 secreted proteins, is utilised in this invention to selectively regulate the genes encoding secreted proteins or their promoters, and to enhance production of chosen proteins. This is achieved by genetically modifying the promoter sequence of a gene coding for a secreted protein to alter its responsiveness to the transcriptional down-regulation. Alternatively, the genes coding for the regulatory factors mediating the down-regulation, or factors in the
25 corresponding signalling pathway, can be modified in a way that the down-regulation is either abolished or enhanced. Inactivation of the down-regulation mechanism is beneficial when production of protein of interest takes place under the regulation of a promoter that is normally subjected to down-regulation during secretion stress. Enhancement of the down-regulation can be utilised to repress production of other proteins when the expression of the
30 protein of interest takes place for instance under a promoter that is not subjected to down-regulation.

In this invention has been found that a specific regulatory region or DNA sequence located in a promoter of a secretable protein is capable of mediating transcriptional down-regulation.

- 5 More specifically, a DNA sequence located in a promoter of a secretable protein in a fungus is mainly characterized by what is stated in the characterizing part of claim 1.

DNA sequences in the promoter mediating transcriptional down-regulation of secreted proteins under secretion stress can be mutated, inactivated or removed to abolish or reduce
10 the down-regulation of the gene or alternatively the down-regulation of the gene can be enhanced by modifying the promoter sequences, e.g. by amplifying the responsive promoter element, subjected to the down-regulation.

More specifically a promoter of a secretable protein is mainly characterized by what is
15 stated in the characterizing part of claim 6.

A method for producing a promoter for improved protein production in a fungal host is stated in the characterizing part of claim 10.

20 The invention can be used to design better strains for protein production by increasing the efficiency of the promoters used for protein production and/or manipulating the regulation system of secreted proteins.

More specifically a fungal host strain for optimised protein production is mainly
25 characterized by what is stated in the characterizing part of claim 12.

A method for producing a fungal host for improved protein production is mainly characterized by what is stated in the characterizing part of claims 26 and 27.

30 The present invention can be used for modification of a homologous or heterologous promoter used for production of a protein, either homologous or heterologous, in a way that the expression is not subject to the down-regulation in a similar manner as the unmodified promoter. In particular, the invention is useful for producing heterologous proteins but can be applied also to production of homologous proteins.

Furthermore the invention can be used for inactivation or reducing the activity or expression of the regulatory factor(s) mediating the down-regulation of the promoter to improve protein production under the promoter, either the regulatory factor(s) binding to the promoter or regulatory factors mediating the response.

A method for optimised protein production of secretable proteins in fungi is mainly characterized by what is stated in the characterizing part of claims 28 and 30.

One possibility to use the invention is overexpression of a regulatory factor to decrease production of homologous secreted proteins during expression of homologous or heterologous proteins under a promoter that is not subjected to the down-regulation. In such case a heterologous protein is expressed and secreted e.g. under a promoter such as *Trichoderma gpd*, which is not affected in stress conditions. Homologous secreted proteins are expressed under a promoter which is down-regulated. Genes encoding proteins mediating the down-regulation are overexpressed.

A method for optimised protein production of secretable proteins in fungi is mainly characterized also by what is stated in the characterizing part of claim 31.

Use of the DNA sequence, promoter or fungal host prepared according to this invention is characterized by what is stated in claim 31.

Other features, aspects and advantages of the present invention will become apparent from the following description and appended claims.

FIGURES

Fig.1. Total protein synthesis and secretion in cultures treated with A23187, DTT and BFA.

A) The amount of radioactivity incorporated into TCA insoluble material in cell extracts prepared from cultures treated with 5uM A23187 (open diamonds, $\diamond$), and from non-treated control cultures (black diamonds, $\blacklozenge$).

- B) The amount of radioactivity incorporated into TCA insoluble material in culture supernatant in cultures treated with 5uM A23187 (open diamonds, ◇), and from non-treated control cultures (black diamonds, ◆).
- C) The amount of radioactivity incorporated into TCA insoluble material in cell extracts
5 prepared from cultures treated with 10 mM DTT (open diamonds, ◇), and from non-treated control cultures (black diamonds, ◆).
- D) The amount of radioactivity incorporated into TCA insoluble material in culture supernatant in cultures treated with 10 mM DTT (open diamonds, ◇), and from non-treated control cultures (black diamonds, ◆).
- 10 E) The amount of radioactivity incorporated into TCA insoluble material in cell extracts prepared from cultures treated with 50µg/ml BFA (open diamonds, ◇), and from non-treated control cultures (black diamonds, ◆).
- F) The amount of radioactivity incorporated into TCA insoluble material in culture supernatant in cultures treated with 50µg/ml BFA (open diamonds, ◇), and from non-
15 treated control cultures (black diamonds, ◆).

Fig.2. 2D gel analysis of CBHI from cultures treated either with 5 µM A23187, 50µg/ml BFA or 10 mM DTT and labelled with ³⁵S-methionine for different time periods.

- A) Labelled CBHI from cell extracts prepared from non-treated control culture and from
20 cultures treated with A23187, DTT or BFA at different time points during the labelling experiment (the time point is indicated above each panel as minutes after addition of the labelled methionine).
- B) Labelled CBHI from culture supernatant after 180 minutes of labelling of the cultures treated with A23187 or BFA and of non-treated cultures.

25

Fig. 3. The synthesis and secretion of CBHI.

- A) The amount of labelled CBHI in the cell extract at different time points of the labelling experiment in cultures treated with 5 µM A23187 (open circles, o) and in non-treated control cultures (black diamonds, ◆)
- 30 B) The amount of labelled CBHI in the culture supernatant at different time points of the labelling experiment in cultures treated with 5 µM A23187 (open circles, o) and in non-treated control cultures (black diamonds, ◆)

C) The amount of labelled CBHI in the cell extract at different time points of the labelling experiment in cultures treated with 10 mM DTT (open circles, o) and in non-treated control cultures (black diamonds, ◆)

5 D) The amount of labelled CBHI in the culture supernatant at different time points of the labelling experiment in cultures treated with 10 mM DTT (open circles, o) and in non-treated control cultures (black diamonds, ◆)

E) The amount of labelled CBHI in the cell extract at different time points of the labelling experiment in cultures treated with 50µg/ml BFA (open circles, o) and in non-treated control cultures (black diamonds, ◆)

10 F) The amount of labelled CBHI in the culture supernatant at different time points of the labelling experiment in cultures treated with 50µg/ml BFA (open circles, o) and in non-treated control cultures (black diamonds, ◆)

15 Fig.4. Northern analysis of *pdi1* and *bip1* expression in cultures treated with A23187, DTT or BFA.

A) The steady-state mRNA level of *bip1* and *pdi1* (the signals normalised with those of *gpd*) at different time points of the treatment with A23187 (the black bars) and in non-treated control cultures (the white bars)

20 B) The steady-state mRNA levels of *bip1* and *pdi1* (the signals normalised with those of *gpd*) at different time points of the treatment with DTT (the black bars) and in non-treated control cultures (the white bars)

C) The steady-state mRNA levels of *bip1* and *pdi1* (the signals normalised with those of *gpd*) at different time points of the treatment with BFA (the black bars) and in non-treated control cultures (the white bars)

25

Fig. 5. Northern analysis of *hac1* mRNA in cultures treated with BFA and A23187 (the black bars) and in the non-treated control cultures (the white bars).

30 Fig. 6. Northern analysis of *cbh1* and *egl1* in cultures treated with A23187, DTT or BFA for different time periods (the black bars) and in the control cultures (the white bars).

Fig. 7. Northern analysis of *xyn1* and *hfb2* proteins in *T. reesei* during DTT treatment (signals normalised with the signal of *gpd*)

Fig. 8. Northern analysis of transcripts that are not down-regulated during treatment with DTT: signals of *ypt1* and *sar1* coding for components of the secretory pathway, *cDNA1* of unknown function, and *bgl2* coding for intracellular β -glucosidase (the signals were normalised with the signal of *gpd*)

5

Fig. 9. The effects of DTT on the transcription of genes from *A. niger*:

A) the glucoamylase gene, *glaA* (average signal of three determinations)

B) the acid protease, aspergillopepsin gene (*pepA*)(average signal of three determinations)

10 C) protein disulfide isomerase (*pdiA*), a foldase resident in the ER, (average signal of three determinations)

D) *bipA*, an ER-resident chaperone, (average signal of three determinations)

(a solid line representing the DTT-treated cultures and a dotted line representing the water treated controls)

15 Fig 10. The effect of exchanging medium containing starch as a carbon source for medium containing xylose as a carbon source on the transcription of *glaA* in *A. niger* AB4.1 (The exchange was accomplished at T=0., and the results represent the average of two determinations)

20 Fig 11.

A) The effect of antisense *pdiA* on the transcription of the glucoamylase gene (*glaA*, average signal of six flasks from two different experiments) (The strain AB4.1 is represented by a solid line, and the strain AS1.1 by a dotted line.)

25 B) The effect of antisense *pdiA* on the transcription of the aspergillopepsin gene (*pepA*, average signal of six flasks from two different experiments). (The strain AB4.1 is represented by a solid line, and the strain AS1.1 by a dotted line.)

C) The dry weight determination for the cultures (average signal of six flasks from two different experiments)

30 Fig 12.

A) Levels of secreted glucoamylase protein for *A. niger* expressing antisense *pdiA* under the control of the *gpdA* promoter. (The data are averages of three determinations for each time-point, the strain AB4.1 is represented by black bars, and the strain ASG67 by a grey bars).

B) Steady-state levels of *glaA* mRNA in *A. niger* expressing antisense *pdiA* under the control of the *gpdA* promoter. (The strain AB4.1 is represented by a solid line, and the strain ASG67 by a dotted line).

5

C) The dry weight determination for the cultures. (The strain AB4.1 is represented by a solid line, and the strain ASG67 by a dotted line).

10 Fig 13. Northern blot analysis of *A. niger* AB4.1 and ASG67 probed with *hacA*. Lanes 1-7 show samples for *A. niger* AB4.1 at 24, 36, 48, 60, 72, 84 and 96 hours while lanes 8 – 14 shown the same time-points for *A. niger* ASG67 (antisense *pdiA* strain).

15 Fig. 14. Bioreactor cultivation of *T. reesei* Rut-C30 and a tPA producing transformant 306/36

A) The expression cassette for production of tPA in *T. reesei* Rut-C30

B) Biomass dry weight and lactose concentration measured during the cultivation to monitor growth.

20 C) Total protein and HEC activity (measuring cellulase, especially endoglucanase, activity) produced in the culture medium.

D) Transcript level of *egl1* (normalised with the signal of actin gene) analysed as an example of a gene coding for a secreted protein.

E) Transcript level of *cbh1* and *cbh1-tPA* fusion (normalised with the signal of actin gene

F) Transcript level of *bip1* (normalised with the signal of actin gene) during the cultivation

25

Fig. 15. Expression of the *lacZ* reporter gene under *cbh1* promoter

A) Schematic view of the *lacZ* expression cassettes used for the expression studies in the presence of 10mM DTT: the *lacZ* expression under the full-length *cbh1* promoter in the strain pMLO16, and under the shortened promoter in the strain pMI34.

30 B) The mRNA levels of *lacZ* and *egl1* (normalised with *gpd* signal) during the treatment with 10mM DTT (the black bars) and in the non-treated control cultures (the white bars) in the strain pMLO16 (the graphs on the left) and in the strain pMI34 (the graphs on the right).

35 Fig. 16. Expression of the *lacZ* reporter gene under *cbh1* promoters deleted to different extent

- A) Schematic presentation of the deletion series of *cbh1* promoter used for the *E. coli lacZ* expression in *T. reesei* for studying the the activity of the promoter under the secretion stress conditions
- 5 B) Northern analysis of the expression of *lacZ*, *egl1* and *gpd1* in DTT treated cultures of the strains pMLO16, pMLO16S, del1(1)S and del0(2)S and in untreated cultures of the same strains. The time point of the treatment is indicated above each lane as minutes from the onset of the treatment. The relative mRNA levels in the DTT treated cultures at each time point (the mRNA level in the untreated control culture was set as 1) are shown in the graphs on the right (*lacZ* signal, the open circles; *egl1* signal, the black diamonds)
- 10 C) Northern analysis of the strains del23, del5(11)S, del5(11) and del6(14) as in B)
- D) Northern analysis of he strains del7(5)S, pMI33 and pMI34 as in B)

Fig. 17. The *cbh1* mRNA level during DTT treatment in cultures of *T. reesei* QM9414 and QM9414 with a deleted *ace1* gene

15

Fig. 18. Screening of fungal mutants defective in the mechanism of down-regulation of genes under secretion stress conditions

- A) Microtiter plate cultures of the strains pMLO16, pMI33 and QM9414 tested for *lacZ* production in the presence and absence of BFA and sophorose. *LacZ* production was
- 20 detected as a darker color reaction
- B) Screening of mutants in microtiter plate cultures for the expression of *lacZ* after sophorose induction in the presence of BFA. *LacZ* producing mutants were detected based on the dark color color reaction after addition of X-gal substrate. The cultures of unmutagenised pMLO16 were were assayed for *lacZ* production after sophorose induction
- 25 in the presence and absence of BFA mutants (the wells indicated by the boxes)

DETAILED DESCRIPTION OF THE INVENTION

- 30 By the term "endogenous proteins" is meant here proteins which are natural products of a microorganism host.

By "recombinant proteins" are meant here proteins that are not natural products of a microorganism. DNA sequences encoding desired homologous or heterologous proteins

35 may be transferred by a suitable method to a host. By "homologous protein" is meant a

protein produced by the same microorganism species. By "heterologous protein" is meant a protein produced by another microorganism species.

By "secretable protein" or "secreted protein" is meant here a protein that is secreted outside
5 of the host cell to the culture medium.

By "improved protein production" is meant protein production which is at least 3 %, preferably at least 5 %, more preferably at least 10 %, still more preferably at least 20 %, most preferably at least 30 % better than protein production by using a fungal host strain
10 which has not been genetically modified to alter their down-regulation.

By "secretion stress" or "secretion stress conditions" we mean here that the secretion capacity of the host is limited or the secretion route overloaded. The limitation may be caused for example by the production of heterologous proteins or increased amounts of
15 homologous proteins, or it may be due to a toxin hindering the synthesis, folding or transport of the protein (e.g. ionophore, DTT or brefeldin A (=BFA)). The limitation can be caused also by modification of the folding or secretion route by genetical means e.g. by enhancing or inactivating the activity of the components required for protein folding or transport. Like UPR, this mechanism of transcriptional down-regulation of secreted protein
20 genes can, however, be considered a natural mechanism for the organism to balance the synthesis of secreted proteins and the folding and secretion capacity, and may (partially) occur in many protein production conditions, such as when the synthesis of secreted homologous proteins is induced.

By "down-regulation", "transcriptional down-regulation" or "feed-back-regulation" we mean here that the mRNA levels corresponding to a protein are lowered due to these cellular responses mentioned above. This down-regulation effect has been shown by measuring the mRNA level of the genes encoding secreted proteins.

30 According to this invention, DNA sequences mediating down-regulation of genes encoding secreted proteins can be found in the promoters of the genes coding for secretable proteins. This means that the promoters comprise regions which are able to down-regulate the gene product, as a response to the action of cellular mechanisms such as regulatory factors.

Various conditions can be used for analysing down-regulation of a gene under secretion stress, as described above. These include e.g. conditions under which secreted proteins are overproduced (heterologous or endogenous proteins), or using toxins, like DTT, BFA or Ca-ionophore, or by modification of the folding or secretion route by genetical means, e.g. by enhancing or inactivating the activity of the components required for protein folding or transport. In this invention the secretion stress was simulated by treating the cultures with DTT, Ca-ionophore A23187, and BFA, or expressing a heterologous secreted protein (tissue plasminogen activator), or reducing by genetic modification the activity of the folding machinery (using the anti-sense technique).

For the purposes of this invention a promoter is defined to comprise DNA sequences mediating transcriptional down-regulation (or down-regulation) if the amount mRNA obtained under the control of the promoter is lower when the host comprising the promoter is grown under secretion stress conditions (as described above) compared with the mRNA amount obtained when the host is grown under non-secretion stress conditions.

In a similar manner for the purposes of this invention a host is defined to comprise mechanisms such as regulatory factors mediating transcriptional down-regulation or down-regulation if the mRNA amount of a gene or genes encoding secreted protein(s) is lower when the host is grown under secretion stress conditions (as described above) compared with the mRNA amount when the host is grown under non-secretion stress conditions.

The down-regulation effect is shown by measuring the mRNA level of the gene under secretion stress as described above. For the purposes of this invention a promoter or host is genetically modified in its response to mechanisms mediating transcriptional down-regulation, if a measurable change can be shown in the mRNA level of the genes encoding secreted protein (s). In other words the expression (mRNA amount) of a selected secretable protein is enhanced or decreased. Preferably the change is 10 % or more, more preferably 20 % or more, still more preferably 30 % or more, most preferably 50 % or more, increase or decrease compared to a non-modified promoter or host.

“A reporter protein” is for the purposes of this invention any gene or protein the expression or amount of which can be analyzed. When testing the capacity of a promoter or a host in

mediating transcriptional down-regulation under secretion stress as described above, the mRNA level of the reporter protein can be analyzed.

The DNA sequences or regions mediating down-regulation of secreted proteins are located
5 in the promoters of various genes coding for proteins such as cellulases, hemicellulases, amylolytic enzymes, hydrophobins, proteases, invertases, phytases, phosphatases, swollenins, and pectinases.

Preferably the DNA sequences are located in the promoters selected from the group
10 comprising *cbh1*, *cbh2*, *egl1*, *egl2*, *hfb1*, *hfb2*, *xyn1*, *swo*, *gla*, *amy*, and *pepA* promoters.

This invention shows that a number of genes encoding secreted proteins by *Trichoderma* and *Aspergillus* are subject to the transcriptional down-regulation mechanism. The promoter of a secretable protein according to this invention is preferably a promoter of an
15 efficiently secreted hydrolase of the genus *Trichoderma*. More preferably the promoter is a cellulase or hemicellulase promoter of *Trichoderma*. Most preferably the promoter is *cbh1* of *Trichoderma*. The promoter of a secretable protein may also be the promoter of an efficiently secreted hydrolase of the genus *Aspergillus*. Preferably the promoter is a protease or a promoter of an amylolytic enzyme gene of *Aspergillus*. More preferably the
20 promoter is *gla*, *amy* or *pepA*.

As exemplified in this invention DNA sequences mediating down-regulation of secretable proteins can be found in *Trichoderma cbh1* promoter upstream of -162 (SEQ ID NO. 5). Alternatively they can be found upstream of -188 (SEQ ID NO. 2), -211 (SEQ ID NO. 3),
25 -341 (SEQ ID NO. 4), -391 (SEQ ID NO. 1), -501 (SEQ ID NO. 8), -741 (SEQ ID NO. 9), -881 (SEQ ID NO. 10). However, they seem to be located downstream of -1031 (SEQ ID NO. 11), -1201 (SEQ ID NO. 7) or -1281 (SEQ ID NO. 6). The DNA sequences mediating down-regulation of secretable proteins in *cbh1* promoter seem therefore to be located between the nucleotides -1031 and -162. The most important area being between -211 and
30 -341 and between the nucleotides -501 and -1031.

According to one embodiment of this invention a promoter of a secretable protein is genetically modified not to be down-regulated or reduced in down-regulation.

In a promoter modified according to this invention the effect of the DNA sequences mediating down-regulation of secreted proteins is diminished by various mutation methods, or the sequences may be inactivated or removed. For example, promoters where the DNA sequence mediating down-regulation of secretable proteins is deleted are
5 promoters lacking the nucleotides upstream of -501 (SEQ ID NO. 16), -188 (SEQ ID NO. 17), -211 (SEQ ID NO. 18), -341 (SEQ ID NO. 119), -391 (SEQ ID NO. 20), -162 (SEQ ID NO. 21), -881 (SEQ ID NO. 22) and -741 (SEQ ID NO. 23) of *Trichoderma cbh1* promoter.

10 In another promoter modified according to this invention the effect of the DNA sequences mediating down-regulation of secreted proteins may be increased by amplifying the sequence responsible for mediating the down-regulation using standard molecular biology methods .

15 For optimised production of secretable proteins fungal host strains may be constructed, in which mechanisms that down-regulate transcription of genes encoding secreted proteins under secretion stress have been genetically modified.

According to one embodiment of this invention the fungal host strain of this invention may
20 comprise a promoter in which the effect of the DNA sequences mediating down-regulation of secreted proteins is diminished or removed or the effect of the DNA sequences mediating down-regulation of secreted proteins is increased.

According to another embodiment of this invention the expression of the regulatory factors
25 mediating transcriptional down-regulation may be genetically modified in the fungal host. If desired, the expression of the regulatory factors may be reduced or abolished, or the expression of the regulatory factors may be increased.

This invention shows that a number of genes encoding extracellular secreted proteins are
30 transcriptionally down-regulated in the conditions used to demonstrate this regulatory mechanisms and it is expected that many, if not most or all, genes encoding secreted proteins are subject to this transcriptional down-regulation. The genes, promoters and proteins subject to the regulatory mechanism may be selected from the group comprising cellulases (such as cellobiohydrolases, endoglucanases and β -glucosidases), hemicellulases

(such as xylanases, mannases, β -xylosidases, and side chain cleaving enzymes, such as arabinosidases, glucuronidases, acetyl xylan esterases), amylolytic enzymes (such as α -amylases, glucoamylases, pullulanases, cyclodextrinases) hydrophobins, proteases (acidic, alkaline, aspergillopepsin), invertases, fytases, phosphatases, various pectinases (such as
5 endo-and exopolygalacturonases, pectin esterases, pectin and pectin acid lyase) and ligninases (such as lignin peroxidases, Mn peroxidases, laccases).

The regulatory mechanisms are mediating transcriptional down-regulation of the proteins selected from the group comprising those encoded by the genes *cbh1*, *cbh2*, *egl1*, *egl2*,
10 *hfb1*, *hfb2*, *xyn1*, *swo*, *gla*, *amy*, and *pepA*.

As an example the regulatory factor is encoded by the *ace1* gene. Other factors than *ace1* are also involved in this down-regulation which is shown in the examples by the fact that *ace1* is not responsible for (the major part) of the regulation in all culture conditions.

15

The regulatory mechanism are preferably regulating the hydrolases of the genus *Trichoderma*. More preferably they are regulating cellulases or hemicellulases of *Trichoderma*. The regulatory factors may be regulating the hydrolases of the genus *Aspergillus*. Preferably they are regulating proteases or amylolytic enzymes of *Aspergillus*.

20

"A fungal production host" denotes here any fungal host strain selected or genetically modified to produce efficiently a desired product and is useful for protein production for e.g. analytical, medical or industrial use. The host strain is preferably a recombinant strain modified by gene technological means to efficiently produce a product of interest.

25

The invention is here exemplified by two fungal species *Trichoderma* and *Aspergillus*, which shows the general nature of the transcriptional down-regulation mechanism. Modification of this mechanism in other fungi will be useful for improved protein production.

30

Fungal host strains of this invention can be selected from the group comprising *Aspergillus* spp., *Trichoderma* spp., *Neurospora* spp., *Fusarium* spp., *Penicillium* spp., *Hemicoloma* spp., *Tolypocladium* geodes, *Schwanniomyces* spp., *Arxula* spp., *Trichosporon* spp., *Kluyveromyces* spp., *Pichia* spp., *Hansenula* spp., *Candida* spp., *Yarrowia* spp.,

Schizosaccharomyces ssp. and *Saccharomyces* ssp. Preferably the host belongs to *Trichoderma* or *Aspergillus* species, e.g. *T. harzianum*, *T. longibrachiatum*, *T. viride*, *T. koningii*, *A. nidulans*, *A. terreus*, *A. ficum*, *A. oryzae* and *A. awamori*. Most preferably it belongs to *T. reesei* (*Hypocrea jecorina*) or *A. niger* species.

5

A method for optimised protein production of secretable proteins in fungi comprises the steps of:

- selecting a gene encoding a secretable protein;
- genetically modifying the promoter of the gene in its response to mechanisms mediating transcriptional down-regulation of secreted proteins under secretion stress;
- producing a desired secretable protein under the regulation of the promoter in a fungal host; and
- recovering the protein product from the culture medium of the host.

15 According to this invention a method for optimised protein production of secretable proteins in fungi may comprise the steps of:

- cultivating a fungal host as defined above in a suitable culture medium; and
- recovering the protein product from the medium.

20 The protein product may be any product originating from bacteria or higher or lower eukaryotes, the protein product may originate from fungal or mammalian origin. The protein product may be a hydrolase, such as cellulase, hemicellulase, amylolytic enzyme, hydrophobin, protease, invertase, fytase, phosphatase, pectinase or it may be any mammalian protein, such as immunoglobulin or tPA.

25

According to one embodiment of this invention the protein product may be expressed from a promoter not subject to transcriptional down-regulation. Other, undesired proteins may be expressed from a promoter regulated by down-regulation. By enhancing the down-regulation, it is possible to direct the production to the protein product expressed from a promoter not subject to transcriptional down-regulation. Such promoter may be a constitutive promoter, such as *gpd*.

30

A method for optimised protein production of secretable proteins in fungi comprises the steps of:

- selecting a gene of a secretable protein;
- operable linking the coding region of the selected secretable protein into a promoter not regulatable by the mechanism of transcriptional down-regulation ;
- culturing the fungal host under suitable culture conditions and overproducing proteins
- 5 mediating down-regulation in the fungal host; and
- recovering the selected secretable protein from the culture medium of the host.

The selected secretable protein may be a heterologous protein and the undesired secretable proteins may be homologous proteins.

10

By “genetically modifying the promoter to be or not to be regulatable by down-regulation” means here that the promoter has been modified by any suitable conventional or molecular biology method well known in the art to be or not to be regulated by down-regulation in a similar manner than the unmodified promoter is, by DNA techniques, such as by site

15 directed mutagenesis or deletion, or by conventional mutagenesis using chemical agents or irradiation, followed by screening or selecting for cells modified in the transcriptional down-regulation mechanism. In this invention the genetic modification has been exemplified by deleting parts of *Trichoderma cbhl* promoter not to be regulated by down-regulation.

20

“Genetically modifying the genes encoding proteins mediating down-regulation in secretion stress” means here that the genes have been modified by any suitable conventional or molecular biology method well known in the art to be overproduced or inactivated or modified in their activity or expression. The modification is preferably made

25 by recombinant DNA techniques, such as by site directed mutagenesis or deletion but also any other method for genetic modification can be used, such as crossing or fusing cells with desired properties, or by conventional mutagenesis using chemical agents or irradiation, followed by screening or selecting for cells modified in the transcriptional down-regulation mechanism.

30

We have demonstrated the existence of down-regulation mechanism of genes coding for the secreted proteins in filamentous fungi in response to secretion stress. Examples are shown from the fungal species *T. reesei* and *A. niger*. Evidence for the novel regulation mechanism has been obtained analysing fungal cultures treated with chemical agents

preventing either protein synthesis, folding or transport, or by analysing fungal strains displaying diminished foldase levels (see Examples 1, 2, 3, 4 and 5). In addition, in strains producing heterologous proteins, the genes coding for endogenous secreted proteins are expressed at lower levels compared to their parental strain (see Example 6). In eukaryotic systems two feedback mechanisms have been reported in recent years that allow the cell to sense the state of the lumen of the ER and respond to secretion stress to alleviate the perturbations. These include the UPR pathway (Shamu *et al.*, 1994) and attenuation of translation initiation (Harding *et al.*, 1999). Our novel finding comprises a third type of feed-back regulation mechanism functioning under secretion stress, which is shown to be mediated by the promoter sequence of the particular gene using a reporter gene system consisting of *lacZ* expression under *cbh1* promoter sequences (Examples 7 and 8).

Based on the results obtained it is possible to continue characterization of the promoter regions involved in the down-regulation of the genes coding for secreted proteins in *Trichoderma*. The promoter regions involved can be localised by studying for example the *lacZ* reporter gene expression under the *cbh1* promoter that has been deleted to different extent and using conditions in which the mRNA level of the genes coding for extracellular proteins is down-regulated, e.g. treatment with DTT. Based on this analysis, selected promoter regions can be used in gel shift assays with cell extracts from stressed and non-stressed cultures (e.g. DTT- treated and non-treated) to identify the specific regions even more in detail, and to characterize possible binding sites for regulatory factors. Comparison of the promoter sequences can be used for identification of sequences mediating the down-regulation in other promoters that are affected in the stress conditions. Using the methods described here or known in the art it is possible to identify regions in any organism and any promoter from a gene encoding a secreted protein, responding to this transcriptional down-regulation.

Cloning and characterization of the regulatory proteins involved in the feed-back regulation and binding to the promoter sequences can be performed using e.g. yeast-one hybrid system taking advantage of the characterised promoter elements in the *cbh1* promoter (and in the other relevant genes showing down-regulation). Cloning systems for DNA binding proteins that can be applied are commercially available (e.g. MatchmakerTM by Clontech) or have been reported (e.g. Saloheimo *et al.* 2000).

The promoter sequence found to be mediating the down-regulation of the gene can be modified in such a way that the down-regulation in stress conditions is abolished or reduced. By these means it is possible to increase the production level of the gene in conditions where it would otherwise be down-regulated, and production of either an homologous or a heterologous gene product under the modified promoter (from which the down-regulating sequences have been modified) can be improved. In addition the regulatory factors involved in the down-regulation can be completely or partially inactivated to improve protein production. Similar approach can be taken with any organism known to possess down-regulation of genes coding for secreted proteins, e.g. other species of fungi, preferably other species of filamentous fungi.

Production of heterologous proteins may cause similar type of stress response as e.g. the treatment with the chemical agents DTT, BFA or A23187. Lower levels of endogenous cellulase transcripts have been observed in *T. reesei* cultures producing human tissue plasminogen activator indicating down-regulation of the genes coding e.g. for *egl1* and *cbh1* during production of tPA (Example 6). If the promoters of the genes coding for the endogenous extracellular proteins are used for expression of the heterologous product or overexpression of a homologous product inducing stress responses, the expression may become subject to the feed-back regulation mediating transcriptional down-regulation during the production. Modification of either the promoter elements or the regulatory factors binding to the promoter or mediating the regulatory signal are means to increase protein production by abolishing the down-regulation process.

In some cases it may be beneficial to enhance down-regulation during secretion stress to diminish production of some of the endogenous proteins, and to produce the protein of interest under a promoter that is not down-regulated during secretion stress. This can be achieved by overexpressing the regulatory factors mediating the down-regulation and/or by modifying the promoters to increase the binding of the repressing regulatory factors, e.g. increasing the number of binding sites for the factors. This invention describes one method how those genes can be identified whose promoters are not down-regulated when the expression of secreted protein genes are, an example being the *T.reesei gpd* promoter.

By these means it is possible to selectively regulate the genes coding for secreted proteins to enhance the production of chosen proteins, either by reducing or inactivating the down-regulation of the production promoter or by enhancing down-regulation to selectively repress expression of other secreted proteins. It is to be noted that the invention can be
5 utilised not only in protein production but that the mechanisms of transcriptional down-regulation described here provides means to modify fungal strains also for other purposes and selectively regulate the expression of certain undesired or desired proteins in the host.

10 EXAMPLES

Example 1. The effects of the Ca^{2+} -ionophore A23187, dithiothreitol (DTT) and Brefeldin A (BFA) in *T. reesei* Rut-C30 cultures

15 *Trichoderma* strains, cultivation conditions and methods used for sampling, metabolic labelling and analysis of RNA and proteins .

The *Trichoderma* strains and cultivation conditions have been essentially described elsewhere (Pakula et al. 2000; Ilmén et al 1996). *T. reesei* strain Rut-C30 (Montenecourt & Eveleigh, 1979) was cultivated on minimal medium ($(\text{NH}_4)_2\text{SO}_4$ 7.6 g l⁻¹, KH_2PO_4 15.0 g l⁻¹,
20 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g l⁻¹, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 0.2 g l⁻¹, CoCl_2 3.7 mg l⁻¹, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5 mg l⁻¹, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.4 mg l⁻¹, $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.6 mg l⁻¹, pH adjusted to 5.2 with KOH) that contained lactose 20 g l⁻¹ as a carbon source. Spore suspension with 2×10^7 spores (stored in -80°C in 20% glycerol) was inoculated into 200 ml of the medium, grown in shake flasks at 28°C with shaking at 210 rpm. After 4 days of cultivation the cultures were diluted 1/10
25 into fresh medium, grown further for 24h, and treated either with 10 mM dithiothreitol (DTT), 50µg/ml brefeldin A (BFA) or 5 µM Ca^{2+} -ionophore A23187. A corresponding volume of the solvent of the stock solution was added into the untreated control cultures (0.2% and 0.5% DMSO for the control cultures for A23187 and BFA treatment, respectively, and double distilled water for the control for DTT treatment). The cultures
30 were divided into aliquots for metabolic labelling of the proteins and for RNA isolation at different time points.

Proteins were metabolically labelled with ³⁵S-methionine using the methods described in (Pakula et al. 2000). The preparation of the samples and analysis of the labelled proteins

were carried out essentially as in (Pakula et al. 2000). The labelling experiment was started after 10 min of addition of DTT or A23187 or after 15 min of addition of BFA. 1 mCi of [³⁵S]-methionine (Amersham SJ 1015, *in vivo* cell labelling grade, 1000Ci mmol⁻¹, 10μCi μl⁻¹) was added to a 50ml aliquot of the cultivation. Untreated cultures were labelled in parallel and in a similar manner. Samples of 2ml were collected during a time course. Labelled total protein in cell extracts and culture supernatant was measured using scintillation counting of TCA insoluble material in the samples, and labelled specific proteins (e.g. CBHI) were analysed using 2D gel electrophoresis, and the proteins were quantified using a phosphorimager (Molecular Dynamics). The rates of protein synthesis and secretion, and the average synthesis time and minimum secretion time were determined as described in Pakula et al. 2000.

For the Northern analysis mycelial samples were collected from cultures treated with DTT, BFA or A23187 and from the untreated control cultures after 0, 15, 30, 60, 90, 120, 240 and 360 minutes of the treatment. The first sample (the time point 0 min was withdrawn immediately before addition of DTT, BFA or A23187). The mycelium was filtered, washed with equal volume of 0.7% NaCl, frozen immediately in liquid nitrogen, and stored at -80°C. Total RNA was isolated using the TrizolTM Reagent (Gibco BRL) essentially according to manufacturer's instructions. Northern blotting and hybridisation was carried out according to standard procedures (Sambrook et al). Full-length cDNA of the genes were used as probes.

The effect of A23187, DTT and BFA on protein synthesis and transport in T. reesei

Feedback regulation of genes coding for secreted proteins was studied in cultures treated with reagents known to interfere either with protein synthesis, folding or transport in other organisms. The Ca²⁺-ionophore A23187 has been reported to reduce protein synthesis as well as to inhibit protein folding and transport by emptying the Ca²⁺ stores of the ER in mammalian cells (Broström et al. 1989, Lodish and Kong, 1990, Lodish et al. 1992). Dithiothreitol is a reducing agent that inhibits formation of the disulphide bridges and protein folding in yeast and in mammals (Jämsä et al. 1994, Alberini et al. 1990, Braakman et al. 1992). Treatment of the cells with BFA is known to disrupt Golgi structure and inhibit transport of proteins from the ER to Golgi e.g. in mammalian systems, but the effect

is dependent on the organism and the specific cell type (Pelham, 1991, Shah and Klausner, 1993).

Metabolic labelling of the proteins was used to characterise the effects of A23187, DTT and BFA on protein synthesis and secretion in *T. reesei* cultures (see Pakula et al. 2000 for the methods used).

The cultures were treated for 10 minutes with 5 μ M A23187 or 10mM DTT or for 15 minutes with 50 μ g/ml BFA before the addition of the labelled methionine. Labelled total protein as well as labelled specific proteins were analysed in cell extract and culture supernatant at different time points of the labelling experiment.

The rate of total protein synthesis and the rate of total protein secretion was measured as the amount of radioactivity incorporated into TCA insoluble material per time unit in cell extracts and in culture supernatant (Fig. 1., the radioactivity in the TCA insoluble material is shown per mg of biomass dry weight, and the time point 0 minutes corresponds to the addition of the labelled methionine). The rates were deduced from the values measured during the first 15-45 minutes of the treatment. In the presence of DTT or BFA the rate of total protein synthesis was not affected, whereas the treatment with the ionophore reduced the protein synthesis rate to 51 % of that in the control cells. Production of extracellular labelled proteins was inhibited rather efficiently in cultures treated with DTT or BFA. In these cultures the secretion rate of total labelled proteins into the culture medium was only 5% of that in the control cells. In addition, in BFA treated cultures the production of extracellular proteins was markedly delayed compared to the control cultures. In cultures treated with the ionophore A23187, the rate of production of labelled proteins the culture medium was reduced to 23% of that in the non-treated cultures. The rates of protein synthesis and secretion are summarised in the Table 1 (in the Table 1 the values for the rates are shown as percentage of the values in the non-treated control cultures). The result indicates that DTT and BFA do not hinder protein synthesis, but block protein transport from the cells, whereas A23187 has also an inhibitory effect on protein synthesis.

30

The effect of the treatments on the synthesis of extracellular proteins, specifically, and on their transport was studied using the major cellulase produced by the fungus, cellobiohydrolase I (CBHI), as a model protein. The synthesis and secretion of the protein as well as changes in the pI pattern of the protein during the transport was monitored using

2D gel electrophoresis (as described in Pakula et al. 2000; Fig.2 shows labelled CBHI at different time points of the labelling experiment analysed in 2D gels, pH range of approx. 3.5-4.5 from left to right in each panel). In cell extracts prepared from cultures treated either with DTT or BFA, only the very first nascent pI forms can be detected indicating that the protein is not fully posttranslationally modified in the biosynthetic pathway. In DTT treated cultures, no production of labelled CBHI into the culture medium was detected, and in BFA treated cultures only a minute amount of CBHI was secreted at the late stages of the labelling experiment (the production rate was 4% of that measured in the control cultures). The result suggests that in these conditions the transport of the protein is blocked before the protein reaches the compartment where the modifications causing the heterogeneity in the pI take place. However, the minute amount of CBHI detected in the culture medium of the BFA treated cultures had gained the full pI pattern indicating that a minor portion of the protein is modified and transported, but the amount of the fully processed forms of the protein is too low to be detected in the cell extracts. The effect of the treatment with the ionophore A23187 on protein transport was less pronounced. Formation of the full pI pattern of CBHI was delayed by 15-20 minutes compared to the control cells, and CBHI with full pattern of the pI forms was secreted into the culture medium but with a delay.

The labelled CBHI from samples of cell extract and culture supernatant at different time points of the labelling experiment was analysed in 2D gels and quantified using a phosphorimager (Molecular Dynamics). Parameters, such as the synthesis and secretion rate of CBHI (the amount of labelled protein produced per time unit) as well as the average synthesis time and the minimum secretion time of CBHI, were determined (for the method see Pakula et al. 2000). The quantification of the labelled CBHI during the labelling experiment is shown in Fig. 3, and the deduced parameters describing the synthesis and secretion of CBHI in these conditions are summarised in the Table 1. The average synthesis time of full-length CBHI was not affected in the DTT and BFA treated cultures, being in accordance with the result that total protein synthesis is not affected by these treatments (see above). The minimum secretion time of the molecule measured in the BFA treated cultures was increased from 11 minutes to 69 minutes, and in the DTT treated cultures the parameter could not be determined because of the very low amount of extracellular protein produced in these conditions. Treatment of the cultures with the ionophore A23187 had an effect on CBHI synthesis as well as on transport of the protein.

The minimum secretion time of CBHI was increased by 10 minutes in cultures treated with A23187 when compared to the control cultures, and the synthesis time of CBHI was 3-4 minutes longer than in the control cultures.

- 5 Surprisingly, although the treatment with DTT or BFA did not reduce the rate of total protein synthesis or prolong the time required for the synthesis of CBHI molecules, it was found out that the rate of CBHI synthesis (the amount of labelled CBHI synthesised per time unit) was reduced in cultures treated with DTT or BFA. (In the Table 1., the rates are shown as percentage of the values measured in the control cultures.). In the DTT treated
- 10 cultures the CBHI synthesis rate was 4-24% of the one measured in control cultures and in the BFA treated cultures 52%. Most of the CBHI synthesised remains intracellular. The rate of CBHI production into the culture medium could not be measured in the DTT treated cultures, and in BFA treated cultures it was 4% of the one measured in the control cultures. In cultures treated with the ionophore A23187 the rate of CBHI synthesis was affected to
- 15 greater extent than the total protein synthesis rate. The rate of CBHI synthesis was 26% of that measured in the control cells, and the total protein synthesis rate 51%. The protein secretion rate into the culture medium was reduced to the same extent as the synthesis rate of CBHI (27% of that measured in the control cultures).
- 20 The results show that the treatment with BFA or DTT clearly hindered protein transport in *Trichoderma*, probably preventing protein transport further from the ER, whereas the treatment with A23187 caused only a slight delay in protein transport. The total protein synthesis activity was not affected in cultures treated either with DTT or BFA, whereas the synthesis rate of the secreted model protein CBHI was reduced specifically concomitant to
- 25 the impairment of protein transport. In cultures treated with A23187, a clear reduction in the total protein synthesis rate was measured, but the synthesis rate of CBHI was affected to greater extent compared to the effects on total protein synthesis.

Table 1. The effect of treatment with A23187, DTT or BFA on protein synthesis and secretion in *T. reesei*.

(n.d. = not detected)

5

	Total protein synthesis rate	Total protein secretion rate	CBHI synthesis rate	CBHI secretion rate
Untreated cells	100%	100%	100%	100%
A23187	51%	23%	26%	27%
BFA	100%	5%	52%	4%
DTT	105%	5%	4-24%	n.d.

	Average time of CBHI synthesis	Minimum secretion time of CBHI
Untreated cells	5.7 min	11 min
A23187	9 min	21 min
BFA	4.6 min	69 min
DTT	4.3 min	n.d.

25 *Transcript levels of genes coding for the foldase PDII, the chaperon BIPI, and the transcription factor HACI mediating the UPR response in T. reesei cultures treated either with the Ca²⁺-ionophore A23187, DTT or BFA*

30 Northern analysis of the samples collected during the treatment with A23187, DTT or BFA was carried out to study the effect of the treatments at transcriptional level. The hindrance in protein transport and folding in cultures treated with DTT or BFA was manifested also as activation of the unfolded response (UPR) pathway as indicated by the induction of *pdi1* and *bip1* genes (Fig.4, the result has been reported earlier for *pdi1*; Saloheimo et al. 1999), as well as by the expression of the shortened, actively translated form of the *hac1* transcript that mediate the UPR response (Fig. 5 shows the signals of the short and longer forms of

the transcript normalised with the total *hac1* signal at each time point). In the cultures treated with A23187 the protein transport was only slightly affected and the total amount of protein synthesised was diminished. In these conditions no induction of *pdi1* and *bip1* was observed (Fig.4). However, a transient and rather weak expression of a short form of *hac1* mRNA was observed also in A23187 treated cells indicating some effect also on the UPR pathway (Fig5).

Transcript levels of genes coding for the endogenous secreted proteins in T. reesei cultures treated either with the Ca²⁺-ionophore A23187, DTT or BFA

10

In cultures treated either with DTT or BFA, CBHI was synthesised with a reduced rate compared to non-treated control cells, whereas total protein synthesis was not affected in these conditions. In cultures treated with A23187, the synthesis of CBHI was retarded to a greater extent compared to the total protein synthesis in the treated cultures. Northern analysis of the samples prepared from the cultures treated with the drugs showed that the mRNA level of *cbh1* decreased markedly during the treatment (Fig.6, the *cbh1* signals normalised with the signals of *gpd* at different time points of the treatment). The reduced mRNA level seem to explain, at least partly, the reduced synthesis rate of the protein in the labelling experiment. In DTT and A23187 treated cultures the reduction in the mRNA level of the gene occurred with kinetics corresponding to the measured half-life of the mRNA. In BFA treated cultures the decrease was somewhat slower. Similar reduction was observed in the *egl1* mRNA level during the treatments (Fig.6, signals normalised with the signals of *gpd* at different time points of the treatment). In addition, a Northern analysis of a broader set of genes was carried out from samples of DTT treated cultures. Transcript level of various other genes coding for extracellular proteins was reduced e.g. *xyn1* and *hfb2* (Fig. 7), indicating that many of the genes coding for extracellular proteins are under the feedback control in conditions where there are limitations in protein synthesis, folding or transport.

It is also evident that the down-regulation does not affect all the genes expressed by the fungus, but is common to a group of genes coding for extracellular proteins. In addition to the upregulated genes under the control of UPR, several examples of genes that were not down-regulated were found (Fig. 8). Interestingly, under these conditions the mRNA level of *bgl2* coding for an intracellular β -glucosidase was not decreased, even though the gene

is regulated in similar manner as cellulases in respect to the carbon source available for the fungus. The expression level of genes coding for proteins functioning in the vesicle transport, e.g. *sar1* (Veldhuisen et al. 1997) and *ypt1*, were not affected by the treatment with DTT (Saloheimo et al. submitted). Other genes whose expression is not apparently
5 affected by the DTT treatment are e.g. *cDNA1* and *gpd* (glyceraldehyde-6-P-dehydrogenase). The *gpd* signal was used for normalisation the signals in the Northern analyses.

10 **Example 2. Transcript levels of genes coding for the endogenous secreted proteins in cultures of *A. niger* treated with DTT**

***A. niger* strains, cultivation conditions and methods used for sampling and analysis of RNA**

15 The *Aspergillus niger* strains used in the experiments were AB4.1 (van Hartingsveldt *et al.*, 1987) and AS1.1 (Ngiam *et al.*, 2000). Spores resuspended in 0.1% Tween 20 (Sigma, UK) were used to inoculate liquid cultures to a final density of 1×10^5 spores per ml of medium. The strains were maintained on potato dextrose agar slopes (Difco, USA) with a supplement of 10mM uridine for *A. niger* AB4.1. Slopes were grown at 30°C until they
20 had sporulated and made fresh for each experiment. ACMS/N/P medium (Archer et al., 1990) was used for all the experiments involving liquid culture. *A. niger* AB4.1 cultures were again supplemented with 10mM uridine. Cultures were grown in 100ml aliquots of medium in 250ml conical flasks at 25°C and 150 rpm. In the DTT stress experiments, AB4.1 cultures were grown for 44 hours before addition of 1ml of 2M DTT solution to
25 give a final concentration of 20mM. Control AB4.1 cultures had an equivalent volume of water added. For the medium exchange experiment, cultures were grown for 44 hours at 25°C and 150rpm in ACMS/N/P. The mycelium was harvested through Miracloth (CalBiochem, USA) and washed with two 100ml aliquots of medium with no carbon source that had been pre-warmed to 25°C. The mycelium was then transferred to pre-
30 warmed flasks containing 100ml ACMX/N/P with supplementation where appropriate and incubation was continued using the same conditions as before. ACMX/N/P differs from ACMS/N/P in containing 10g xylose per litre instead of 10g of soluble starch per litre.

Mycelia were harvested through two layers of Miracloth and flash frozen in liquid nitrogen. The mycelia were then ground under liquid nitrogen to a fine powder which was freeze dried in an Edwards Modulyo freeze drier for two days. Dry weights were established by weighing the mycelia after two days in the freeze drier and then drying for a
5 further day. If no decrease in weight was observed over this period the culture was assumed to be completely dry.

Total RNA was extracted from 100mg of freeze dried, ground mycelia using the RNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's instructions. RNA was
10 quantified by reading absorbances at 230, 260 and 280nm on a Uvikon 850 spectrophotometer (Kontron Instruments, UK). Ratios of over 2.0 for the 260nm:280nm readings were accepted as being indicative of good quality RNA. RNA quality was also assessed by running samples on 7% formaldehyde gels (Sambrook *et al.*, 1989). For northern blotting, 10µg of RNA per lane was run on a 7% formaldehyde gel in MOPS
15 running buffer (Sambrook *et al.*, 1989) for 16 hours at 25V in a Life Technologies Horizon 11-14 submarine gel electrophoresis tank. Samples were prepared using Sigma RNA loading dye (Cat.# R4268). After electrophoresis, the gel was washed in 5 changes of DEPC-treated water (Sambrook *et al.*, 1989) for 20 minutes each wash and then soaked in 50mM NaOH for 10 minutes. Transfer to Hybond XL nylon membrane (Amersham Intl.,
20 UK) was achieved using an Appligene vacuum blotter according to the manufacturer's instructions with 10xSSC (Sambrook *et al.*, 1989) as transfer buffer. Transfer time was 2.5 hours. After transfer, the blot was soaked in 50mM NaOH for 5 minutes and then rinsed in 2xSSC for 30 seconds before being allowed to air dry overnight.

25 Probes for the northern blots were labelled using the Megaprime labelling kit and α -³²P dATP (both Amersham Intl., UK) according to the manufacturer's instructions. The *glaA* probe was a 637bp fragment corresponding to co-ordinates +1059 to +1696 in the sequence of the *A. niger* glucoamylase gene (Boel *et al.*, 1984). The actin probe was a 765bp fragment corresponding to co-ordinates +889 to +1654 in the γ -actin gene of *A.*
30 *nidulans* (Fidel *et al.*, 1988). The *pdiA* probe was a 303bp fragment corresponding to co-ordinates +63 to +365 in the sequence of the *pdiA* gene of *A. niger* (Ngiam *et al.*, 1997). The *pepA* probe was a 445bp fragment corresponding to co-ordinates +1186 to +1631 in the *A. awamori* aspergillopepsin gene (Berka *et al.*, 1990). The *bipA* probe was a 445bp fragment corresponding to co-ordinates +712 to +1156 of the *A. niger* *bipA* gene (van

Gemerén *et al.*, 1997) All of the probes were amplified by PCR from *A. niger* genomic DNA and purified from agarose-TAE gels using the Qiaquick gel extraction kit (Qiagen, UK).

5 Blots were pre-hybridised at 65°C in Hyb9 hybridisation solution (Puregene, USA) for 30 minutes prior to the addition of the probe DNA. The hybridisation was then carried out overnight at 65°C. Blots were washed twice in 2xSSC, 0.1%SDS for 15 minutes at 65°C and then once in 0.1xSSC, 0.1%SDS for 30 minutes at 65°C. Blots were visualised and the band intensities quantified using a FujiFilm BAS1500 phosphorimaging system. RNA
10 loadings were normalised using the γ -actin probe. The figures shown in the graphs represent the ratio between the target mRNA signal and that of γ -actin. This is dependent on the time of exposure for the blots on each phosphorimage plate. Because the values on the different graphs do not represent absolute levels of the transcripts they are not directly comparable.

15

The effect of DTT on transcript levels of genes *glaA*, *pepA*, *pdiA* and *bipA* in *A. niger* cultures

Figure 9. shows the results from a DTT time course experiment running over 10 hours (from the addition of the stress agent, average signals of three determinations). Part (A)
20 shows the effect on the steady state RNA levels for the *glaA* gene over this period. It can be seen clearly that in the DTT-treated cultures the amount of mRNA drops steadily over time, with a half-life of about 70 minutes. This correlates well with data from a medium exchange experiment carried out in this lab (Figure 10.) which shows that the $T_{1/2}$ of *glaA* mRNA is ca. 70 minutes in the absence of *glaA* mRNA synthesis. The result in Figure 9A
25 therefore suggests that DTT treatment inhibits the transcription of *glaA* and that the decline in the level of the *glaA* mRNA is due to its normal degradation within the organism. Figure 9B shows the effect of DTT stress on another secreted protein, aspergillopepsin (*pepA*). This gene is only induced when the pH of the medium becomes more acidic and so transcription does not occur until late in the time course. The data show that, though there
30 is an increase in the levels of *pepA* mRNA in the control cultures, there is no significant increase in the DTT treated cultures. Figure 9C and D show the effects of DTT on genes involved in the unfolded protein response. Both of the genes shown, *pdiA* and *bipA*, show a rapid response to the addition of the stress agent. This response does not appear to be transient but, conversely, is long lived. It is not known whether this is due to the

production of messenger RNA for an extended period after addition of the DTT or due to long half lives for the mRNAs involved.

Example 3. The transcript levels of genes *glaA* and *pepA* in cultures of *A. niger* expressing *pdiA* antisense transcript under the control of glucoamylase promoter

The expression of *glaA* and *pepA* has been compared in *A. niger* strain expressing *pdiA* antisense construct and in its parental strain. The methods for cultivation of the strains and RNA analysis have been described in the Example 2.

Figure 11 show data obtained from a comparison of *A. niger* AS1.1, which contains multiple copies of a *pdiA* antisense sequence under the control of the glucoamylase promoter, to the parental strain *A. niger* AB4.1 when grown on medium containing starch as a carbon source. Panel (a) shows the effect on the mRNA levels for the *glaA* gene. It can be seen that from the first time-point at 24 hours the levels of *glaA* mRNA in the AS1.1 strain show a gradual decline while those for AB4.1 increase. From this and Panel (a) in Figure 1 it can be seen that the levels of *glaA* mRNA in the parental strain (AB4.1) are actually increasing in relation to the level of γ -actin which is used for normalisation. This may be due in part to the long half-life of the *glaA* mRNA that would mean that the rate of breakdown of the mRNA is significantly slower than its rate of production giving rise to an ever-increasing population for this mRNA. In panel (b) the effects on the transcription of the *pepA* gene are shown. Again there are significantly lower levels of mRNA in the AS1.1 strain than in the parent, AB4.1. Panel (c) shows the dry weight determinations for the experiments, which show that there is no significant effect on the growth of the fungus when the antisense construct is expressed.

25

Example 4. The transcript levels of the gene *glaA* and the levels of secreted glucoamylase in cultures of *A. niger* constitutively expressing *pdiA* antisense transcript under the control of the *gpdA* promoter

The expression of *glaA* has been compared in an *A. niger* strain constitutively expressing *pdiA* antisense cDNA under the control of the *gpdA* promoter (strain ASG67) and in its parental strain. The methods for cultivation of the strains and RNA analysis have been described in Example 2. For analysis of secreted glucoamylase protein levels, a 7ml sample of culture filtrate from each flask was collected and stored at -20°C until required.

The method used for determination of glucoamylase was that of MacKenzie *et al.*, 1994.

Figure 12 shows data obtained from a comparison of *A. niger* ASG67, which contains multiple copies of a *pdiA* antisense sequence under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter, to the parental strain AB4.1 grown on medium containing starch as a carbon source. Panel (a) shows the effect on the levels of secreted glucoamylase. It can be seen that, although the levels of secreted glucoamylase increase in both strains over time, the levels for the antisense strain are lower than those for the parental strain (AB4.1), especially later in the growth of the fungus. In panel (b) the effects on the transcript levels for the *glaA* gene can be seen. After initially reaching the same transcript level at 36 hours, there is a gradual increase in transcript levels in the parental strain (AB4.1) which is not mirrored in the *pdiA* antisense strain (ASG67). Panel (c) shows the dry weight determinations for the experiments which demonstrate that there is no significant effect on the growth of the fungus when the antisense construct is expressed.

Example 5. The splicing of the *hacA* transcript in *A. niger* constitutively expressing *pdiA* antisense transcript.

The splicing of the *hacA* transcript, which encodes the positively acting regulatory factor for the unfolded protein response, has been analysed in an *A. niger* strain which constitutively expresses a *pdiA* antisense sequence and in its parental strain. The methods for cultivation of the strains and RNA analysis have been described in Example 2. The *hacA* probe used in the experiment was the *hacA* cDNA isolated at VTT. The same cultivations were used to provide the data in Example 4.

Figure 13 shows a northern blot for *hacA* over time. If there was induction of the unfolded protein response (UPR) there would be evidence for a second mRNA species slightly lower on the gel than the species which is present. The mRNA present is of the correct size for unspliced *hacA*. These data suggest that there is no induction of the UPR which implies that the transcriptional down-regulation mechanism is distinct from the UPR and is controlled in a different manner.

Example 6. The expression level of genes coding for endogenous secreted proteins in *T. reesei* strains producing heterologous proteins

Strains, cultivation conditions and methods used in the analysis of the cultures.

- 5 *T. reesei* Rut-C30 strain producing human tissue plasminogen activator (tPA, Verheijen et al. 1986) was constructed by transforming the parental strain with the expression cassette shown in Fig. 14A using the methods described in Penttilä et al. 1987.

10 The tPA producing strain and the parental strain Rut-C30 were cultivated in bioreactors in parallel. The culture medium used was lactose-based buffered medium used at VTT Biotechnology (lactose 40 g/l, peptone 4 g/l, yeast extract 1 g/l, KH_2PO_4 4 g/l, $(\text{NH}_4)_2\text{SO}_4$ 2.8 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.8 g/l, supplemented with trace elements). Dry weight of the biomass was measured as described in Example 7. Lactose concentration in the culture medium was determined using a kit obtained from Boehringer Mannheim, total
15 protein in the culture medium was measured using the Protein Assay obtained from BioRad, HEC activity was measured as described (in Bailey and Nevalainen, 1981; IUPAC, 1987) and the tPA concentration was measured using the EIA kit provided by TNO (the Netherlands). RNA isolation and Northern analysis was performed as described in the Examples 1, 7, 8, and 9.

20

Expression of the endogenous extracellular proteins in a tPA producing strain and its parental strain

Production of endogenous secreted proteins and the expression of the corresponding genes was studied in *T. reesei* Rut-C30 and in a transformant producing a tPA (human tissue
25 plasminogen activator), which is an example of a heterologous protein that is very poorly produced by the fungus, and expected to induce various stress responses in its host. The transformant has been estimated to harbour approximately five copies of the expression cassette, from which tPA is produced as a CBHI-fusion protein under *cbh1* promoter.

- 30 To compare protein production and expression of the corresponding genes in the two strains, parallel cultivations in bioreactors were carried out. Formation of biomass and consumption of the carbon source, lactose, was measured during the cultivation to monitor growth (Fig. 14B). Total protein and cellulase activity (activity against the substrate HEC, measuring mainly endoglucanase activity) produced into the culture medium were

measured throughout the cultivation (Fig. 14C). Northern analysis was carried out to analyse *egl1* (Fig. 14D), *cbh1* (Fig. 14E) and *bip1* (Fig. 14F) expression in the cultures. The signal of actin was used for normalisation of the signals in the Northern.

5 Even though, the two strains grew rather similarly during the cultivation, it was obvious that the tPA producing strain produced much less total protein and cellulase activity into the culture medium compared to the parental strain. The tPA produced by the transformant only a minor proportion of the total protein produced, the highest yield obtained is 25 mg/l. In accordance with the low protein production in the tPA producing culture, the expression
10 levels of *egl1*, coding for the extracellular endoglucanase I, and *cbh1*, coding for cellobiohydrolase I, were lower in the culture producing tPA. Expression of the chaperon gene *bip1* was induced in the tPA producing culture indicating activation of stress responses, such as UPR, by production of the heterologous protein. Thus the low expression levels of endogenous genes coding for secreted proteins in the transformant
15 could be due to the down-regulation mechanism active during secretion stress.

Example 7. Expression of the reporter gene lacZ under full-length *cbh1* promoter and a shortened minimal *cbh1* promoter in DTT treated cultures of *T. reesei* - the role of the promoter sequence in the down-regulation

20

Cultivation conditions, and methods used for analysis of the RNA samples

The strain QM9414 (Mandels et al. 1971) and its derivatives pMI34 and pMLO16 expressing *Escherichia coli lacZ* under *cbh1* promoter (Ilmén et al 1996) were cultivated on the minimal medium containing 0.05% proteose peptone and 20 g/l sorbitol or glycerol.
25 8×10^7 spores were inoculated per 200ml of growth medium and the cultures were grown in conical flasks at 28°C with shaking at 210 rpm. α -Sophorose (1mM) was added after 23h and after 32h of cultivation to induce cellulase gene expression on sorbitol medium. Treatment of the cultures with 10mM DTT was started after 40h of cultivation. Mycelial samples for RNA isolation were collected and subjected to Northern analysis as described
30 in the Example 1. Dry weight of the cultures was measured before and after the sophorose induction and the treatment with DTT by filtering and drying mycelium samples at 105°C to constant weight (24 h). The dry weight in the cultures was 1.1-1.4 g/l at the beginning of the treatment with DTT.

To study whether the feedback regulation of the mRNA level was mediated by the promoter sequence of the gene involved, a reporter gene system was used. A schematic view of the reporter gene expression cassettes is shown in Fig. 15A. The *E. coli lacZ* gene was expressed under a *cbh1* promoter in the strain *T. reesei*, either under a full-length *cbh1* promoter of 2.2 kb or under a minimal promoter of 161 bp, and the expression levels were studied during DTT treatment of the strains. The quantification of the *lacZ* signal normalised with the signal of *gpd1* is shown in Fig. 15B. The *lacZ* transcript level is down-regulated during DTT treatment only when expressed under the full-length *cbh1* promoter. However, no down-regulation was observed if a minimal *cbh1* promoter containing the putative TATA-box and the transcription start sites was used for *lacZ* expression, even though the short promoter is functional and even inducible with sophorose. The transcript level of *egl1* was analysed in both of these strains to control that the down-regulation mechanism is functional in these strains under these conditions. The result indicates that sequence elements in the *cbh1* promoter are required for the down-regulation, and a mechanism other than the instability of the mRNA is involved in the process.

T. reesei strains harbouring *E.coli lacZ* gene under shortened *cbh1* promoters were cultivated and treated with DTT as described in the Example 7, and the expression of the *lacZ* gene was analysed (as in the example 7). Figure 16A shows the schematic presentation of the *cbh1* promoter constructs used for *lacZ* expression in the different strains. The Northern analysis of *lacZ*, *egl1* and *gpd1* mRNA level in the cultures treated with DTT and in the non-treated cultures is shown in the Figures 16B, C and D. The mRNA level of *egl1* was analysed as an example of an endogenous gene subjected to the down-regulation under secretion stress conditions (e.g. in DTT treated cultures), and the signal for *gpd1* was used as a control for loading of the samples. The signals of *lacZ* and *egl1* mRNA were quantified and normalised with the signal of *gpd1*, and the ratio of the signal in the DTT treated sample to the signal in the control samples at different time points of the treatment is shown as graphs. In the strains harbouring the constructs with

cbh1 promoters of 1029 bp in length or longer (shown in Fig. 16B), the expression of *lacZ* was decreased during the treatment with DTT to a similar extent as in the strains expressing the gene under the full-length *cbh1* promoter of 2.2kb. In strains expressing the *lacZ* gene under *cbh1* promoters of 339bp to 499bp in length (shown in Fig. 16C), the level of *lacZ* mRNA was clearly decreased during the treatment with DTT, but not to the same extent as if expressed under the full-length *cbh1* promoter, and not to the same extent as the mRNA level of *egl1* that was used as an internal control for down-regulation in the strain. In strains expressing the *lacZ* gene under the shortened promoters of 161 bp to 209 bp in length (shown in Fig. 16D), a strong expression of *lacZ* (as compared to the signal in the non-treated cultures) was detected during the treatment with DTT. The results indicate that in the case of the *cbh1* promoter, the regions involved in the decrease in the expression level during the DTT treatment are located within the 1029bp region upstream of the translation start codon, the most important regions being located in the regions 500-1029bp and 209-339bp upstream of the start codon.

15

Example 9. Expression of *cbh1* in DTT treated cultures of *T. reesei* QM9414 and its derivative harbouring a deletion in the gene *ace1*

To study the possible role of the cellulase regulator *ace1* in the down-regulation of the cellulase promoters under the secretion stress conditions, cultures of *T. reesei* QM9414 and a derivative of the strain with a deletion in the gene *ace1* (Saloheimo et al. 2000) were treated with DTT and analysed for cellulase expression. The strains were cultivated on sorbitol containing medium, induced with sophorose, and treated with 10 mM DTT as described in the Example 7. Sampling of the mycelium for RNA analysis as well as the Northern analyses have been described in the Examples 1 and 7 as well. The transcript level of *cbh1* was quantified during the treatment and the signals were normalised with the ones of *gpd1* (Fig. 17).

The *cbh1* is subjected to down-regulation during DTT treatment in cultures of QM9414 in a similar manner as has been shown for the strain *T. reesei* Rut-C30 (Example 1). However, in cultures of the QM9414 strain harbouring a deletion in *ace1* grown on sorbitol containing medium, the *cbh1* is constitutively expressed also during treatment with DTT. In these specific conditions the *ace1* activity seem to be required for the down-regulation of the *cbh1* promoter. However, we have also evidence that in other culture conditions (e.g.

on glycerol containing medium), the *acel* activity is not required, indicated that other factors, not yet known, are involved in this regulation mechanism.

Example 10. Isolation of fungal mutant strains defective in the mechanism of transcriptional down-regulation of genes under secretion stress conditions

T. reesei strain pMLO16 expressing the *E. coli lacZ* reporter gene under the full-length *cbh1* promoter was mutagenised using UV irradiation, and mutants capable of expressing *lacZ* under secretion stress conditions, in the presence of BFA, were screened for based on color reaction.

A spore suspension containing 10^7 spores/ml was subjected to UV radiation leading to 15-46% viability of the spores. The mutagenised spores were cultivated on minimal medium containing sorbitol as a carbon source (as in the Example 7. except that pH 7.0 was used in this case) on microtiter plates, approx. 3 spores per well. After cultivation of 7 days, sophorose and brefeldin A were added to induce *lacZ* expression and to generate secretion stress conditions at the same time. Induction of LacZ production in the presence of BFA was detected by the color reaction caused by addition of X-gal in the cultures. The *lacZ* expressing cultures were purified on PD plates, and the ability of the mutants for induction of the *cbh1* promoter (controlling *lacZ* expression) in the presence of BFA was confirmed. The Figure 18A. shows the *lacZ* activity in the control cultures of pMLO16 expressing *lacZ* under the down-regulatable full-length *cbh1* promoter, in the strain pMI33 expressing the *lacZ* under a minimal promoter of *cbh1* (not down-regulated in the secretion conditions, see also example 8), and in the *lacZ* negative strain QM9414. After sophorose addition, there is no *lacZ* production in the presence of BFA, whereas in the absence of BFA, *lacZ* is produced, as indicated by the color reaction. The Figure 18B. shows an example of screening of the mutants in the microtiter plate cultures. The mutants expressing *lacZ* under the secretion stress conditions can be isolated based on the color reaction. As a control, the unmutagenised spores of pMLO16 were cultivated on the plates in the presence and absence of BFA (see the boxed wells; positive color reaction indicating *lacZ* production in the absence of BFA, and lack of color reaction in the presence of BFA)

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<210> 2

<211> 2027

<212> DNA

<213> Trichoderma reesei

<400> 2

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<210> 3

<211> 2003

<212> DNA

<213> *Trichoderma reesei*

<400> 3

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<210> 4

<211> 1874

<212> DNA

<213> *Trichoderma reesei*

<400> 4

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<210> 5

<211> 2053

<212> DNA

<213> *Trichoderma reesei*

<400> 5

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<210> 6

<211> 934

<212> DNA

<213> *Trichoderma reesei*

<400> 6

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 <211> 1014
 <212> DNA
 <213> *Trichoderma reesei*

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<210> 8
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 <213> *Trichoderma reesei*

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<210> 9
 <211> 1474
 <212> DNA
 <213> *Trichoderma reesei*

<400> 9
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 tagattgggg agaagttgac ttccgcccag agctgaaggt cgcacaaccg catgatatag 180
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 agtccgagct gtaactacct ctgaatctct ggagaatggt ggactaacga ctaccgtgca 480
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<210> 10
 <211> 1344
 <212> DNA
 <213> *Trichoderma reesei*

<400> 10
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 tagattgggg agaagttgac ttccgcccag agctgaaggt cgcacaaccg catgatatag 180
 ggtcggcaac ggcaaaaaag cacgtggctc accgaaaagc aagatgtttg cgatctaaca 240
 tccaggaacc tggatacatc catcatcacg caccgaccact ttgatctgct ggtaaactcg 300
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<210> 11

<211> 1184

<212> DNA

<213> *Trichoderma reesei*

<400> 11

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tagattgggg	agaagttgac	ttccgcccag	agctgaagg	cgcacaaccg	catgatatag	180
ggtcggcaac	ggcaaaaaag	cacgtggctc	accgaaaagc	aagatgtttg	cgatctaaca	240
tccaggaacc	tggatacatc	catcatcacg	cacgaccact	ttgatctgct	ggtaaactcg	300
tattcgccct	aaaccgaagt	gcgtggtaaa	tctacacgtg	ggcccccttc	ggtatactgc	360
gtgtgtcttc	tctaggtggc	attccttttc	cttcctctag	tgttgaattg	tttgtgttgg	420
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cctgcatcat	gtatataata	gtgacccctg	gaaggggggt	ttggagcaat	gtgggacttt	540
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aatctattca	aacaccaagc	ttgctctttt	gagctacaag	aacctgtggg	gtatatactc	720
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cgacaagcaa	agcgttccgt	cgcagtagca	ggcactcatt	cccgaaaaaa	ctcggagatt	960
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<210> 12

<211> 1281

<212> DNA

<213> *Trichoderma reesei*

<400> 12

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gcgaatgtgt	atatataaag	gttcgaggtc	cgtgcctccc	tcagtctctc	cccatctact	1200
catcaactca	gacccctcag	gagacttgta	caccatcttt	tgaggcacag	aaacccaata	1260
gtcaaccgcg	gactgcgcac	c				1281

<210> 13
 <211> 1031
 <212> DNA
 <213> *Trichoderma reesei*

<400> 13
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 gttcacggca agggaaacca ccgatagcag tgtctagtag caacctgtaa agccgcaatg 180
 cagcatcact ggaaaataca aaccaatggc taaaagtaca taagttaatg cctaaagaag 240
 tcatatacca gcggctaata attgtacaat caagtggcta aacgtaccgt aatttgccaa 300
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 aagaagacag aggtagaagt gtctgactcg gagcgttttg catacaacca agggcagtga 480
 tggaagacag tgaaatgttg acattcaagg agtatattagc cagggatgct tgagtgtatc 540
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 aagtgtggta ggatcgaaca cactgctgcc ttaccaagc agctgagggt atgtgatagg 780
 caaatgttca ggggccactg catggtttcg aatagaaaga gaagcttagc caagaacaat 840
 agccgataaa gatagcctca ttaaaccgaa tgagctagta ggcaaagtca gcgaatgtgt 900
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 gatcctccag gagacttgta caccatcttt tgaggcacag aaaccaata gtcaaccgcg 1020
 gactgcgcat c 1031

<210> 14
 <211> 1201
 <212> DNA
 <213> *Trichoderma reesei*

<400> 14
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 gactgaccgg acgtgttttg cccttcattt ggagaaataa tgtcattgcy atgtgtaatt 180
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 agggaaacca ccgatagcag tgtctagtag caacctgtaa agccgcaatg cagcatcact 360
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 gcggctaata attgtacaat caagtggcta aacgtaccgt aatttgccaa cggcttgtgg 480
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 ctcagctggg gatcccccaa ttgggtcgct tgtttgttcc ggtgaagtga aagaagacag 600
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<210> 15
 <211> 2215
 <212> DNA
 <213> *Trichoderma reesei*

<400> 15

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<210> 16

<211> 501

<212> DNA

<213> Trichoderma reesei

<400> 16

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<210> 17

<211> 188

<212> DNA

<213> *Trichoderma reesei*

<400> 17

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tataaagggt cgagggtccgt gcctccctca tgctctcccc atctactcat caactcagat 120
cctccaggag acttgtacac catcttttga ggcacagaaa cccaatagtc aaccgcggac 180
tgcgcatc                                     188
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<210> 18

<211> 211

<212> DNA

<213> *Trichoderma reesei*

<400> 18

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ggcaaagtca gcgaatgtgt atatataaag gttcgaggtc cgtgcctccc tcatgctctc 120
cccatctact catcaactca gatcctccag gagacttgta caccatcttt tgaggcacag 180
aaaccaata gtcaaccgcg gactgcgcat c                                     211
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<210> 19

<211> 341

<212> DNA

<213> *Trichoderma reesei*

<400> 19

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<210> 20

<211> 391

<212> DNA

<213> *Trichoderma reesei*

<400> 20

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gaagcttagc caagaacaat agccgataaa gatagcctca ttaaaccggaa tgagctagta 240
ggcaaagtca gcgaatgtgt atatataaag gttcgaggtc cgtgcctccc tcatgctctc 300
cccatctact catcaactca gatcctccag gagacttgta caccatcttt tgaggcacag 360
aaaccaata gtcaaccgcg gactgcgcat c                                     391
```

<210> 21

<211> 162

<212> DNA

<213> *Trichoderma reesei*

<400> 21

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ctcatgctct ccccatctac tcatcaactc agatcctcca ggagacttgt acaccatctt 120
ttgaggcaca gaaaccaat agtcaaccgc ggactgcgca tc                                     162
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<210> 22
 <211> 871
 <212> DNA
 <213> *Trichoderma reesei*

<400> 22
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 aatagaaaga gaagcttagc caagaacaat agccgataaa gatagcctca ttaaacggaa 720
 tgagctagta ggcaaagtca gcgaatgtgt atatataaag gttcgaggtc cgtgcctccc 780
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<210> 23
 <211> 741
 <212> DNA
 <213> *Trichoderma reesei*

<400> 23
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 ggtgaagtga aagaagacag aggtagaat gtctgactcg gagcgttttg catacaacca 180
 agggcagtga tggaagacag tgaaatgttg acattcaagg agtatttagc cagggatgct 240
 tgagtgtatc gtgtaaggag gtttgtctgc cgatacgacg aatactgtat agtcacttct 300
 gatgaagtgg tccatattga aatgtaagtc ggcactgaac aggcaaaaga ttgagttgaa 360
 actgcctaag atctcgggcc ctcgggcctt cggcctttgg gtgtacatgt ttgtgctccg 420
 ggcaaagtca aagtgtggta ggatcgaaca cactgctgcc tttaccaagc agctgagggg 480
 atgtgatagg caaatgttca ggggccactg catggtttcg aatagaaaga gaagcttagc 540
 caagaacaat agccgataaa gatagcctca ttaaacggaa tgagctagta ggcaaagtca 600
 gcgaatgtgt atatataaag gttcgaggtc cgtgcctccc tcatgctctc cccatctact 660
 catcaactca gatcctccag gagacttgta caccatcttt tgaggcacag aaaccaata 720
 gtcaaccgcg gactgcgcat c 741

CLAIMS:

1. A method for producing a promoter for protein production in a fungal host, characterized in that the method comprises the steps of:

- 5 - selecting a promoter of a secretable protein,
- genetically modifying the promoter,
- operable linking the promoter to the coding region of a reporter protein,
- expressing the selected reporter protein under the regulation of the modified promoter in a fungal host under suitable culture conditions in secretion stress,
10 - screening or selecting for cells showing enhanced or decreased protein expression of the selected reporter protein compared with the expression obtained with the non-modified promoter under the same conditions; and
- recovering the fungal host comprising the promoter having modification in its transcriptional down-regulation mechanism.

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2. The method according to claim 1, characterized in that the coding region of the selected reporter protein is the coding region of a secretable protein.

3. A method for producing a fungal host for protein production, characterized in that the method comprises the steps of:

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- selecting a promoter modified as in claims 1 or 2,
- operable linking the modified promoter to the coding region of a gene encoding a selected secretable protein,
- expressing the selected secretable protein under the regulation of the modified promoter
25 in a fungal host under suitable culture conditions in secretion stress,
- screening or selecting for cells showing enhanced or decreased protein expression of the selected secretable protein compared to the expression of secretable proteins under a non-modified promoter under the same conditions; and
- recovering the fungal host comprising the promoter having modification in its
30 transcriptional down-regulation mechanism.

4. The method according to any one of the preceding claims, characterized in that the promoter is selected from the group comprising a cellulase, a hemicellulase, an

amylolytic enzyme, a hydrophobin, a protease, an invertase, a phytase, a phosphatase, a swollenin, a ligninolytic enzyme and a pectinase promoter.

5. The method according to claim any one of the preceding claims, characterized in that the promoter is selected from the group comprising *cbh1*, *cbh2*, *egl1*, *egl2*, *hfb1*, *hfb2*, *xyn1*, *swo*, *gla*, *amy*, and *pepA* promoter.

6. The method according to any one of the preceding claims, characterized in that the modified region is located upstream of -162 of the *Trichoderma cbh1* promoter.

7. The method according to any one of the preceding claims, characterized in that the modified region is located between the nucleotides -1031 and -162 in *Trichoderma cbh1* promoter.

8. The method according to any one of the preceding claims, characterized in that the modified region is located between the nucleotides -1031 and -501 in *Trichoderma cbh1* promoter.

9. The method according to any one of claims 1 to 8, characterized in that the modified region is located between the nucleotides -341 and -211 in *Trichoderma cbh1* promoter.

10. A method for producing a fungal host for protein production, characterized in that the method comprises the steps of:

- genetically modifying regulatory factors binding to the promoter of a gene encoding a secretable protein or mediating the regulatory signal in the expression of a selected secretable protein in a fungal host,
- expressing the selected secretable protein in the modified fungal host in secretion stress conditions,
- screening or selecting for cells showing enhanced or decreased protein expression of the selected secretable protein compared to the expression of the secretable protein in a non-modified host under the same conditions; and
- recovering the fungal host.

11. The method according to claim 10, characterized in that the regulatory mechanisms are mediating the transcriptional down-regulation of the genes encoding proteins selected from the group comprising cellulases, hemicellulases, amylolytic enzymes, hydrophobins, swollenin, proteases, invertases, fytases, phosphatases, ligninolytic enzymes, and pectinases.

12. The method according to claim 10 or 11, characterized in that the regulatory mechanisms are mediating transcriptional down-regulation of the genes encoding proteins selected from the group comprising those encoded by the genes *cbh1*, *cbh2*, *egl1*, *egl2*, *hfb1*, *hfb2*, *xyn1*, *swo*, *gla*, *amy*, and *pepA*.

13. The method according to any one of claims 10 to 12, characterized in that the fungal host is a host obtained by the method of any one of claims 3 to 9.

14. The method according to any one of claims 10 to 13, characterized in that the regulatory factor is encoded by the *ace1* gene and that the expression or activity of *ace1* gene is reduced or abolished.

15. The method according to any one of claims 10 to 13, characterized in that the regulatory factor is encoded by the *ace1* gene and that the expression or activity of *ace1* gene is amplified or increased.

16. The method according to any one of claims 10 to 15, characterized in that the fungal host strain is selected from the group comprising *Aspergillus* ssp., *Trichoderma* ssp., *Neurospora* ssp., *Fusarium* ssp., *Penicillium* ssp., *Humicola* ssp., *Tolypocladium geodes*, *Kluyveromyces* ssp., *Pichia* ssp., *Hansenula* ssp., *Candida* ssp., *Yarrowia* ssp., *Schizosaccharomyces* ssp., *Saccharomyces* spp.

17. The method according to any one of claims 10 to 16, characterized in that the strain belongs to *Aspergillus* ssp. or *Trichoderma* ssp.

18. The method according to any one of claims 10 to 17, characterized in that the strain belongs to *A. niger* or *T. reesei*.

19. A method for overproduction of homologous secretable proteins or production of heterologous secretable proteins in fungi, c h a r a c t e r i z e d in that the method comprises the steps of:

- operable linking the promoter, obtained by the method of claim 1 or 2 or by the method of any one of claims 3 to 9, to the coding region of a gene encoding a selected secretable protein, said promoter being screened or selected on the basis of enhanced protein expression; and

expressing the selected secretable protein under the regulation of the promoter in a fungal host under suitable culture conditions; or

- expressing the selected secretable protein in the fungal host obtained by the method of any one of claims 10 to 17, said fungal host being screened and selected on the basis of enhanced protein expression; and

- recovering the protein product from the culture medium of the fungal host.

20. A method for decreased protein production of homologous secretable proteins in fungi, c h a r a c t e r i z e d in that the method comprises the steps of:

- operable linking the promoter, obtained by the method of claim 1 or 2 or by the method of any one of claims 3 to 9 to the coding region of a gene encoding a selected secretable protein, said promoter being screened or selected on the basis of decreased protein expression; and

expressing the selected secretable protein under the regulation of the promoter in a fungal host under suitable culture conditions; or

- expressing the selected secretable protein in the fungal host obtained by the method of any one of claims 10 to 17, said fungal host being screened or selected on the basis of decreased protein expression.

21. The method according to claim 19 or 20, c h a r a c t e r i z e d in that the protein product is selected from the group comprising proteins originating from bacteria or lower or higher eucaryotes or from fungal or mammalian origin, such as cellulase, hemicellulase, amylyolytic enzyme, hydrophobin, protease, invertase, phytase, phosphatase, a ligninolytic enzyme, pectinase, immunoglobulin or tPA.

22. A method for optimised protein production of secretable proteins in fungi, c h a r a c t e r i z e d in that the method comprises the steps:

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- selecting a gene of a secretable protein,
- operable linking the coding region of the gene encoding the selected secretable protein into a promoter not regulated by transcriptional down-regulation,
- producing the selected protein under suitable culture conditions in a fungal host that overproduces proteins mediating down-regulation or produces these regulatory factors with enhanced activity; and
- recovering the selected secretable protein from the culture medium of the host.

23. The method according to claim 22, characterized in that the promoter is obtained by the method of claim 1 or 2 or by the method of any one of claims 3 to 9 and screened or selected on the basis of enhanced protein expression.

24. The method according to claim 23, characterized in that the promoter is selected from the group of *Trichoderma gpd1*, *cDNA1*, *ypt1*, *sar1*, *bgl2* promoter and *Aspergillus gpdA* promoter.

25. The method according to any one of claims 22 to 24, characterized in that the protein mediating down-regulation is ACEI.

26. A DNA sequence located between -1031 and -162 upstream of *Trichoderma cbh1* promoter (nucleotides 1186 to 2053 in SEQ ID NO: 5) mediating transcriptional down-regulation of secreted proteins under secretion stress, or a DNA sequence having the same function in a promoter selected from the group comprising *cbh1*, *cbh2*, *egl1*, *egl2*, *hfb1*, *hfb2*, *xyn1*, *swa*, *gla*, *amy*, and *pepA* promoter.

27. The DNA sequence according to claim 26, wherein the DNA sequence is located between nucleotides -1031 and -501 upstream of *Trichoderma cbh1* promoter (nucleotides 1186 to 1714 in SEQ ID NO: 5).

28. The DNA sequence according to claim 26, wherein the DNA sequence is located between nucleotides -341 and -211 upstream of *Trichoderma cbh1* promoter (nucleotides 1876 to 2004 in SEQ ID NO: 5).

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29. Use of any of the DNA sequences of claims 26 to 28 in genetically modified form to enhance or decrease the expression of a selected protein in a fungal host in secretion stress conditions.

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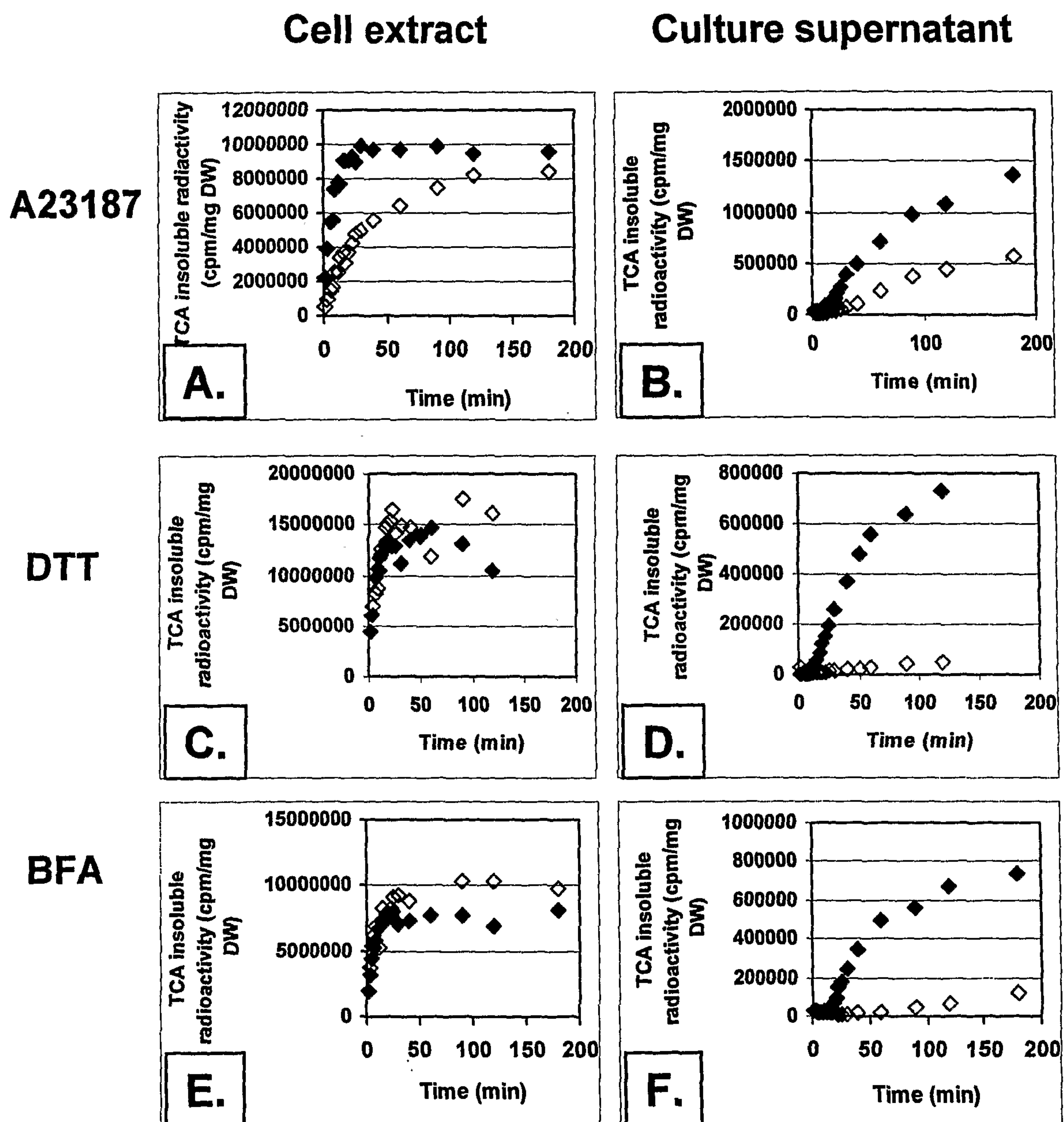


Fig. 1

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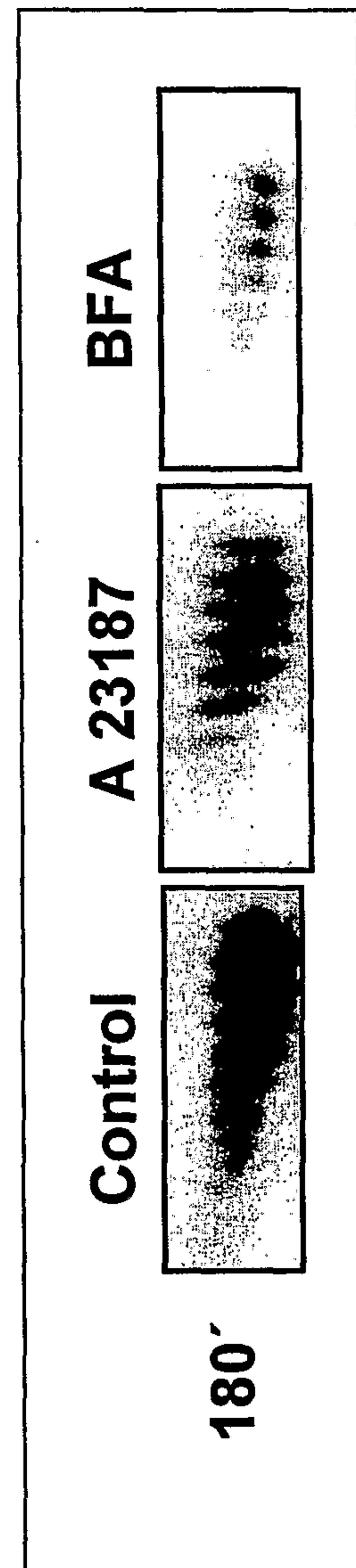
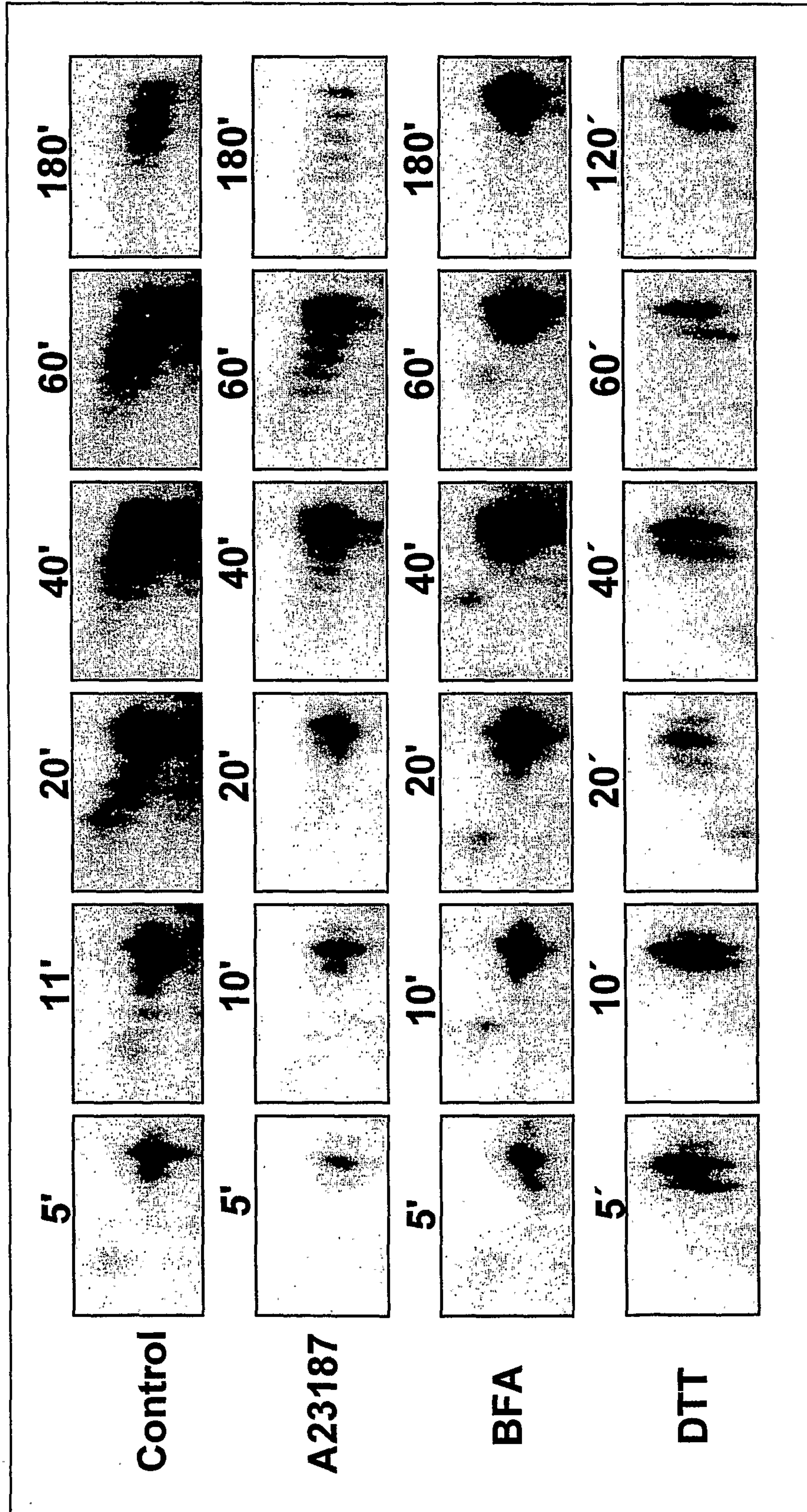
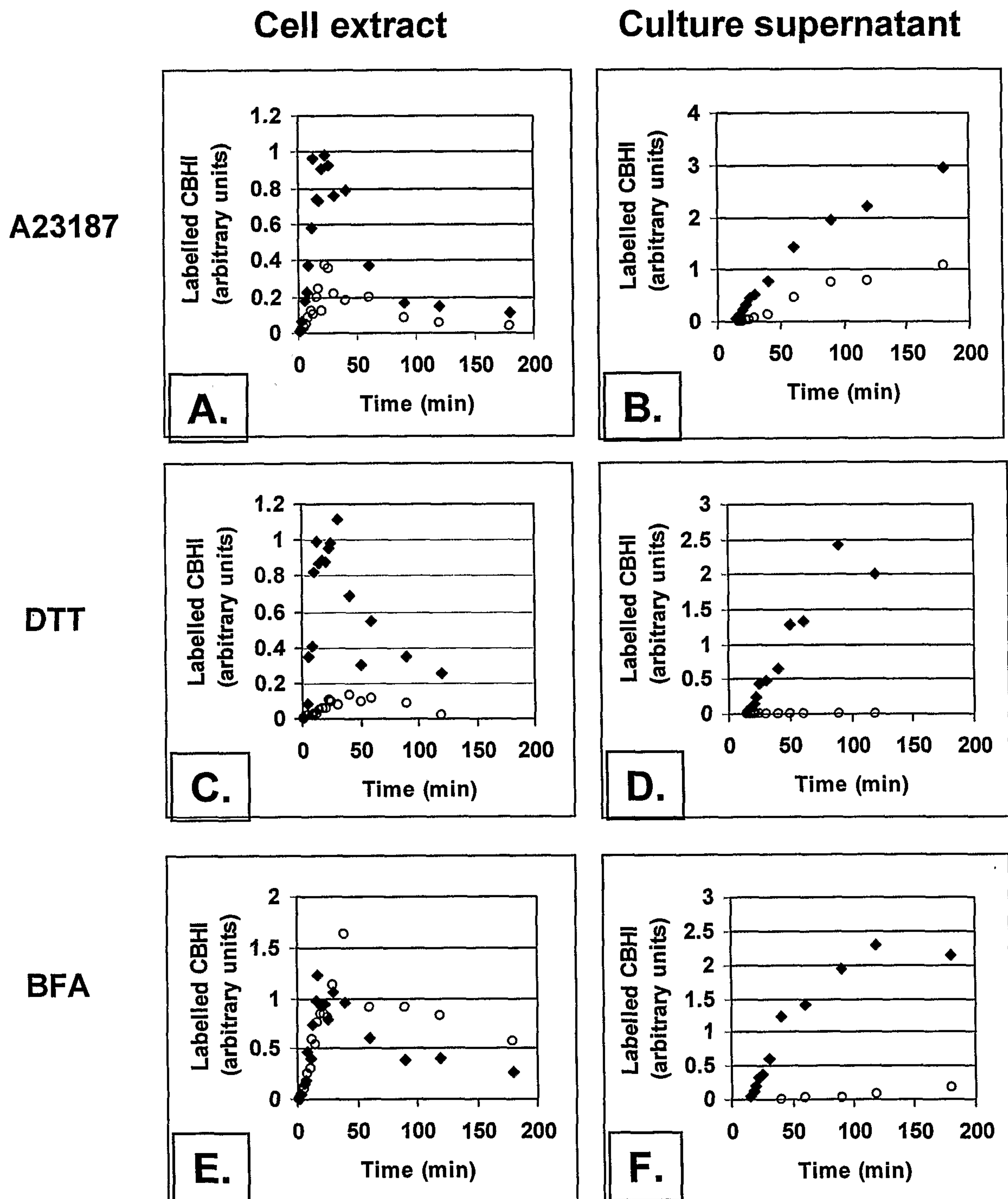


Fig. 2

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**Fig. 3**

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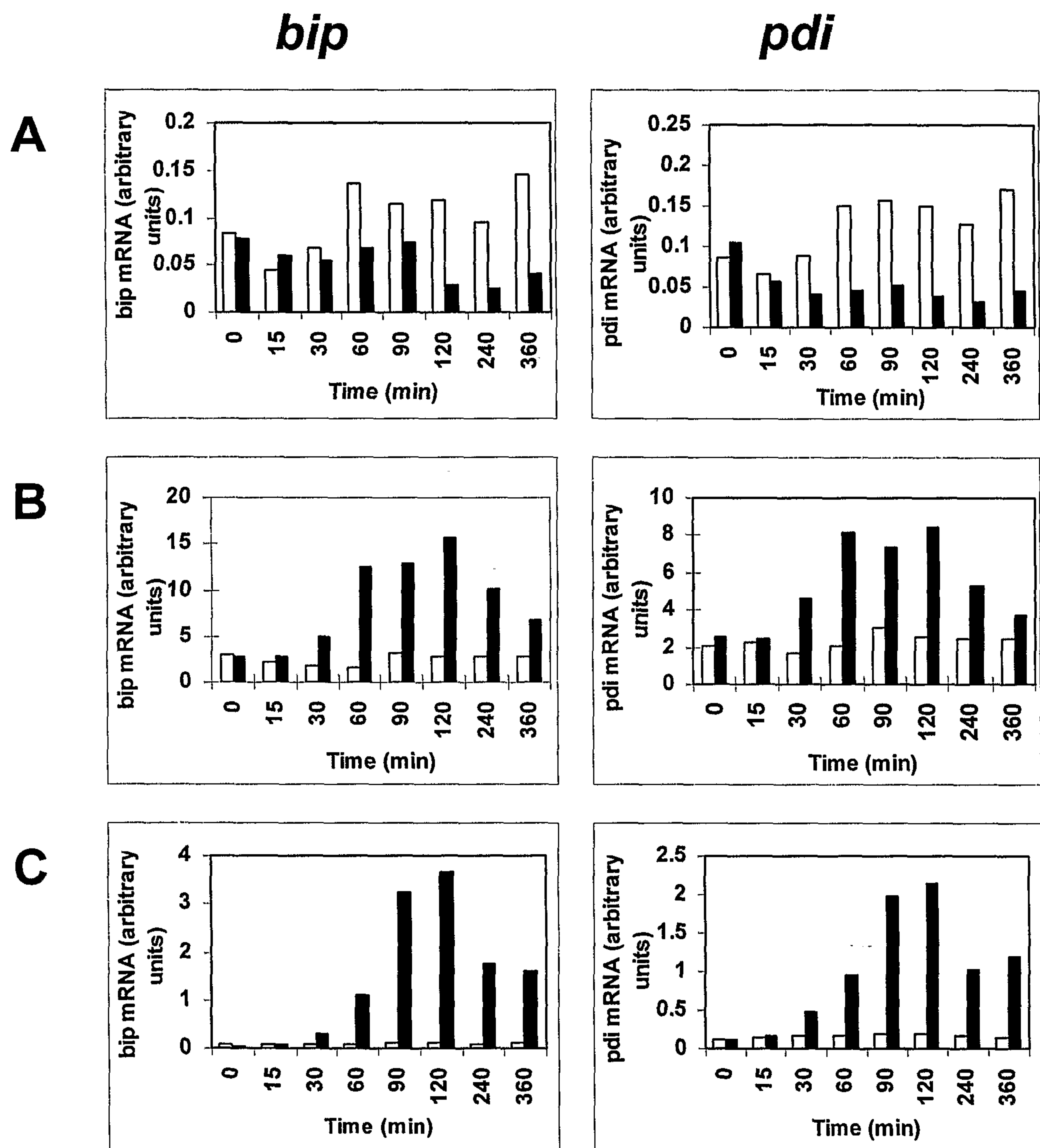


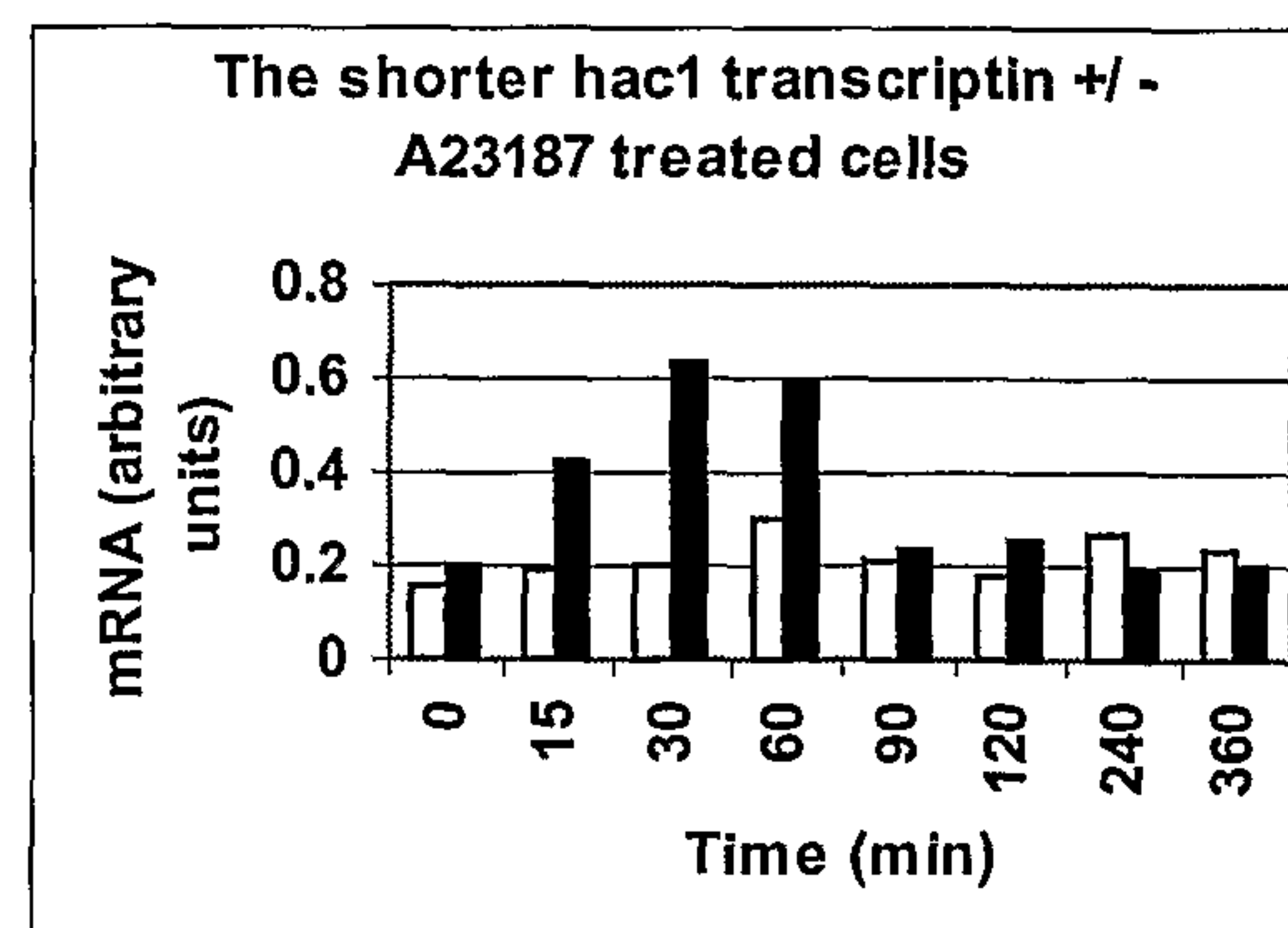
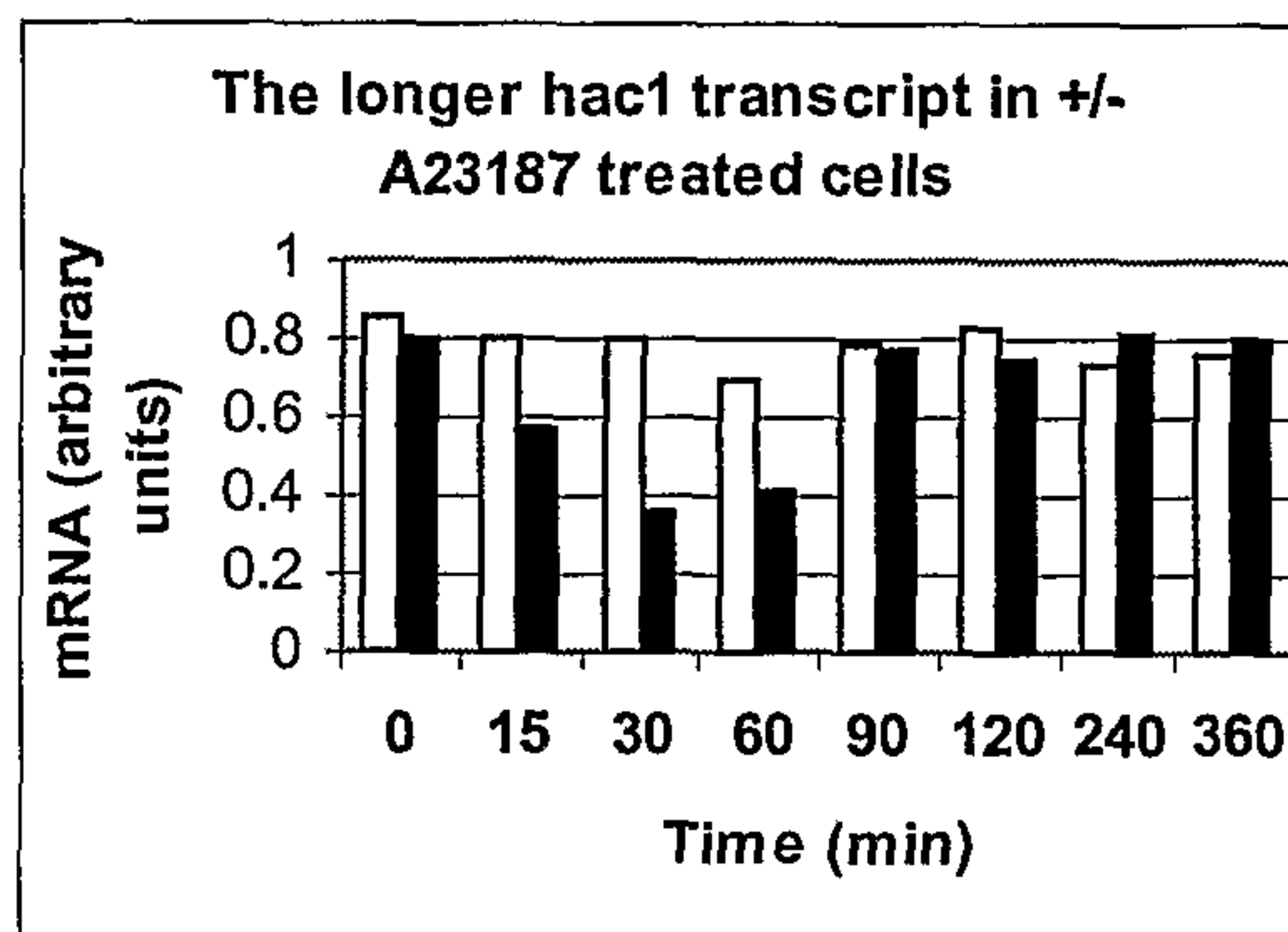
Fig. 4

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The longer hac1 transcript

The shorter hac1 transcript

A23187



BFA

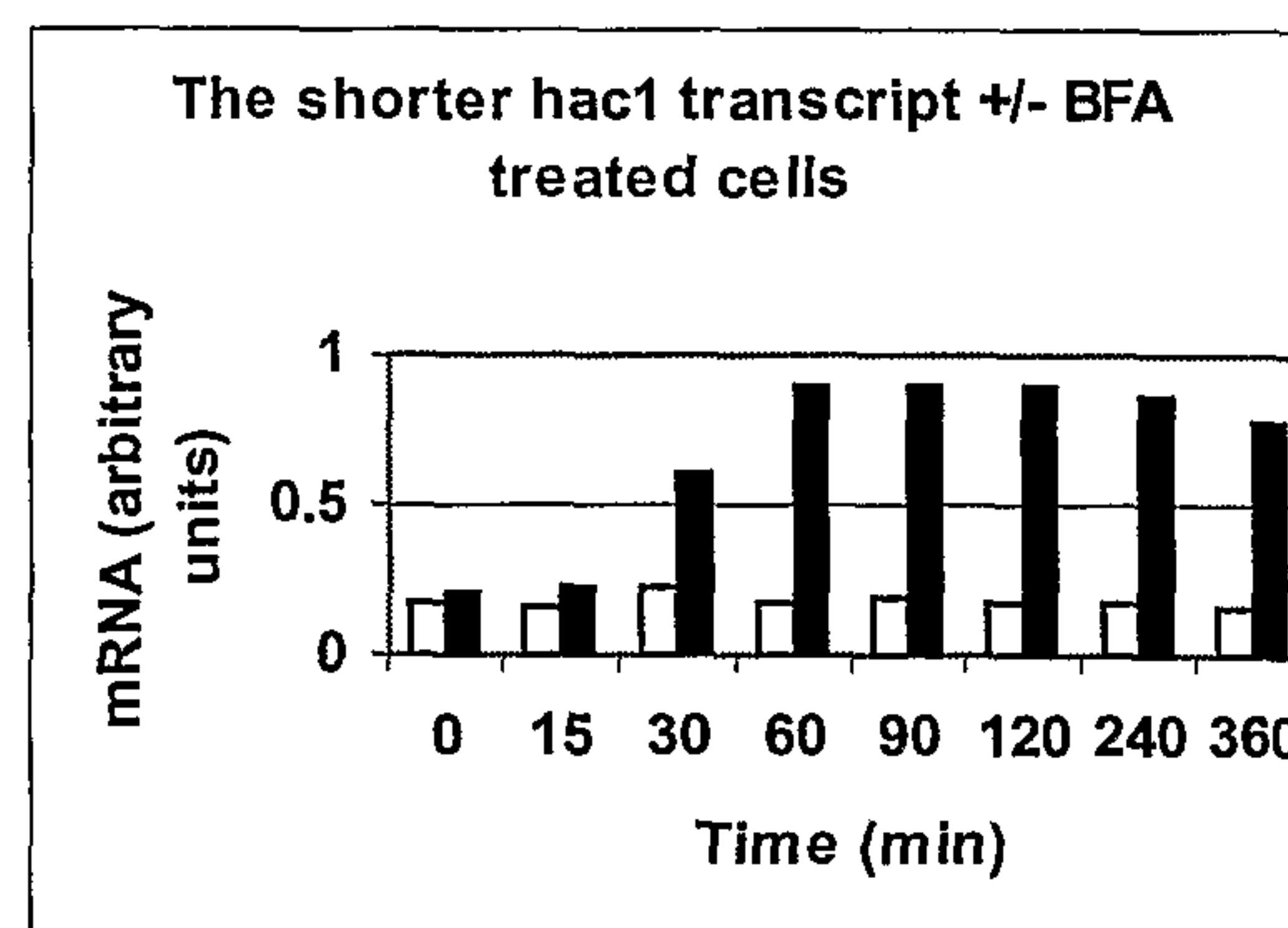
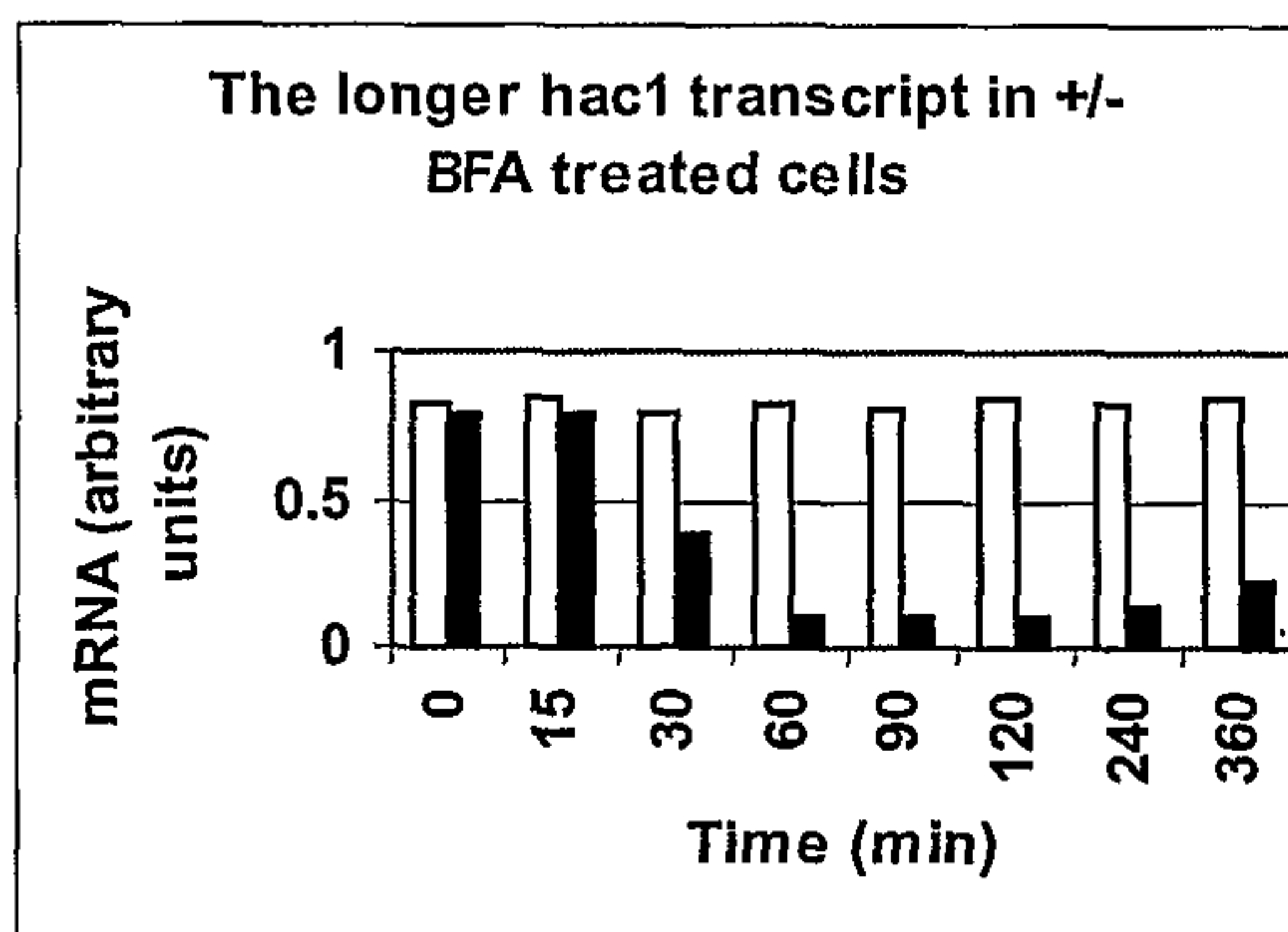
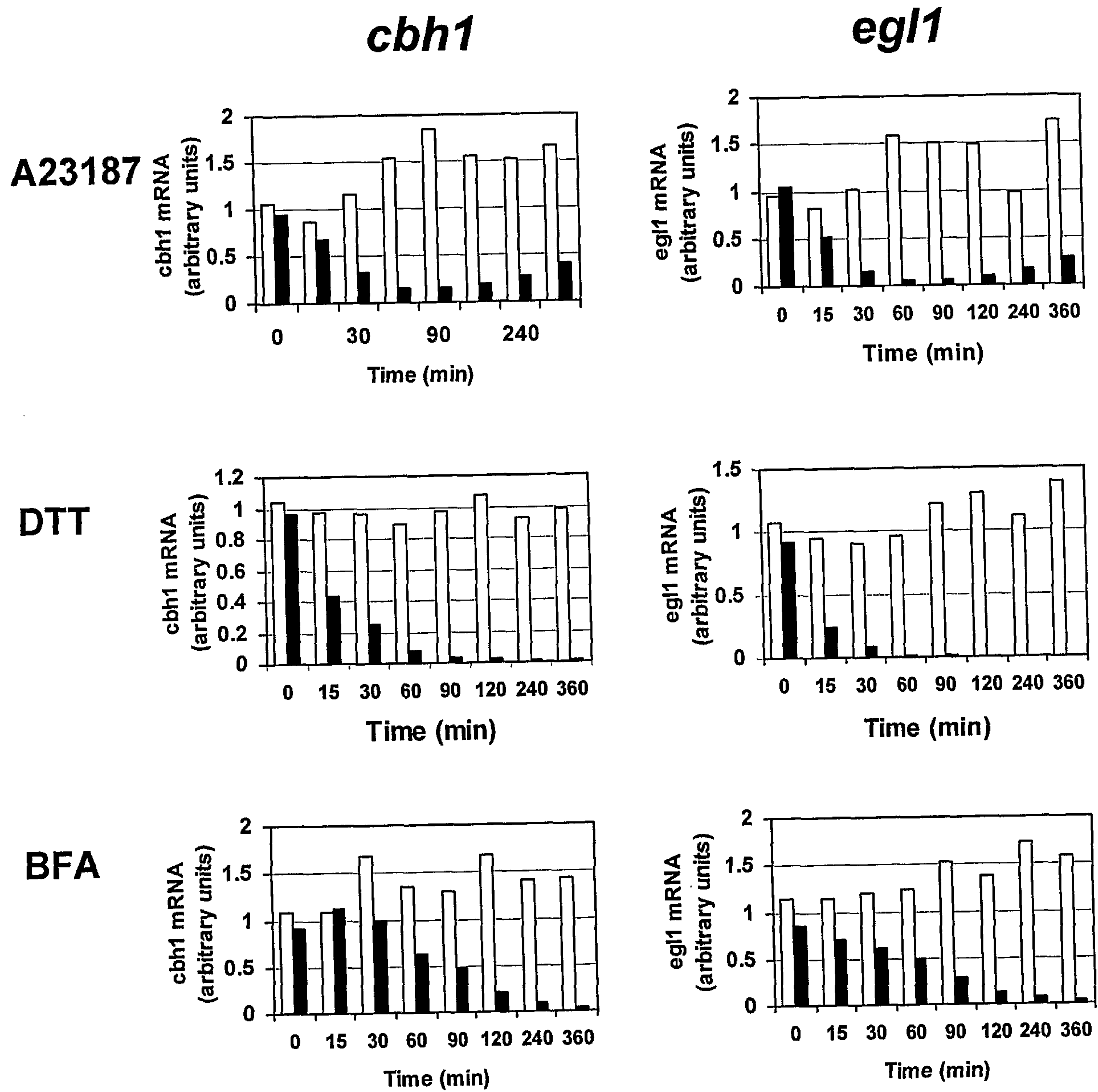
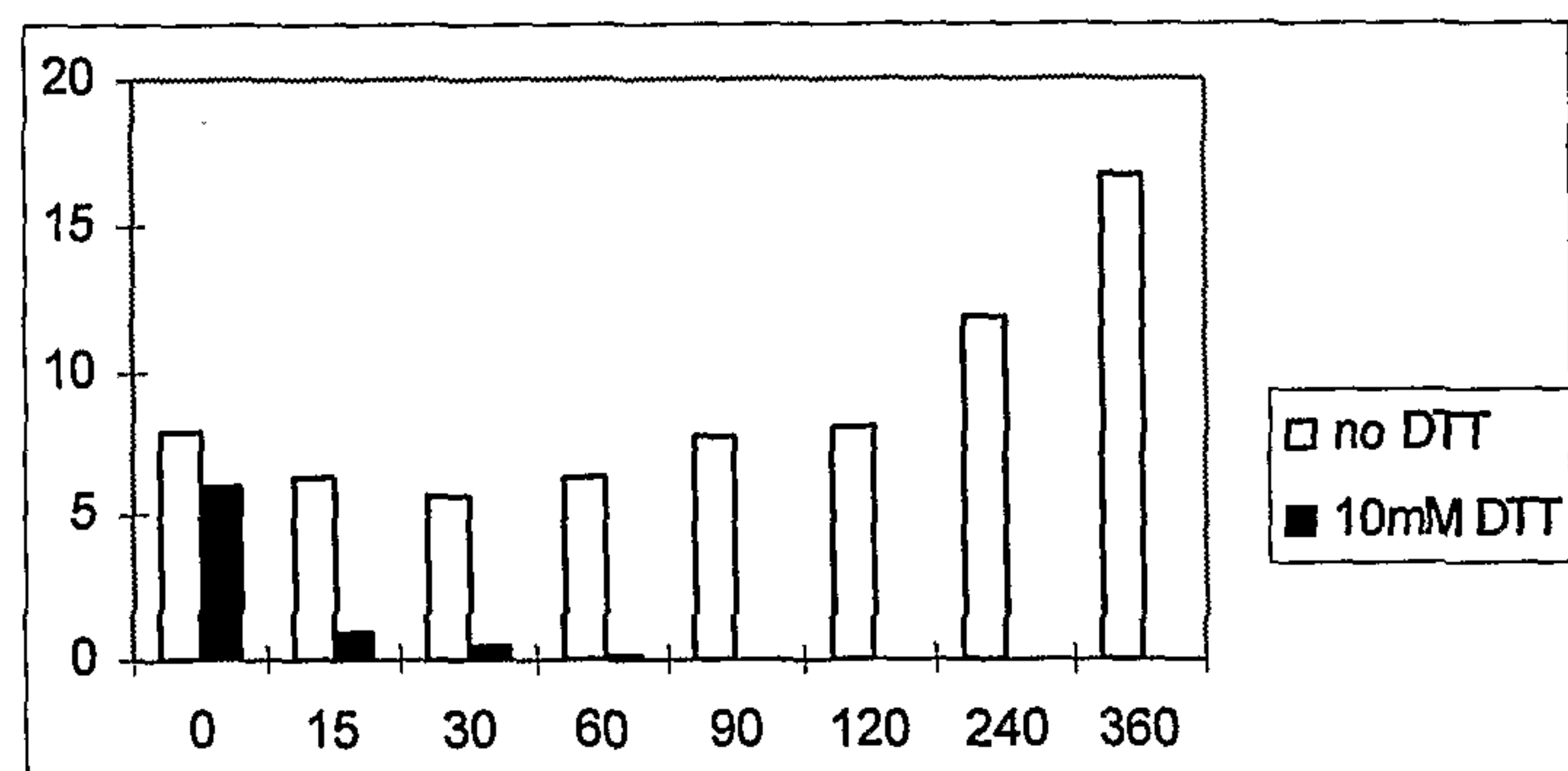
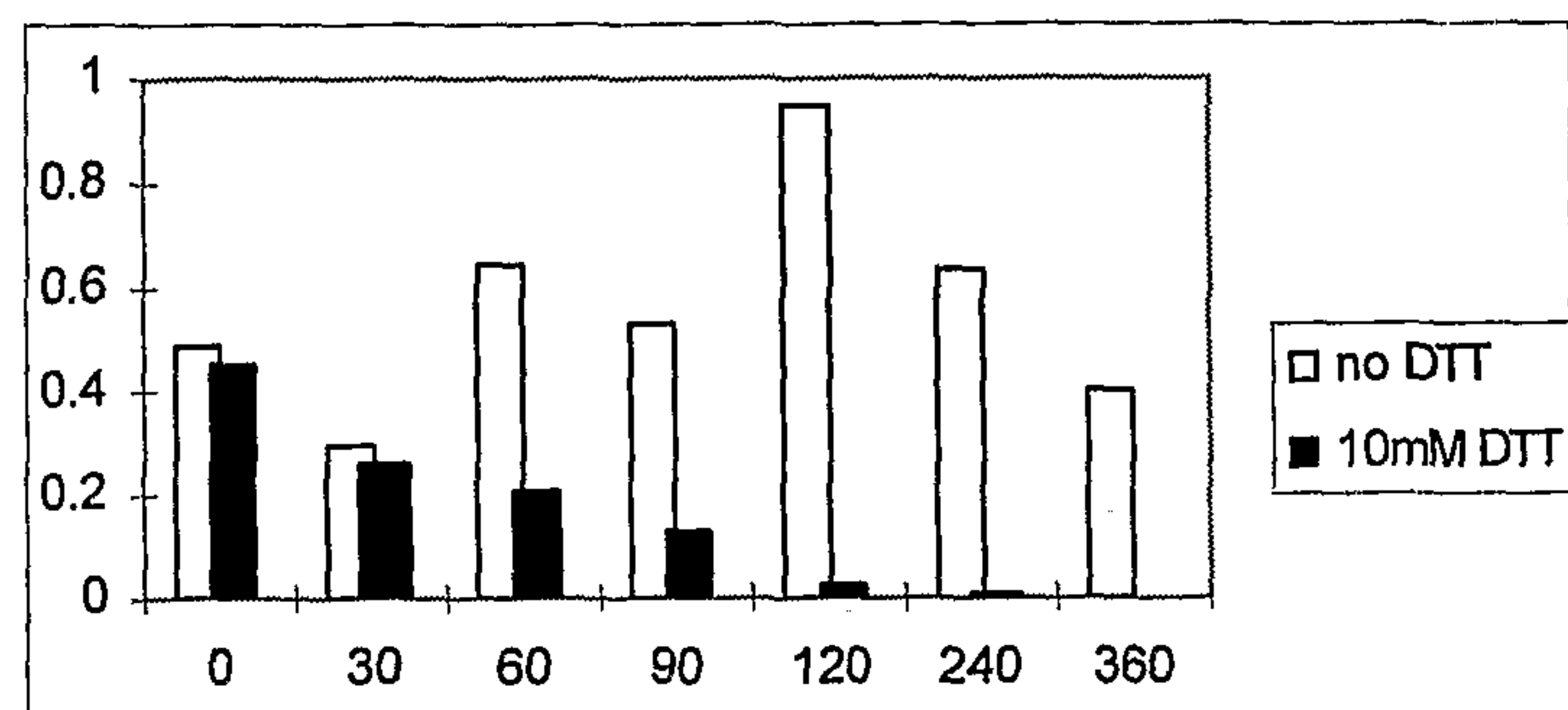


Fig.5

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**Fig. 6**

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xyn1*hfb2***Fig.7**

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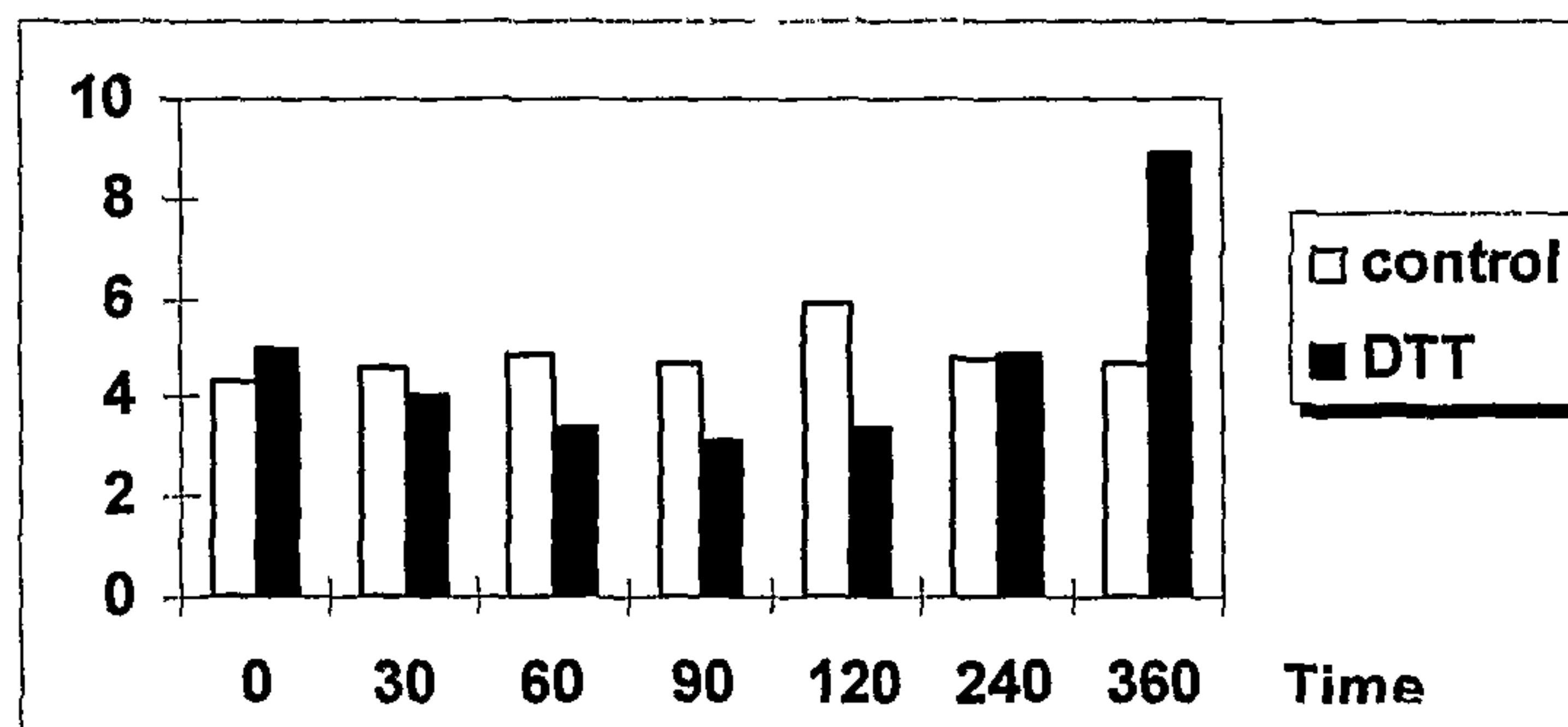
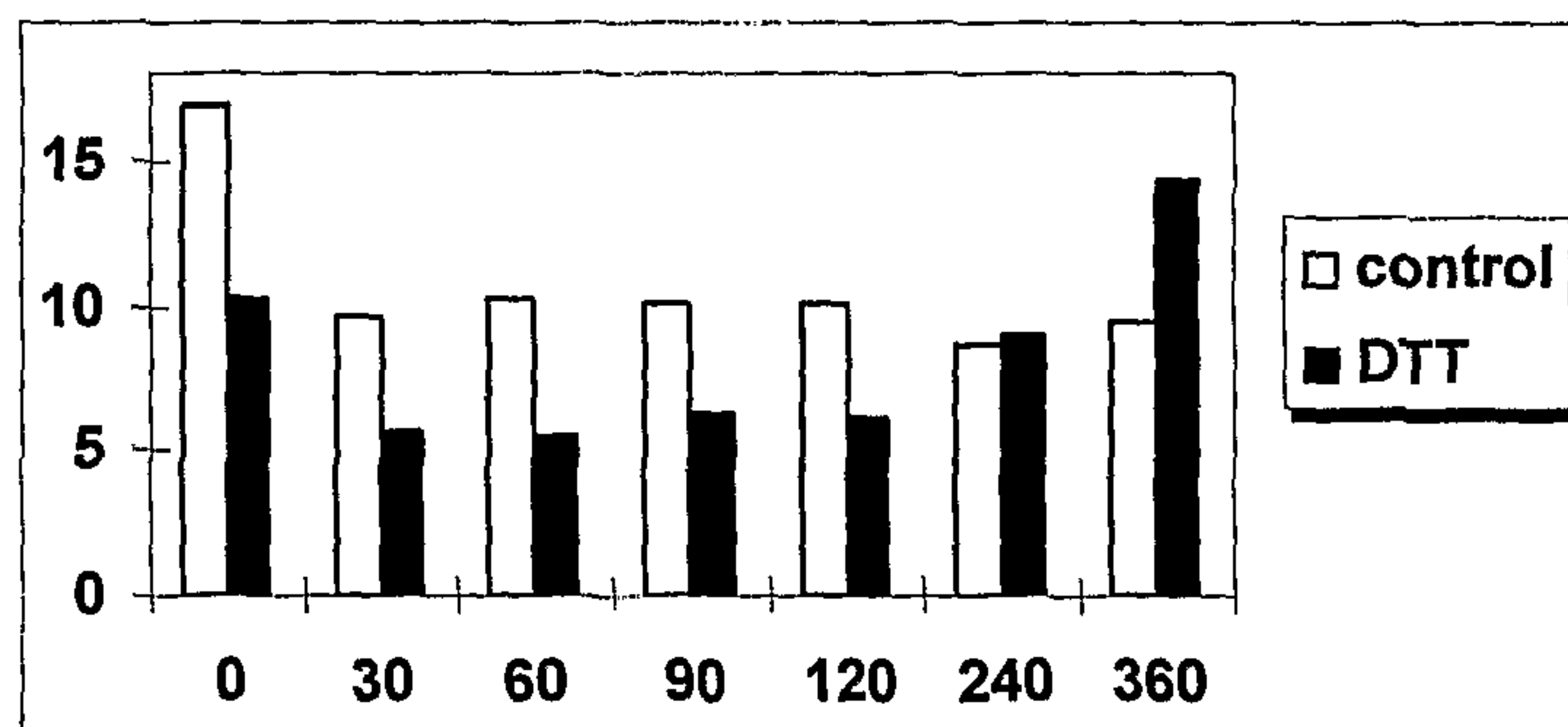
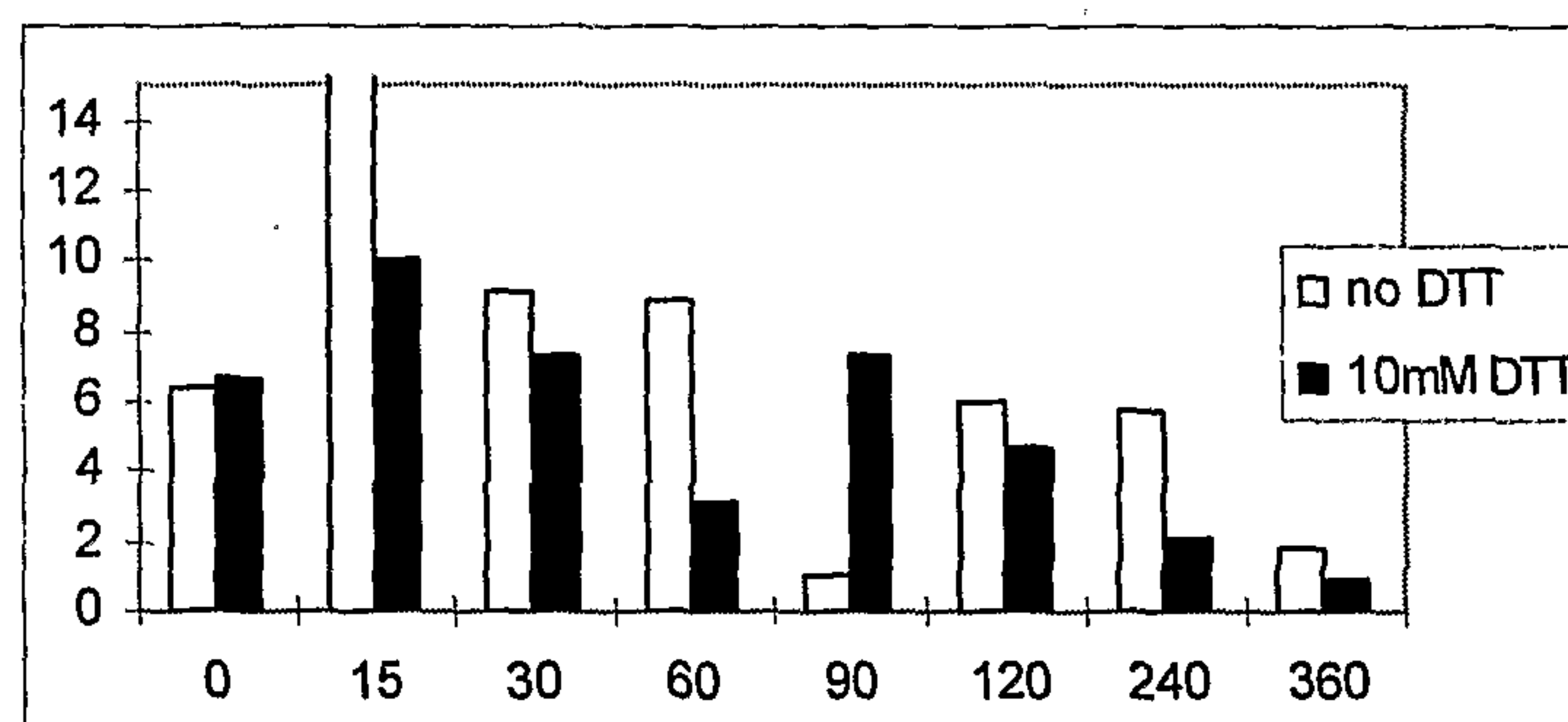
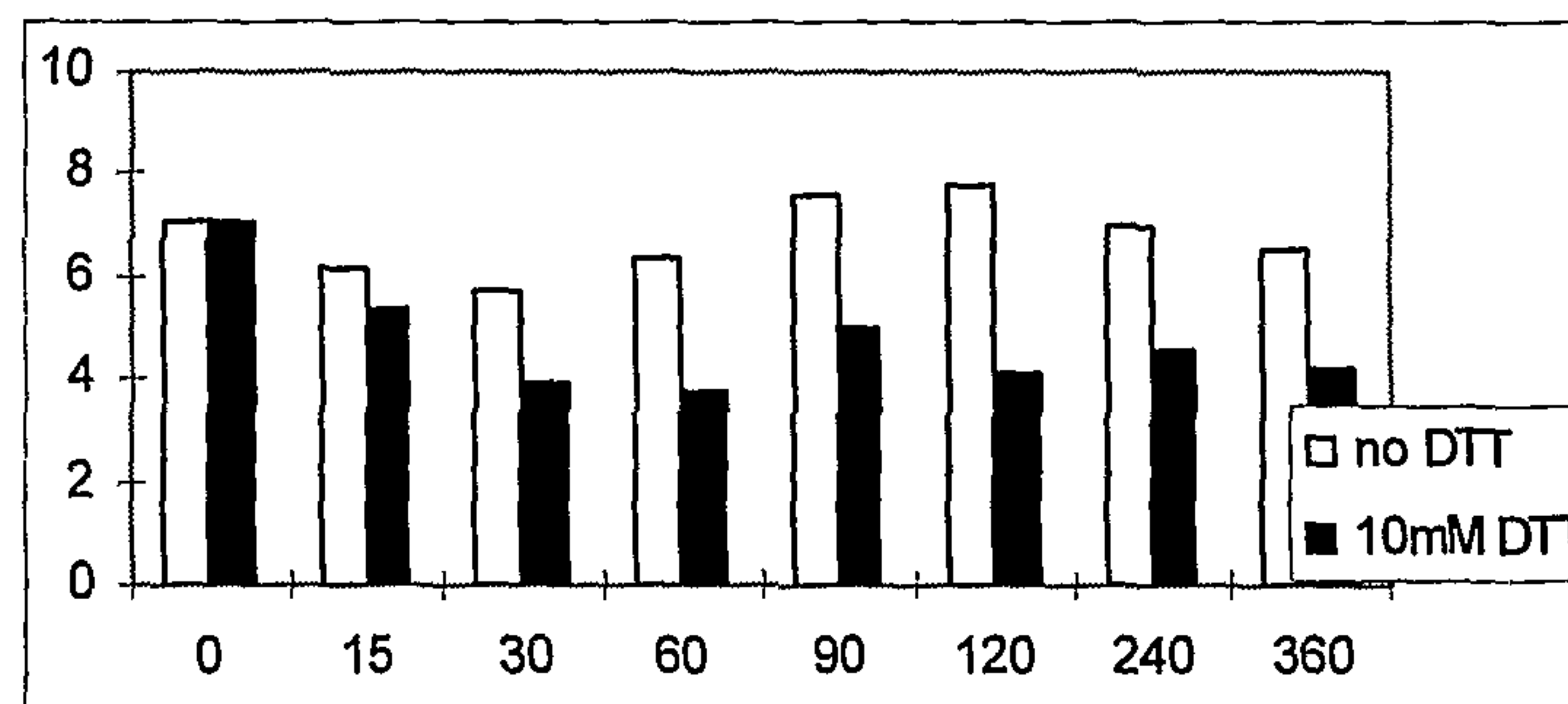
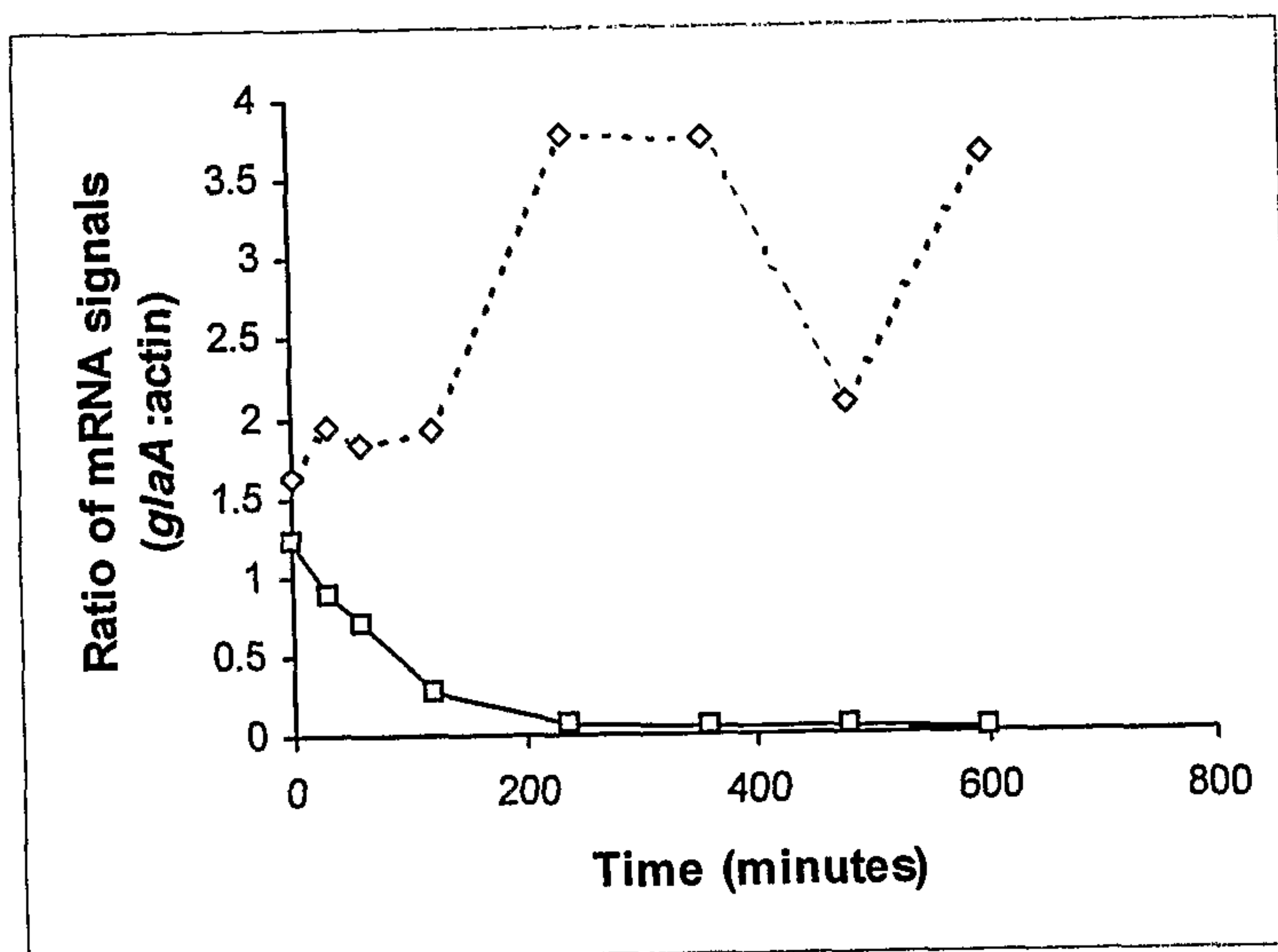
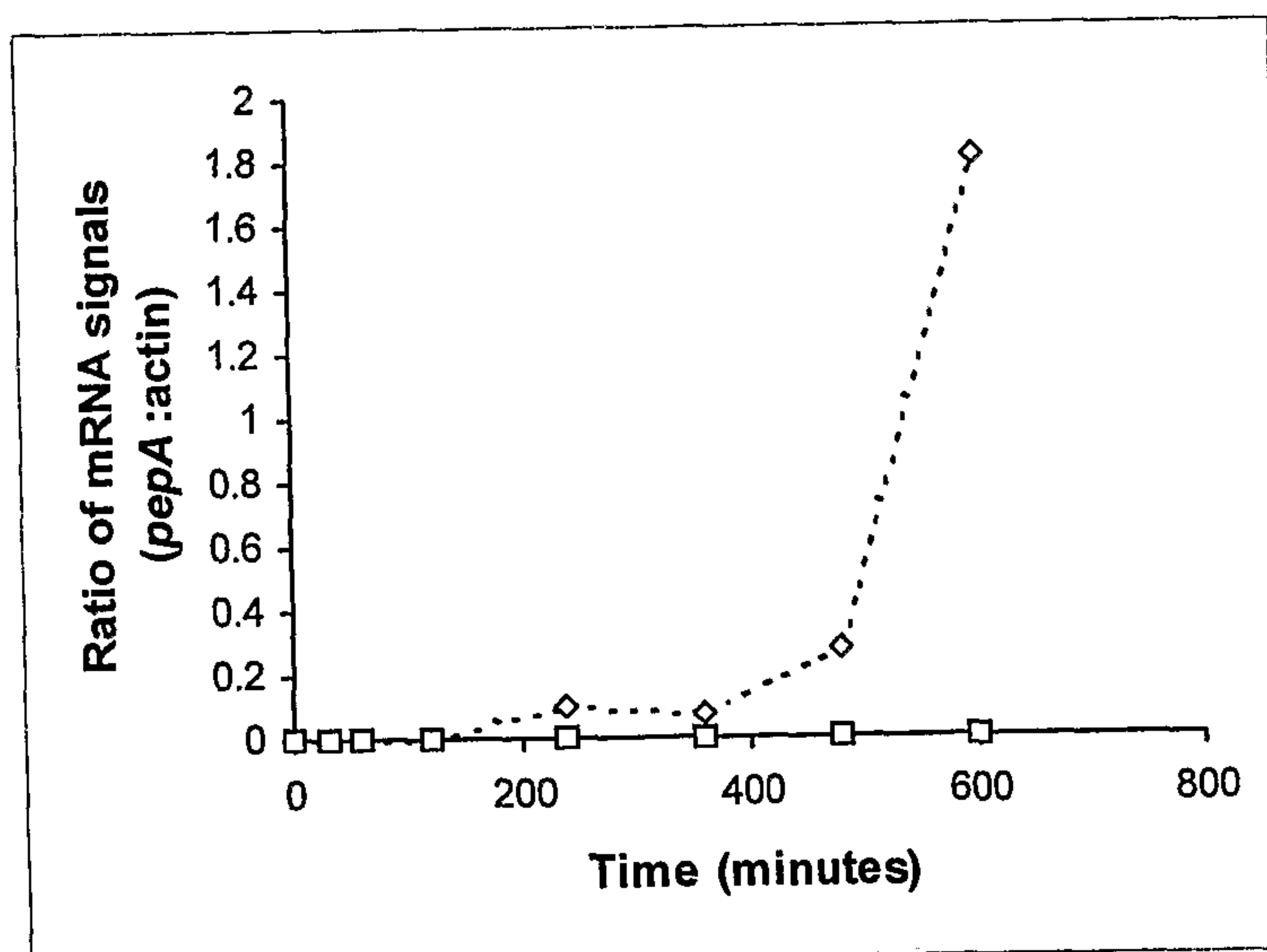
ypt1*sar1**cDNA1**bgl2*

Fig. 8

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Fig. 9A**Fig. 9B**

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Fig. 9C

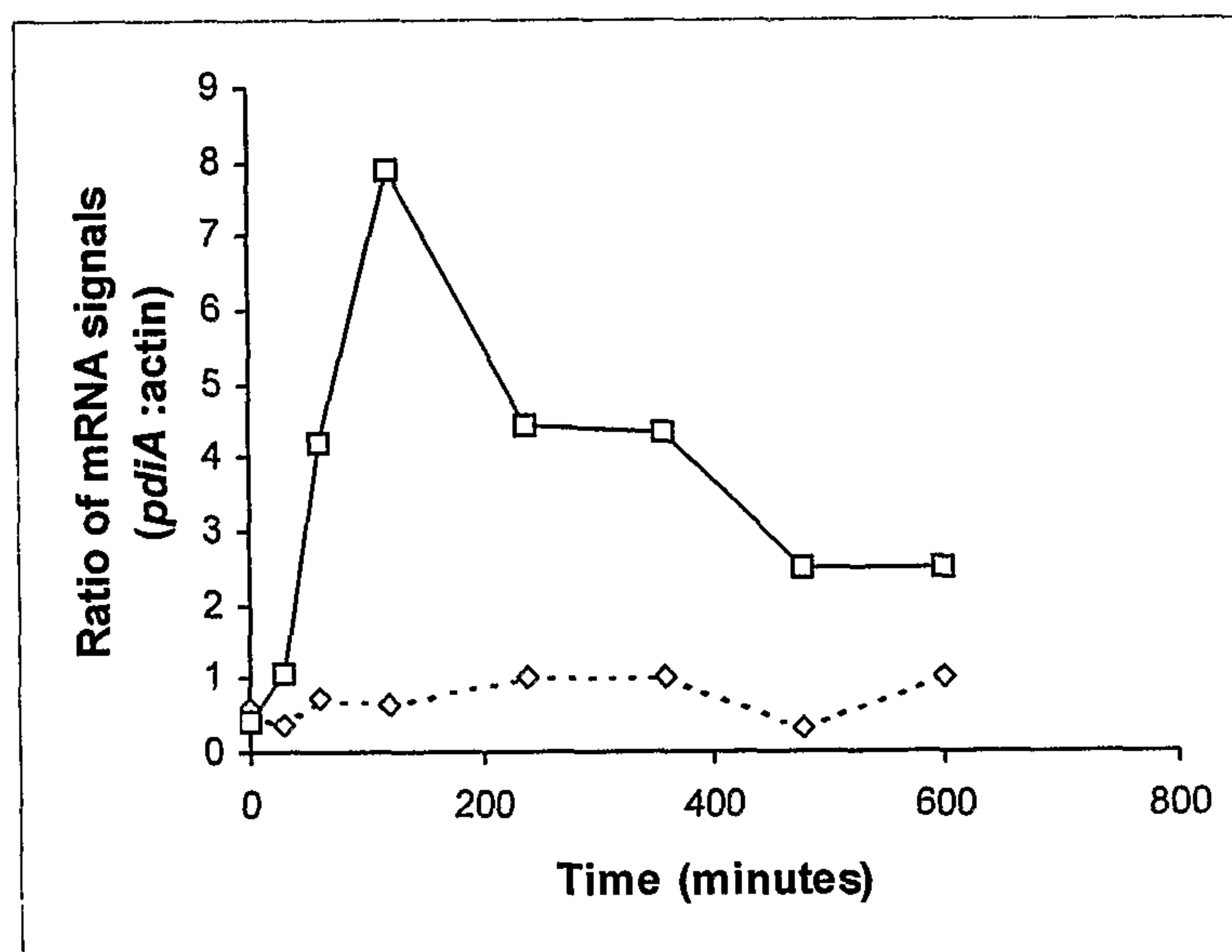
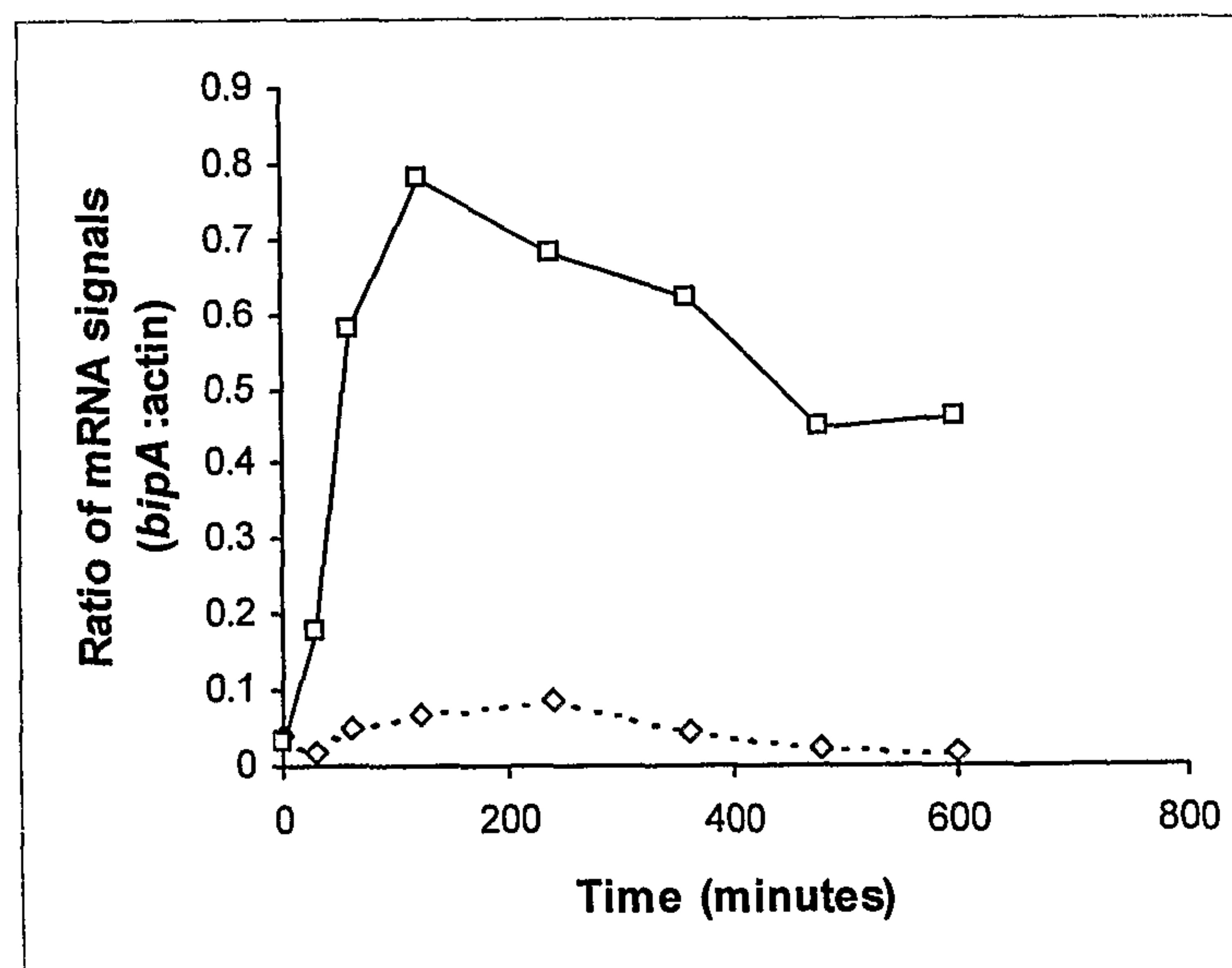
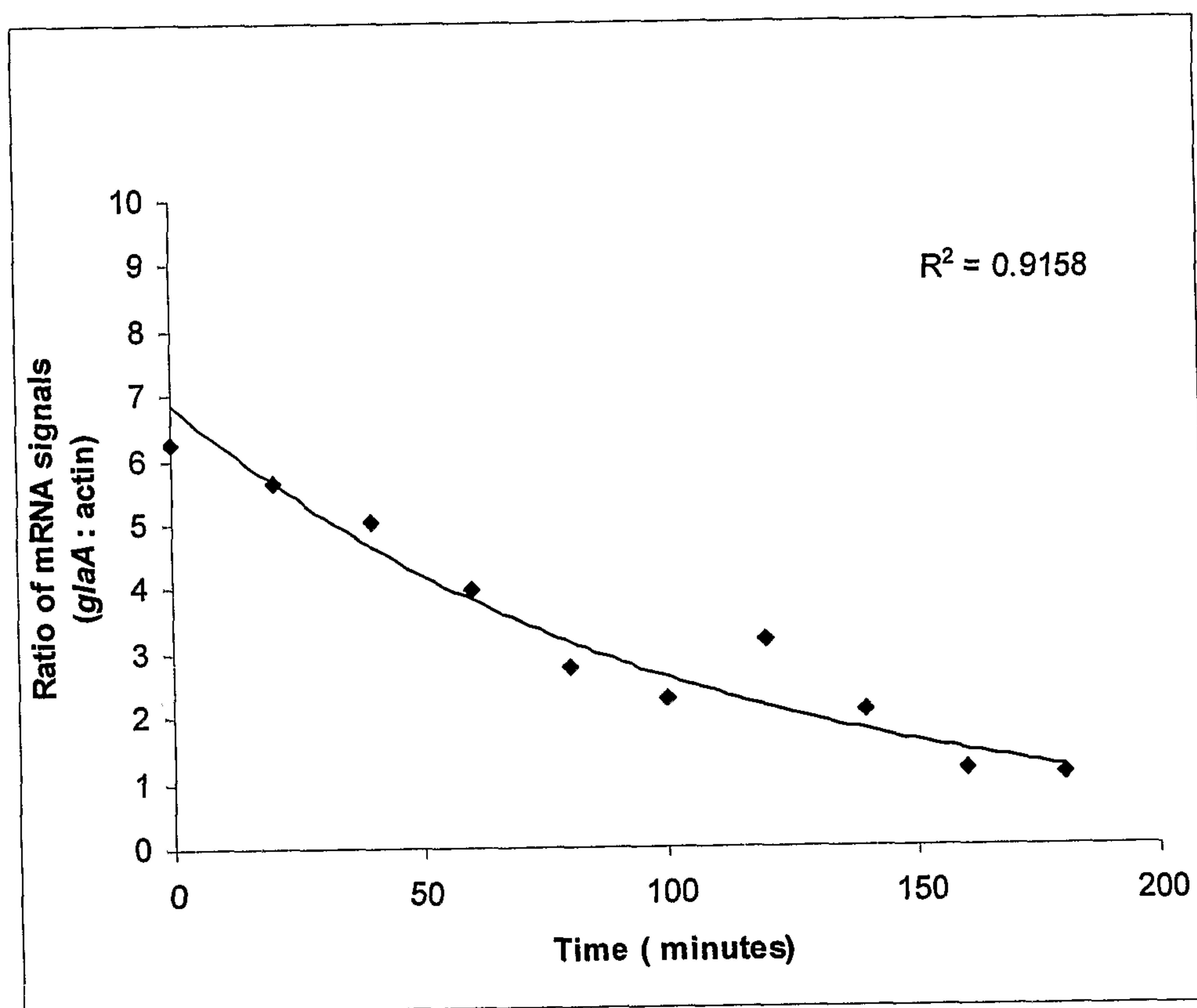


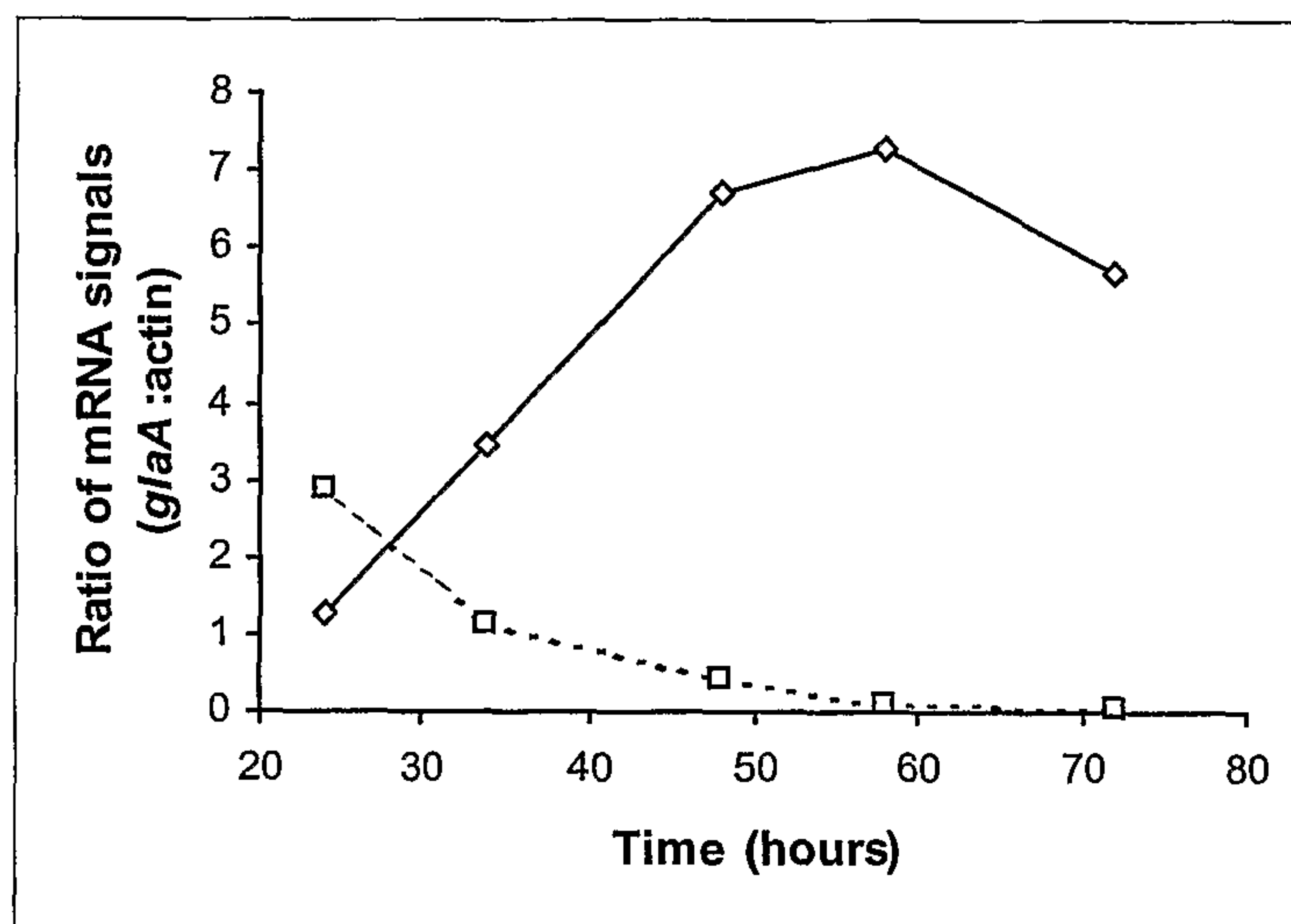
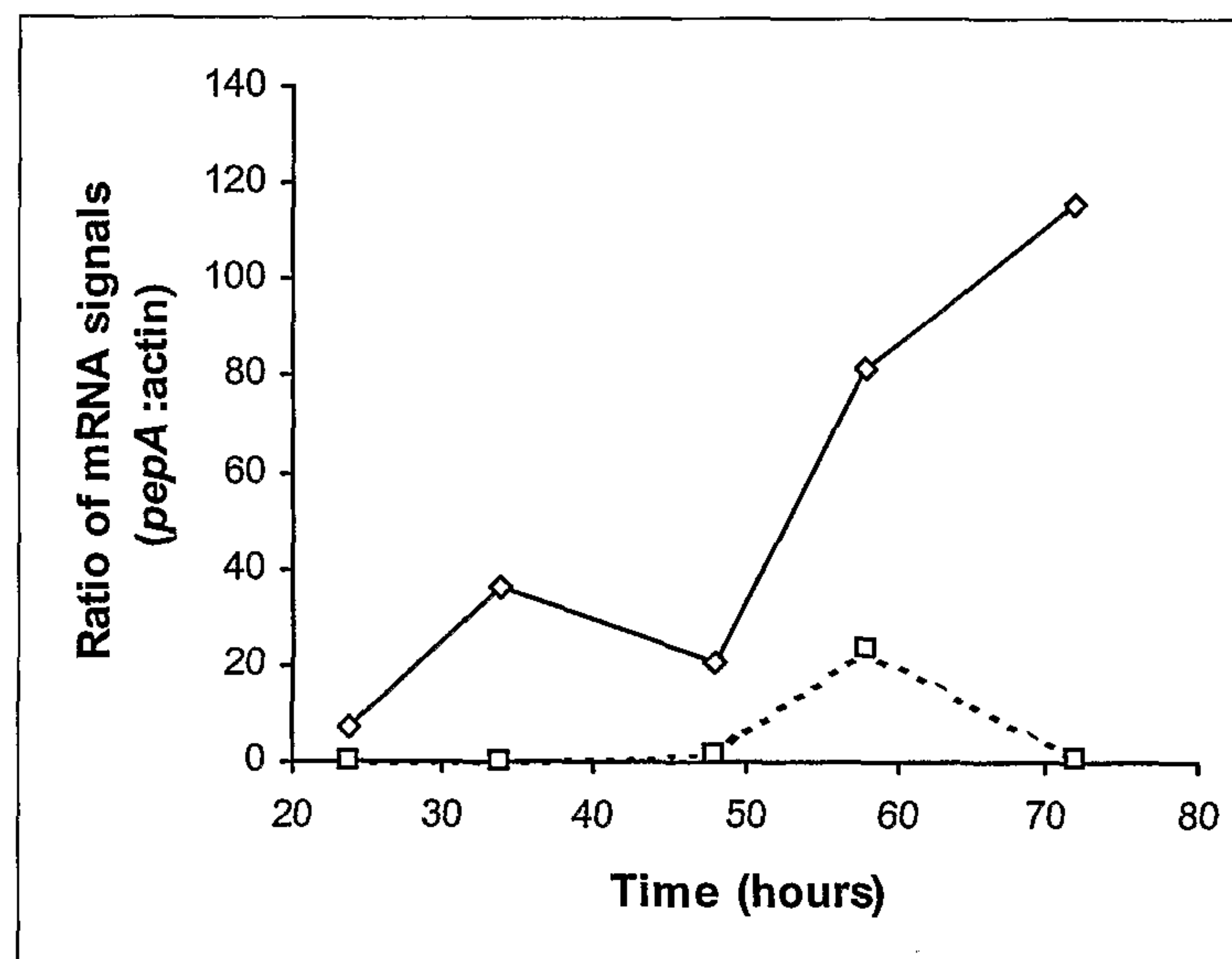
Fig. 9D



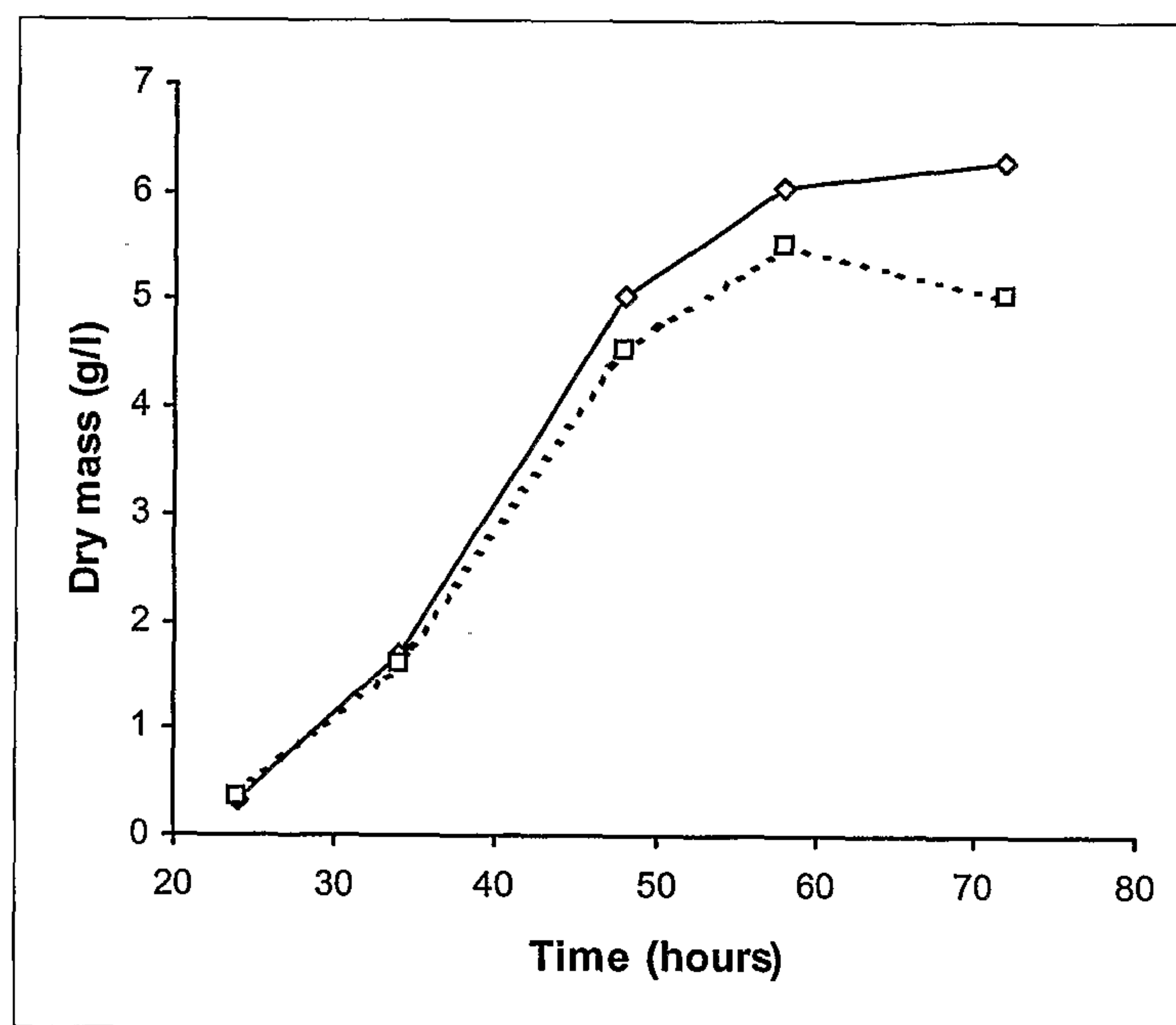
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**Fig. 10**

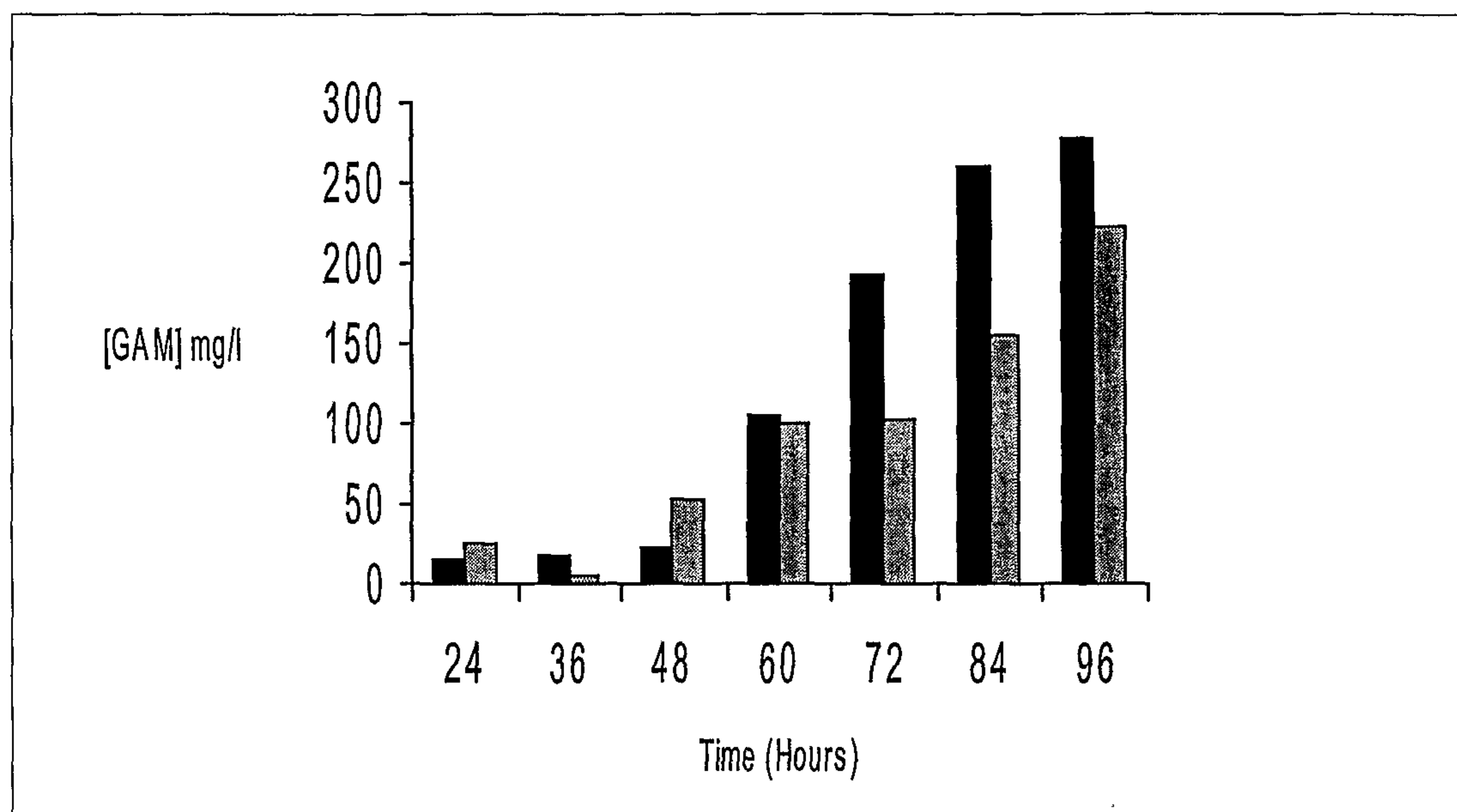
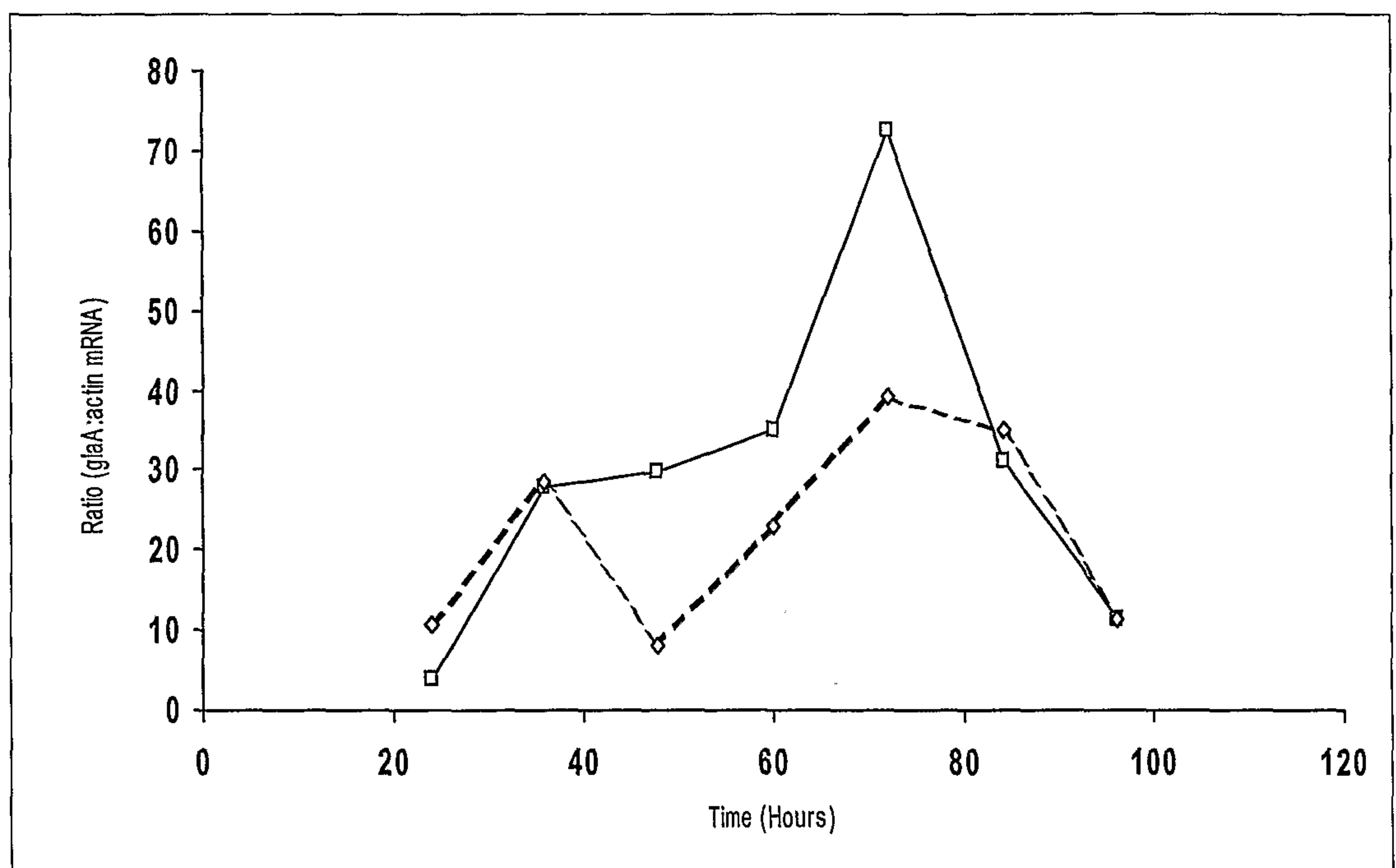
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Fig. 11A**Fig. 11B**

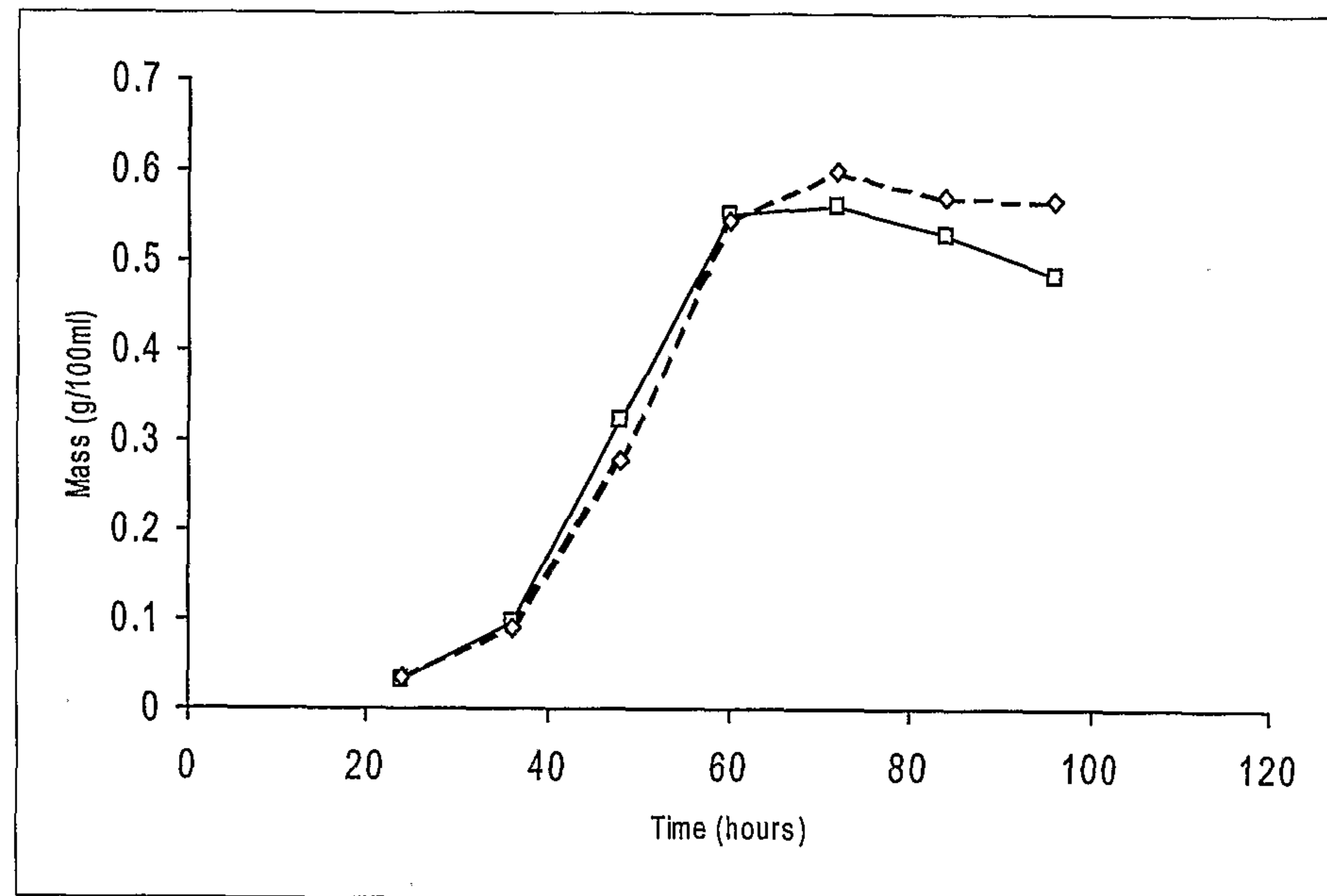
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**Fig. 11C**

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Fig. 12A**Fig. 12B**

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Fig. 12C

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1 2 3 4 5 6 7 8 9 10 11 12 13 14

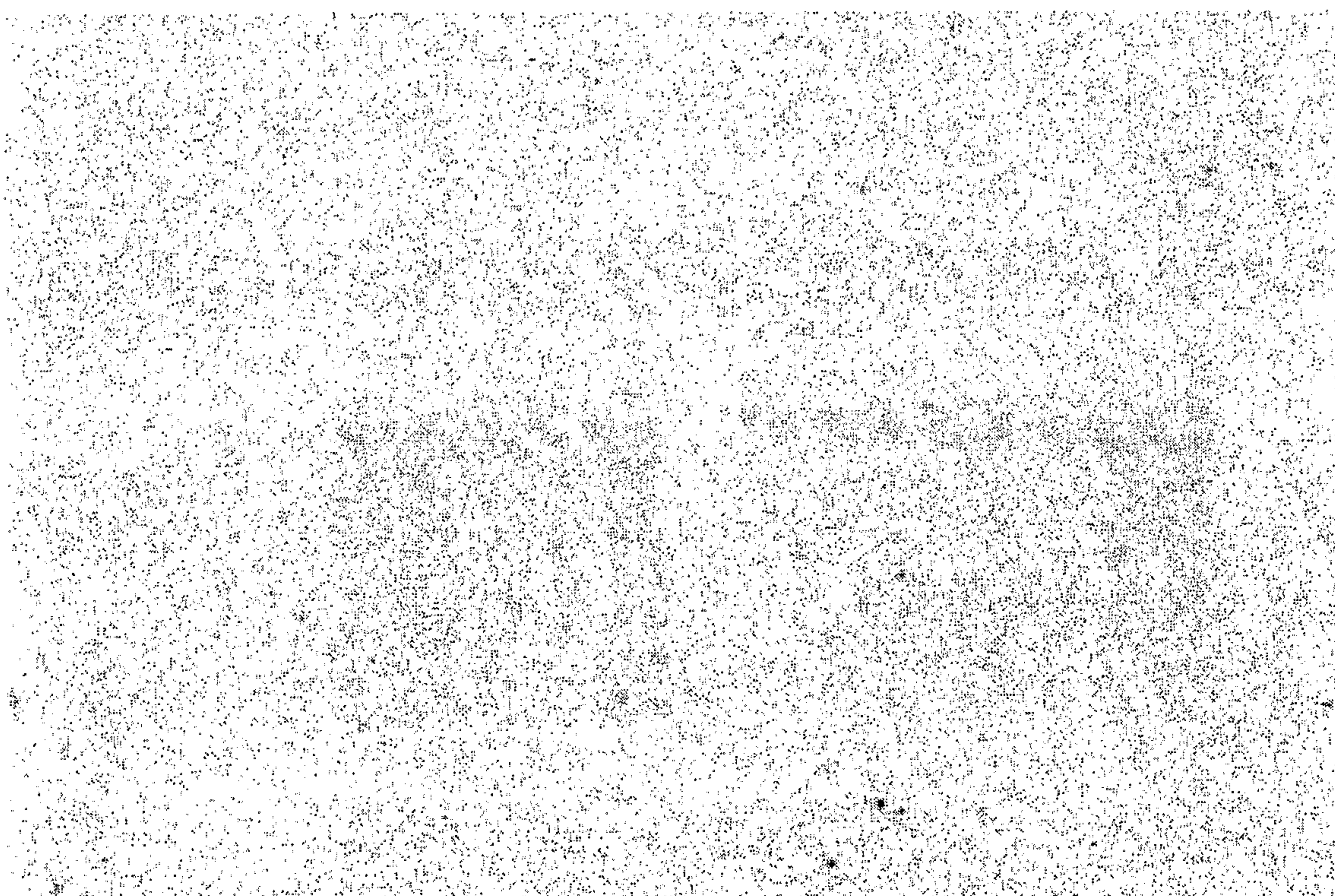
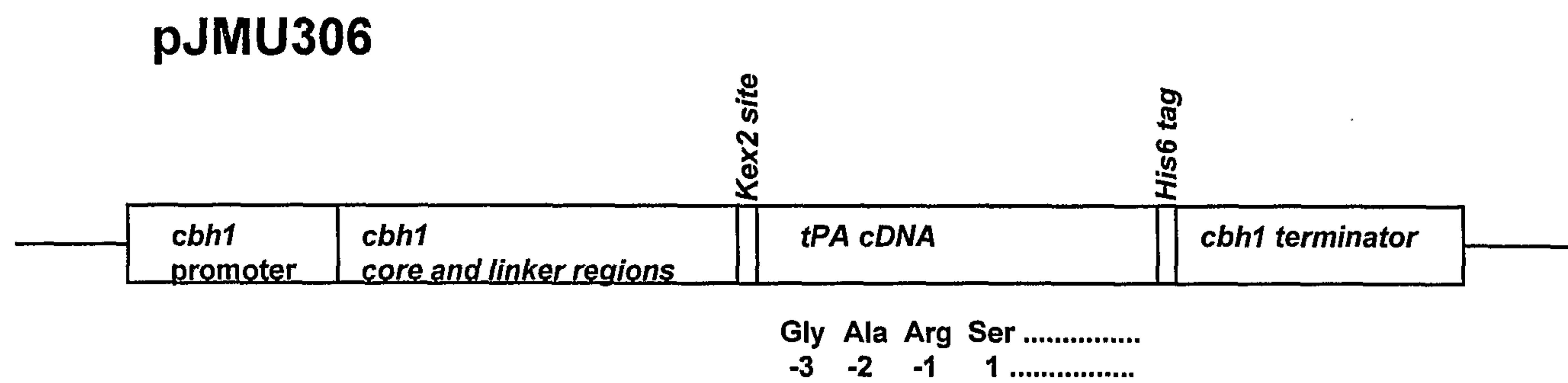


Fig. 13

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Fig 14A



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Fig 14B

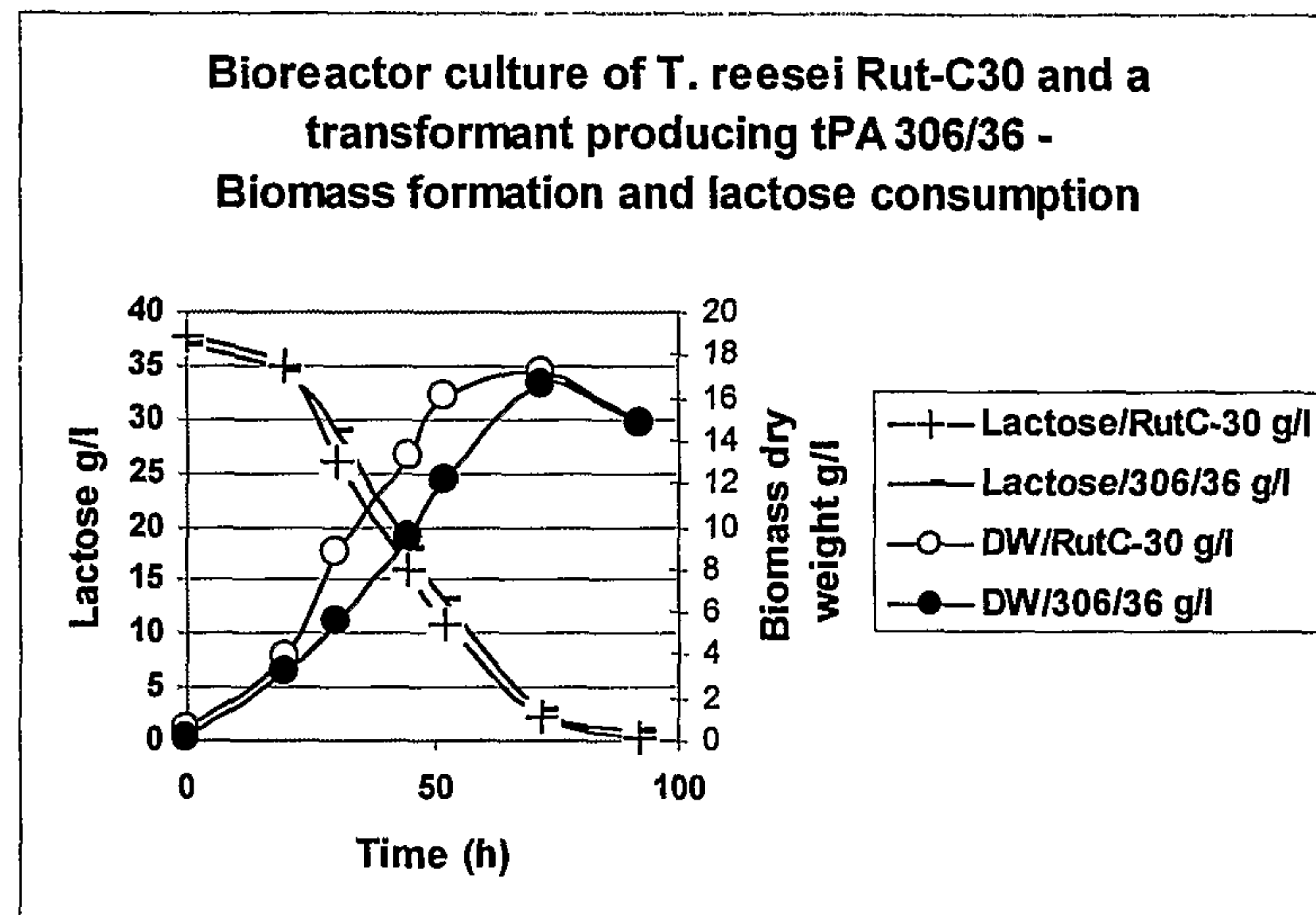
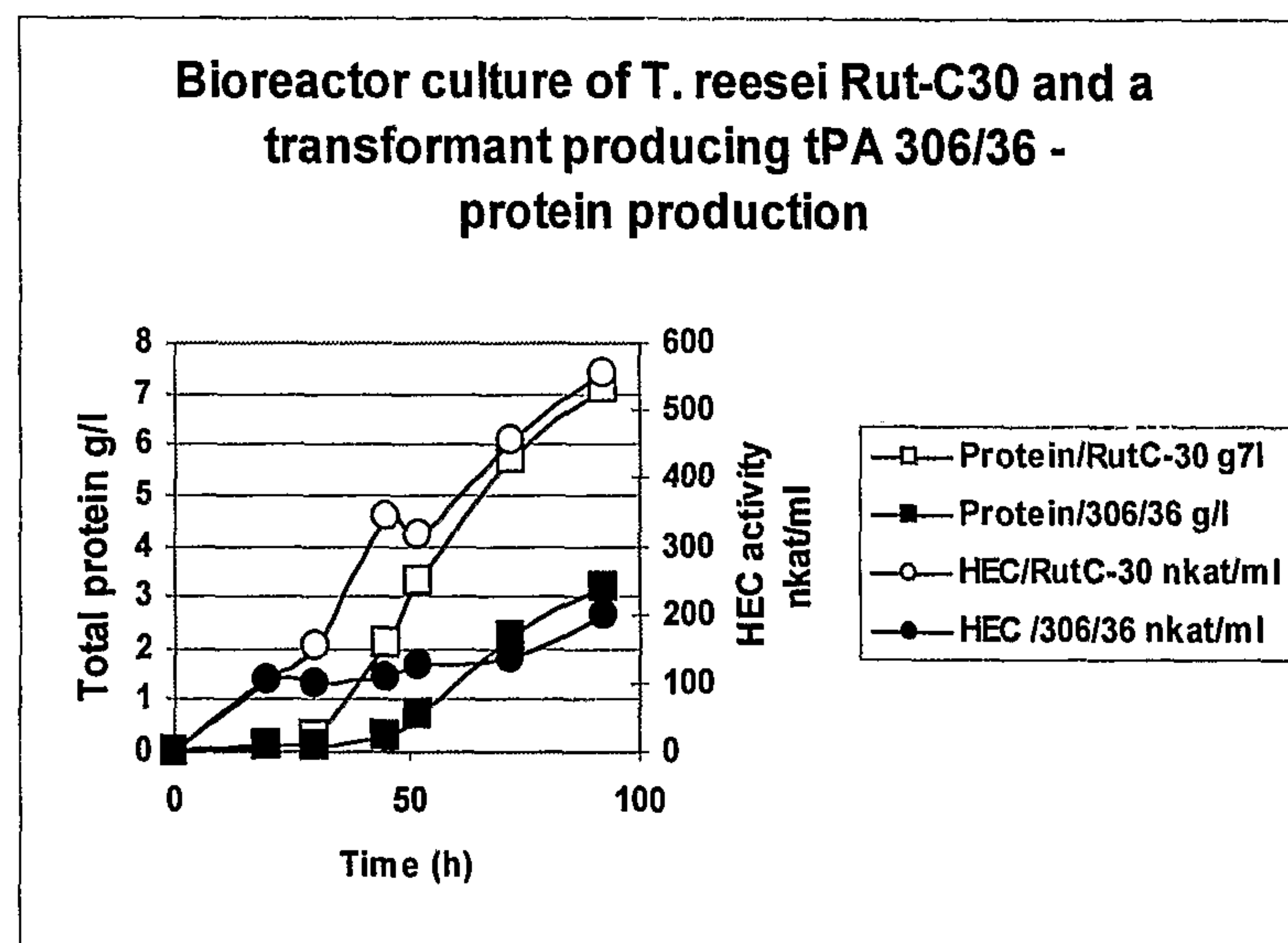


Fig 14C



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Fig 14D

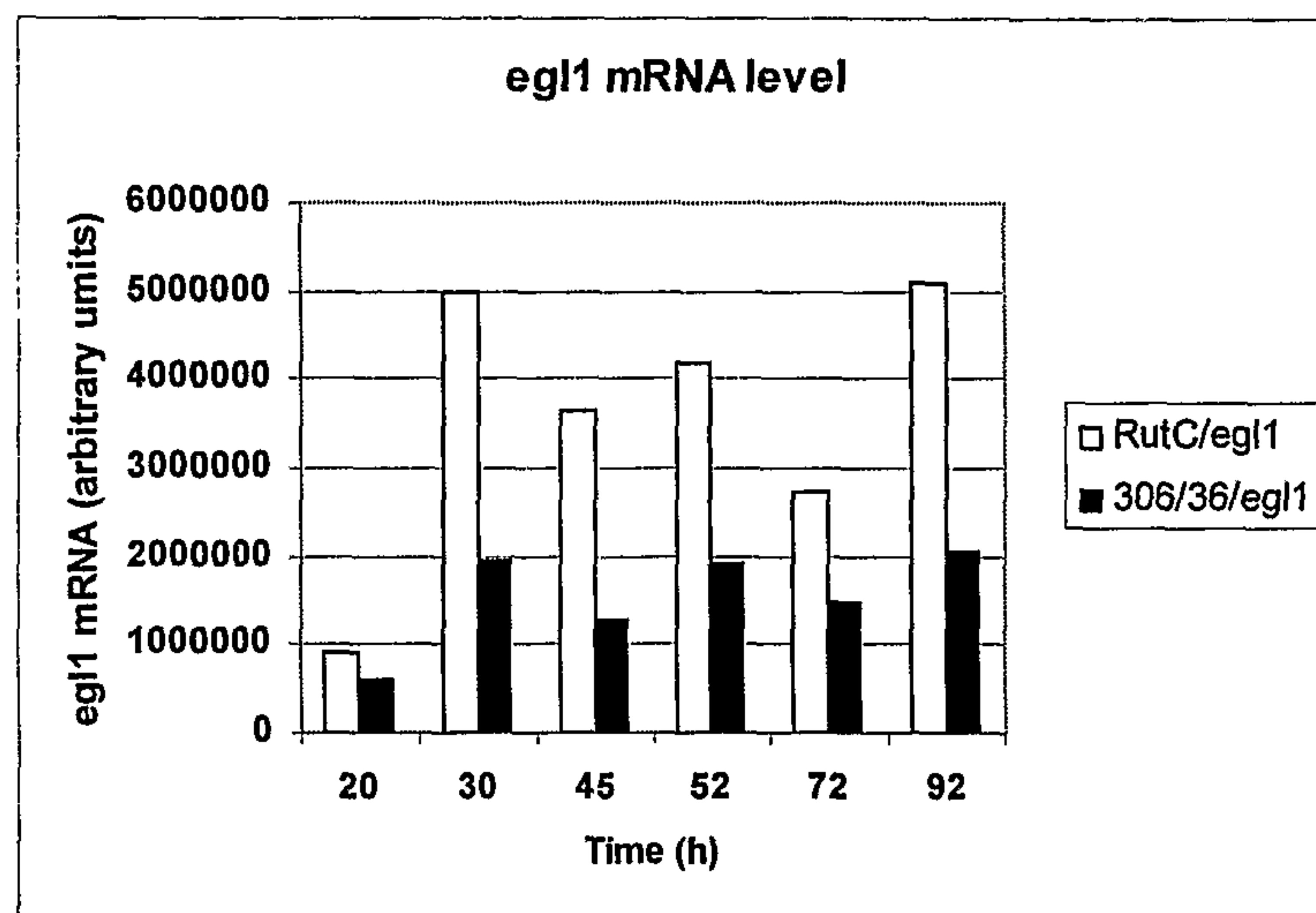


Fig 14E

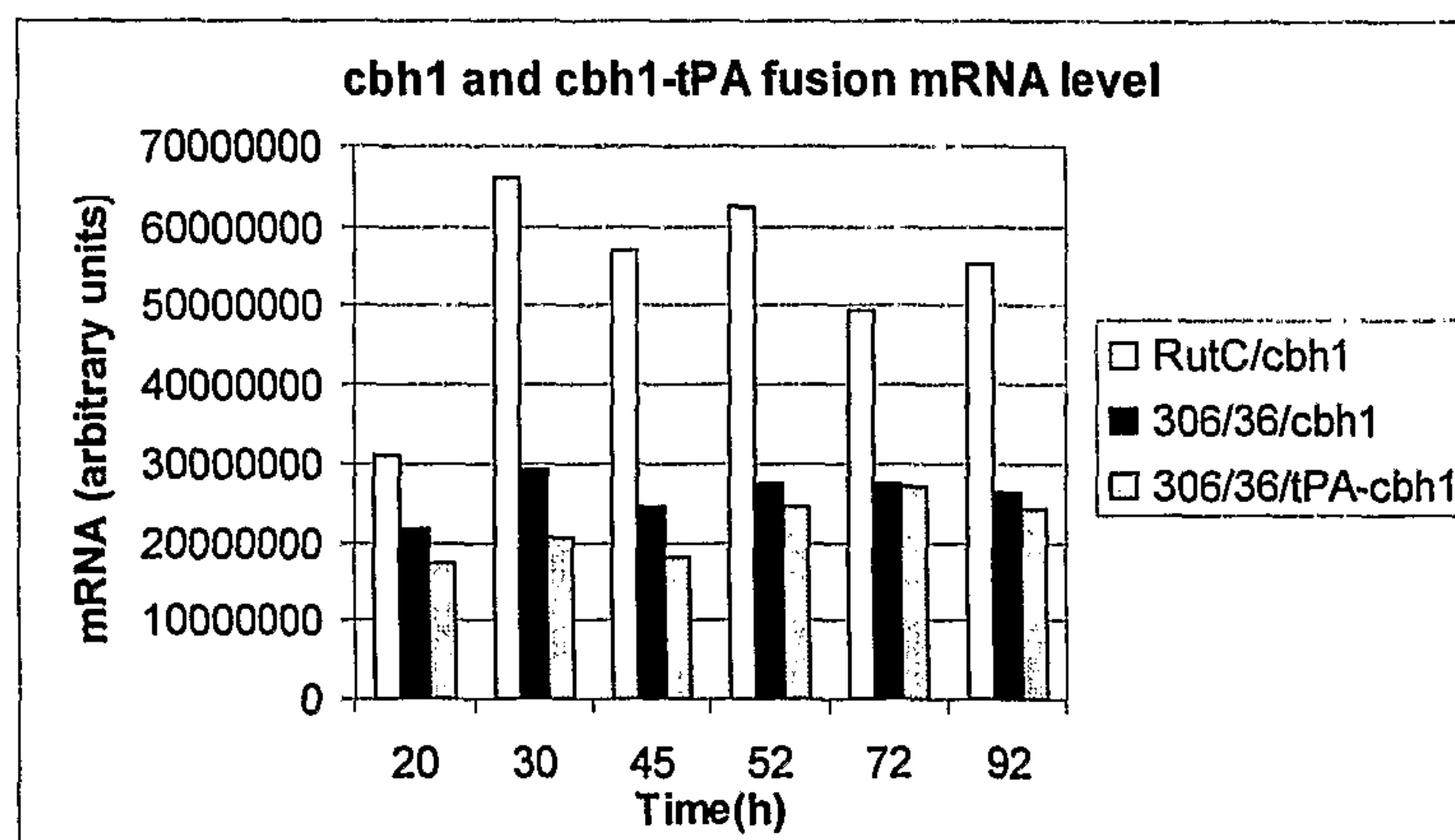
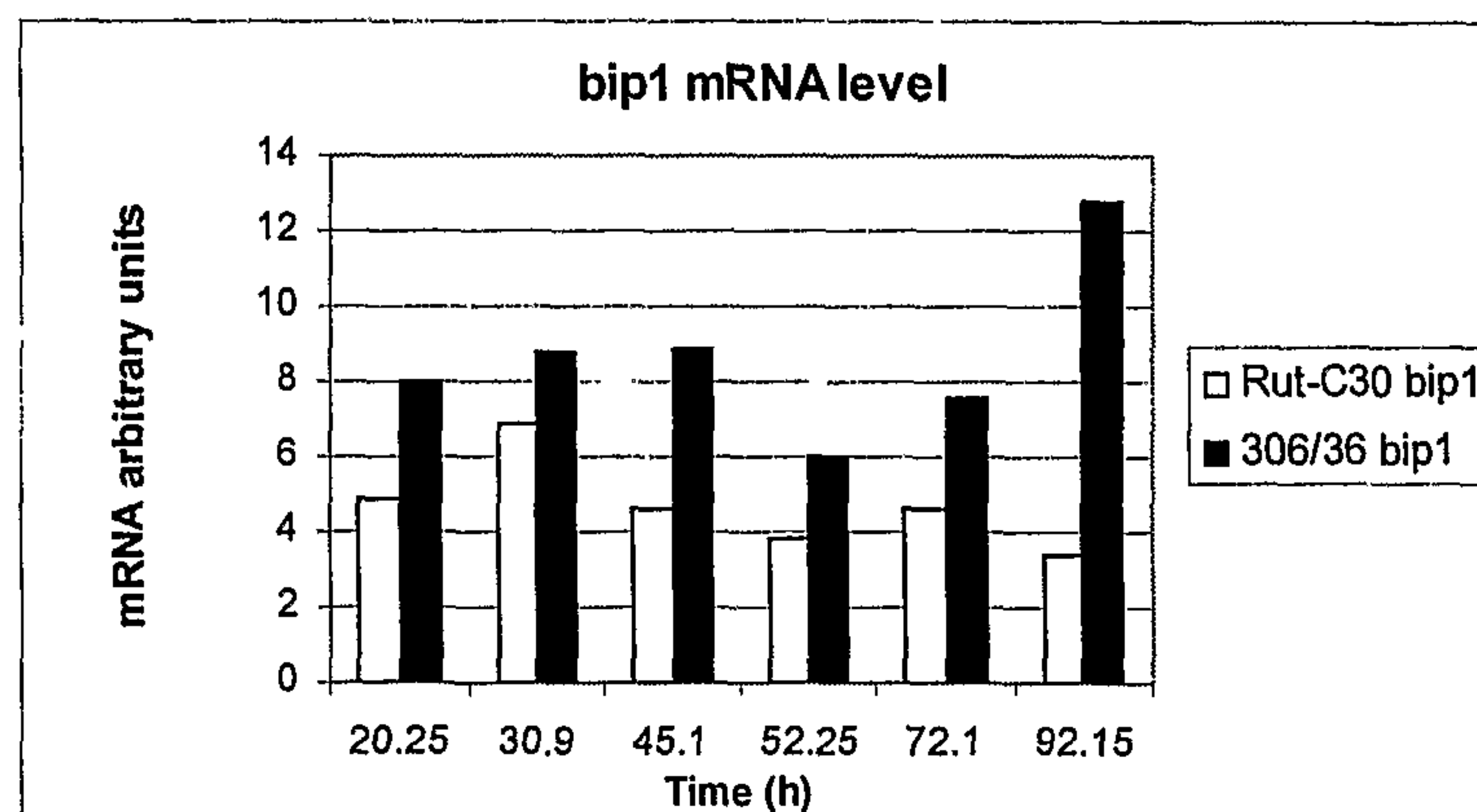
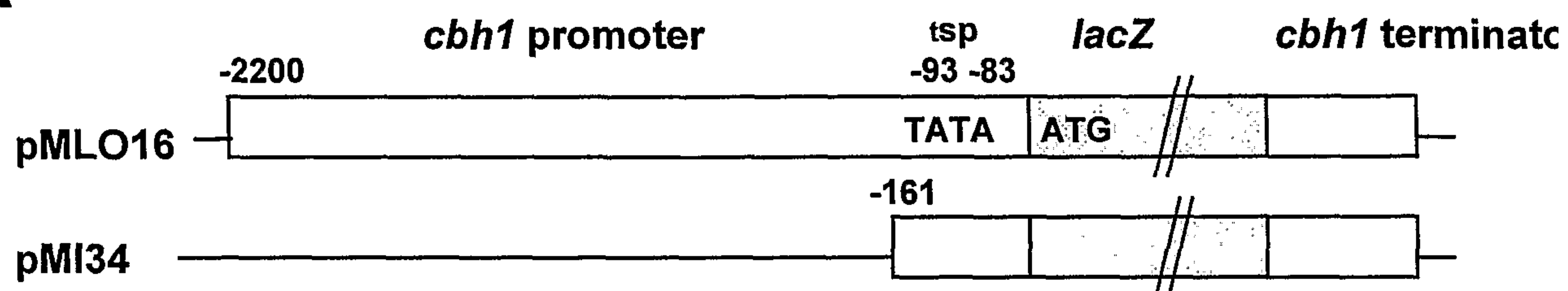
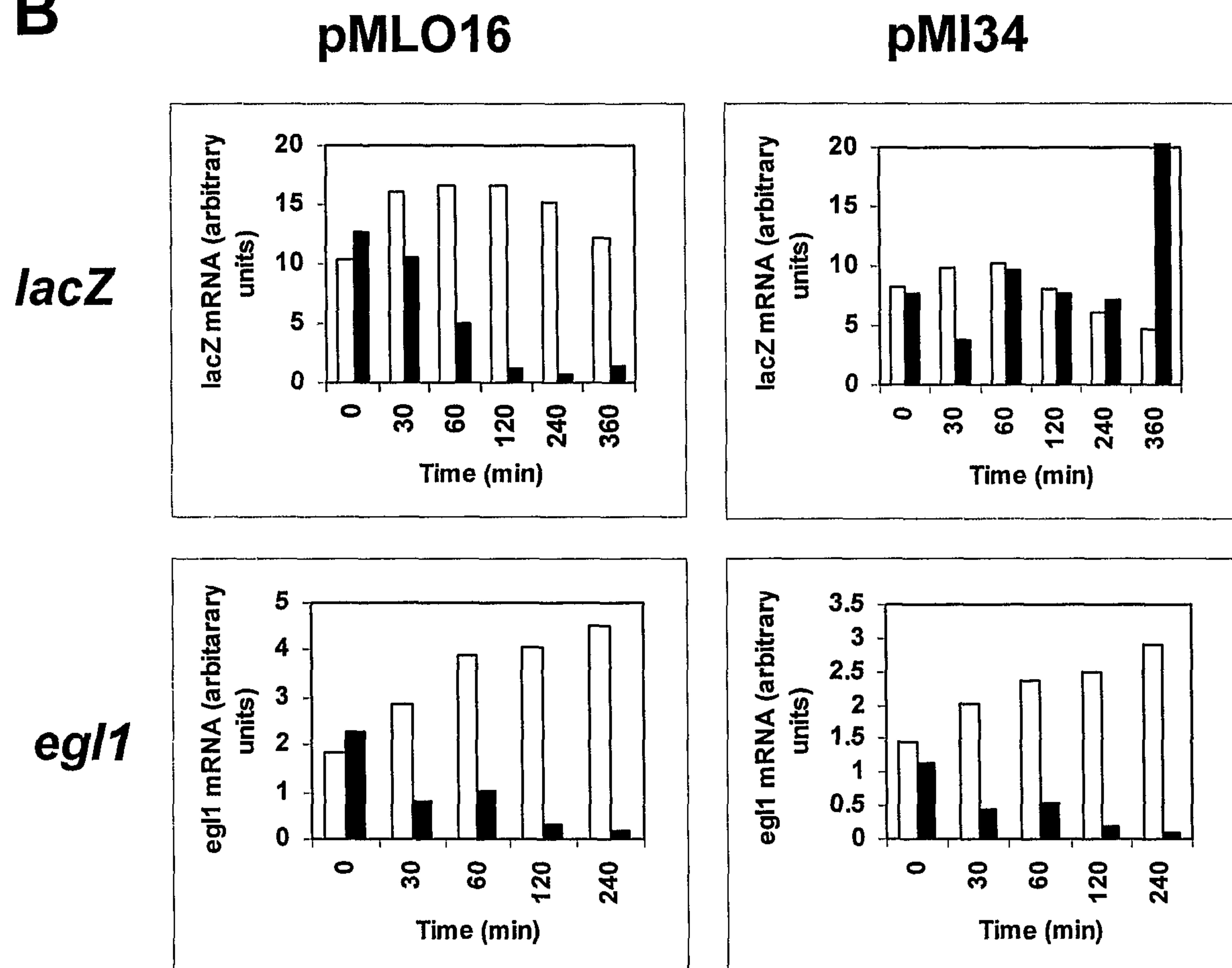


Fig 14F



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A**B****Fig. 15**

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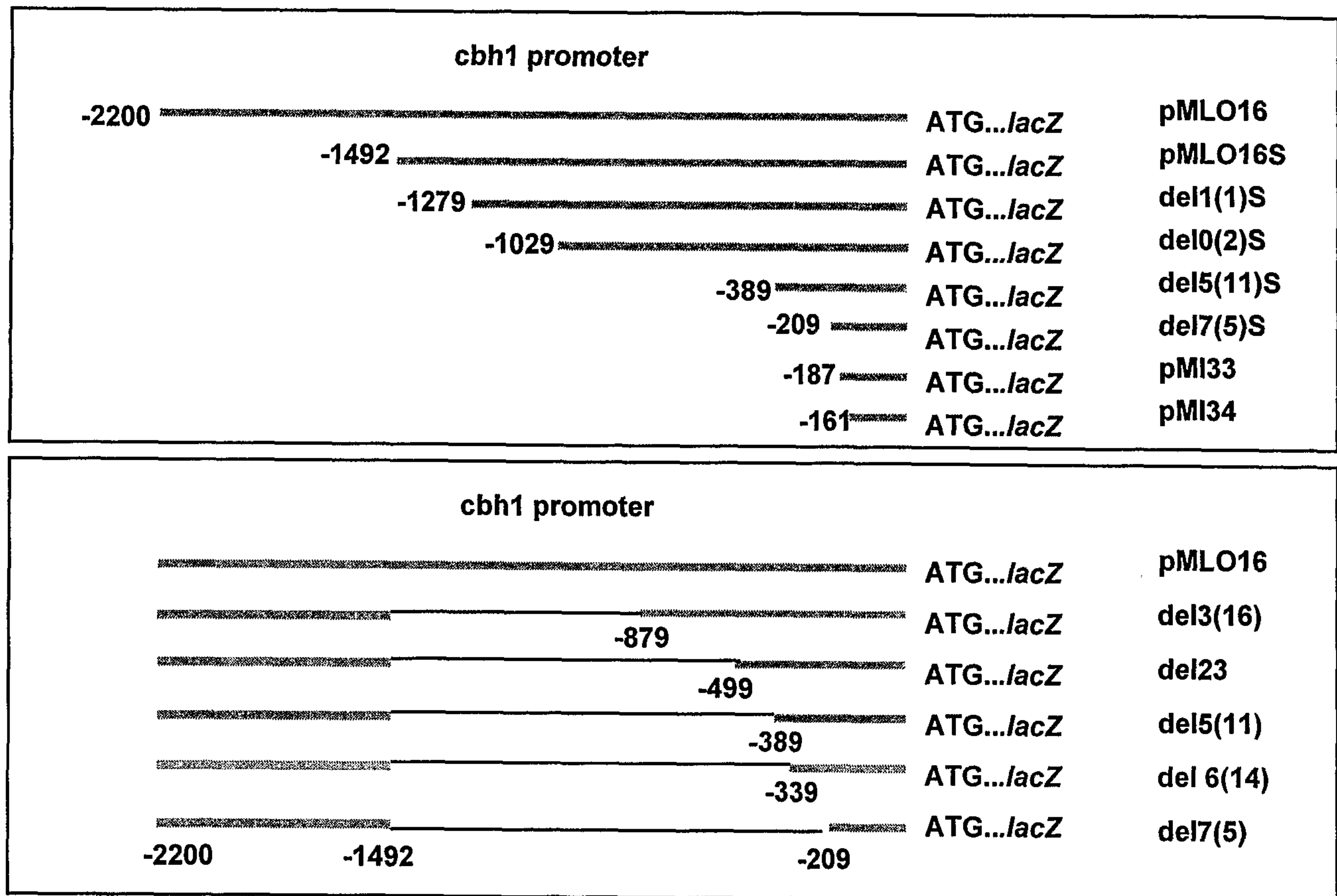


Fig. 16A

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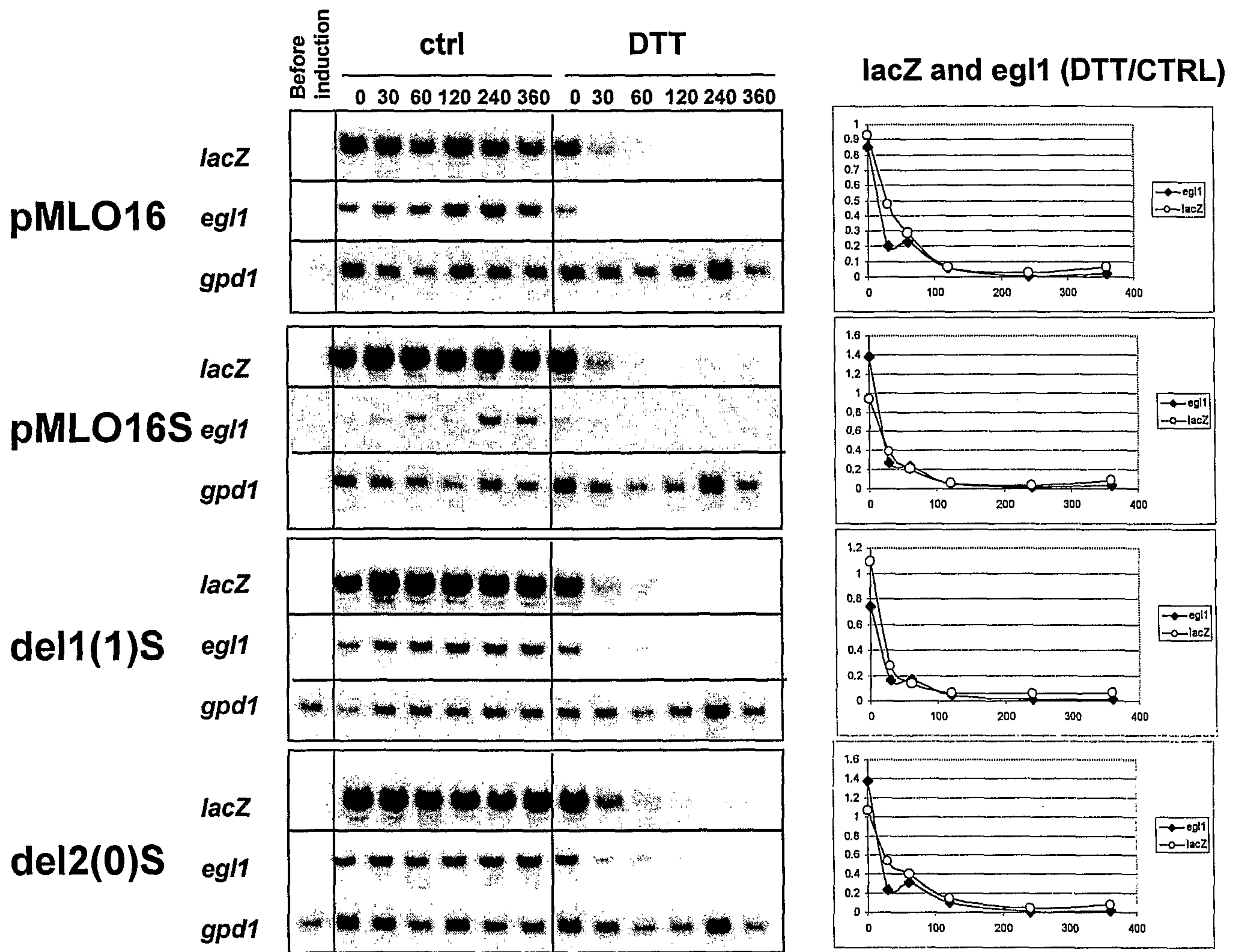


Fig. 16B

	Before induction	ctrl							DTT						
		0	30	60	120	240	360	0	30	60	120	240	360		
<i>lacZ</i>															
<i>egl1</i>															
<i>gpd1</i>															
<i>lacZ</i>															
<i>egl1</i>															
<i>gpd1</i>															
<i>lacZ</i>															
<i>egl1</i>															
<i>gpd1</i>															

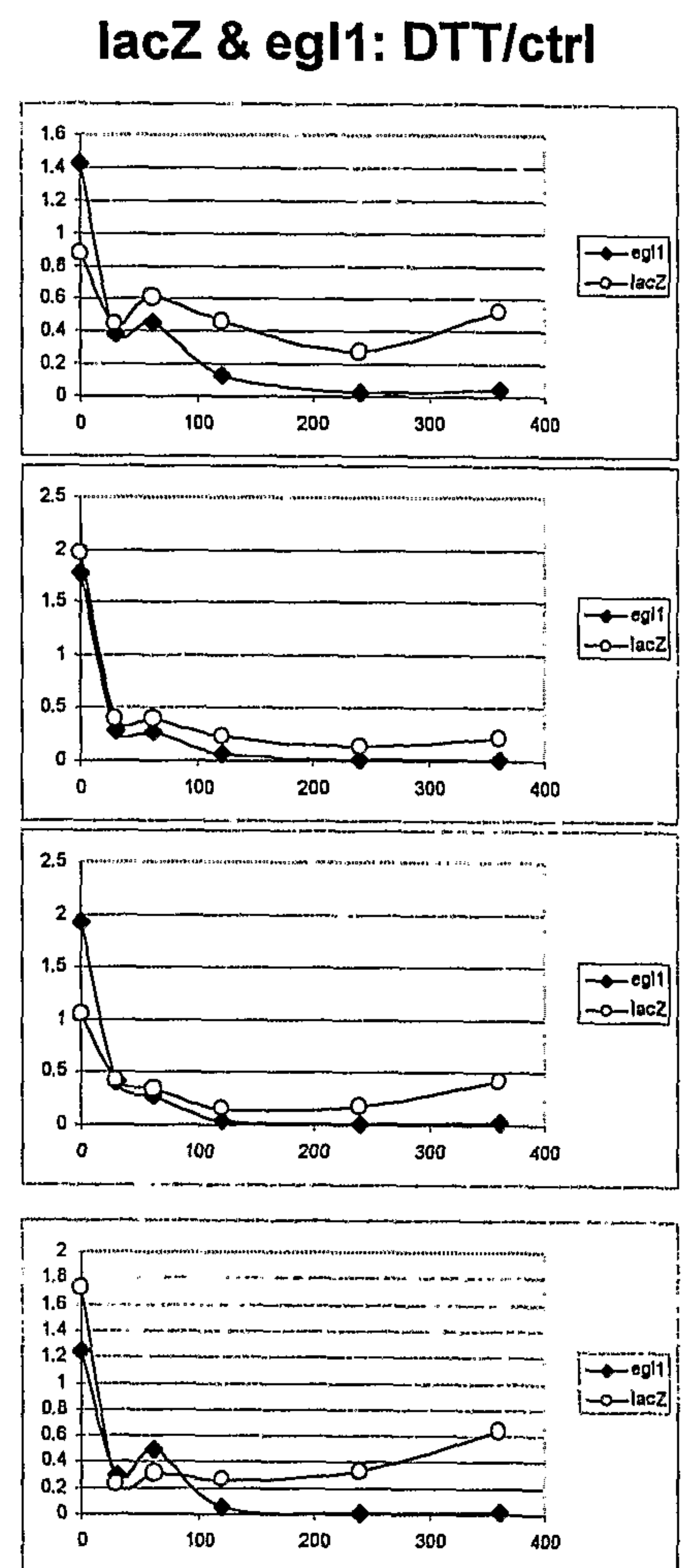


Fig 16C

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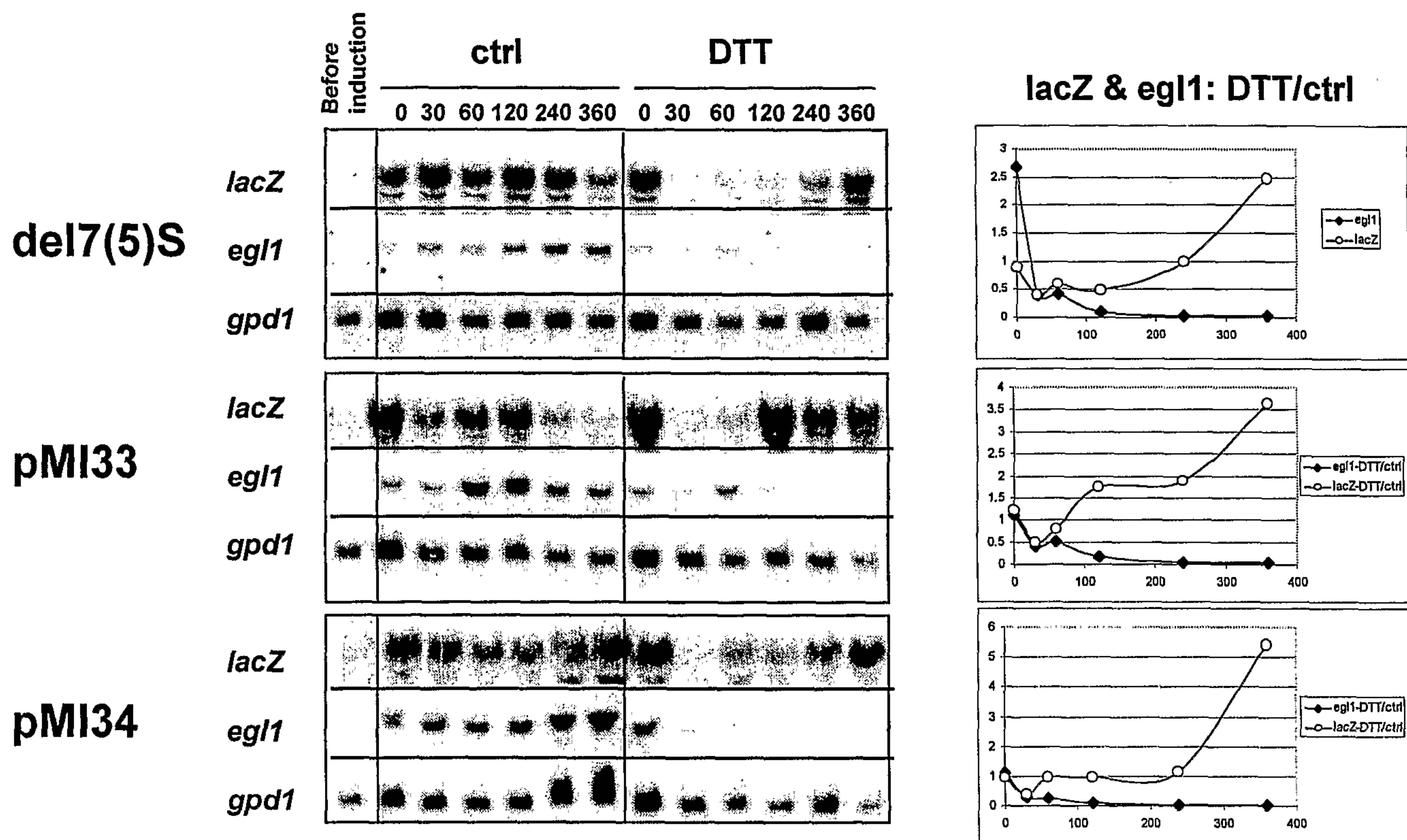
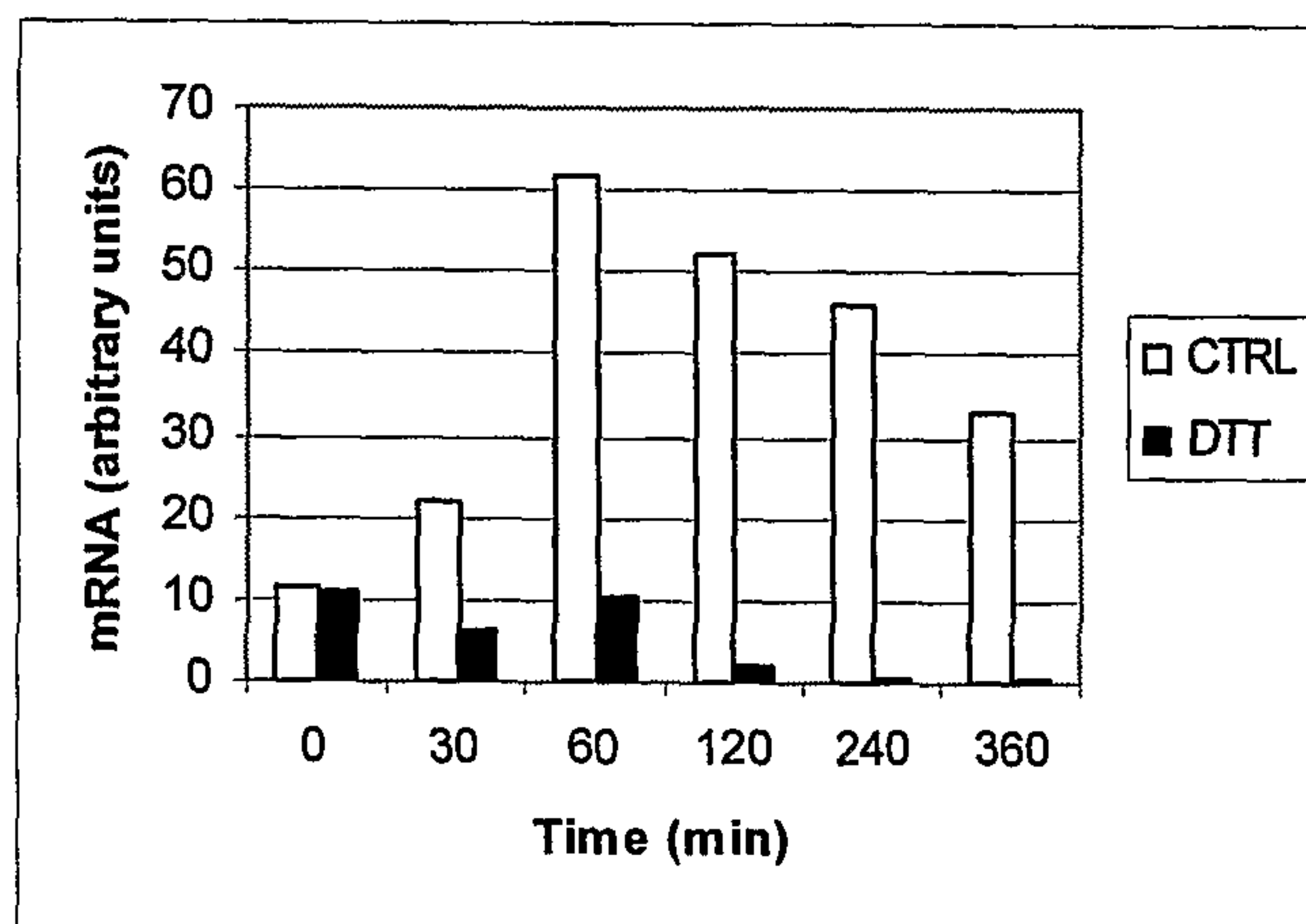
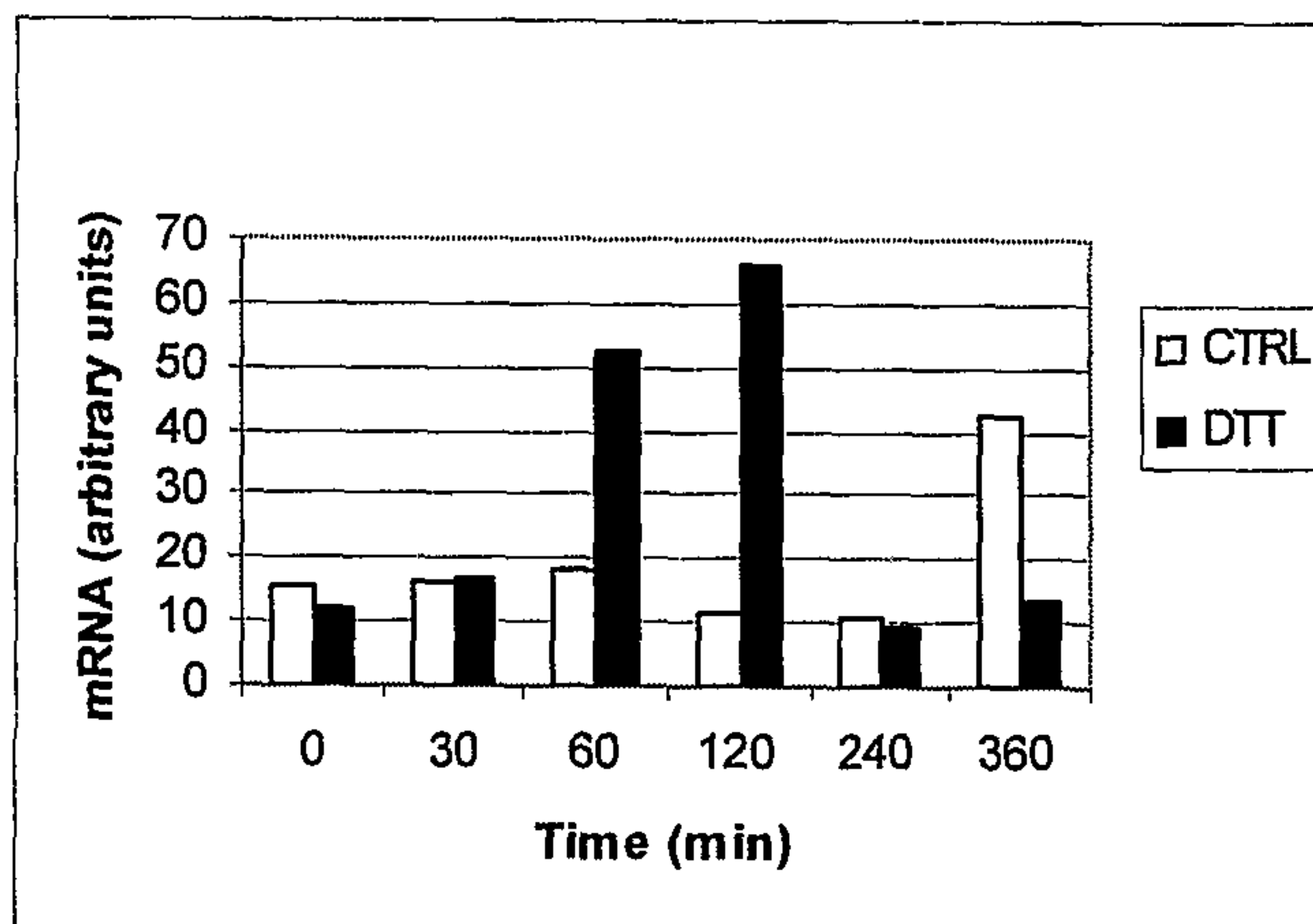


Fig. 16D

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***cbh1* mRNA level
(normalised with *gpd* signal)**

QM9414**QM9414
 $\Delta ace1$** **Fig. 17**

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Fig. 18A

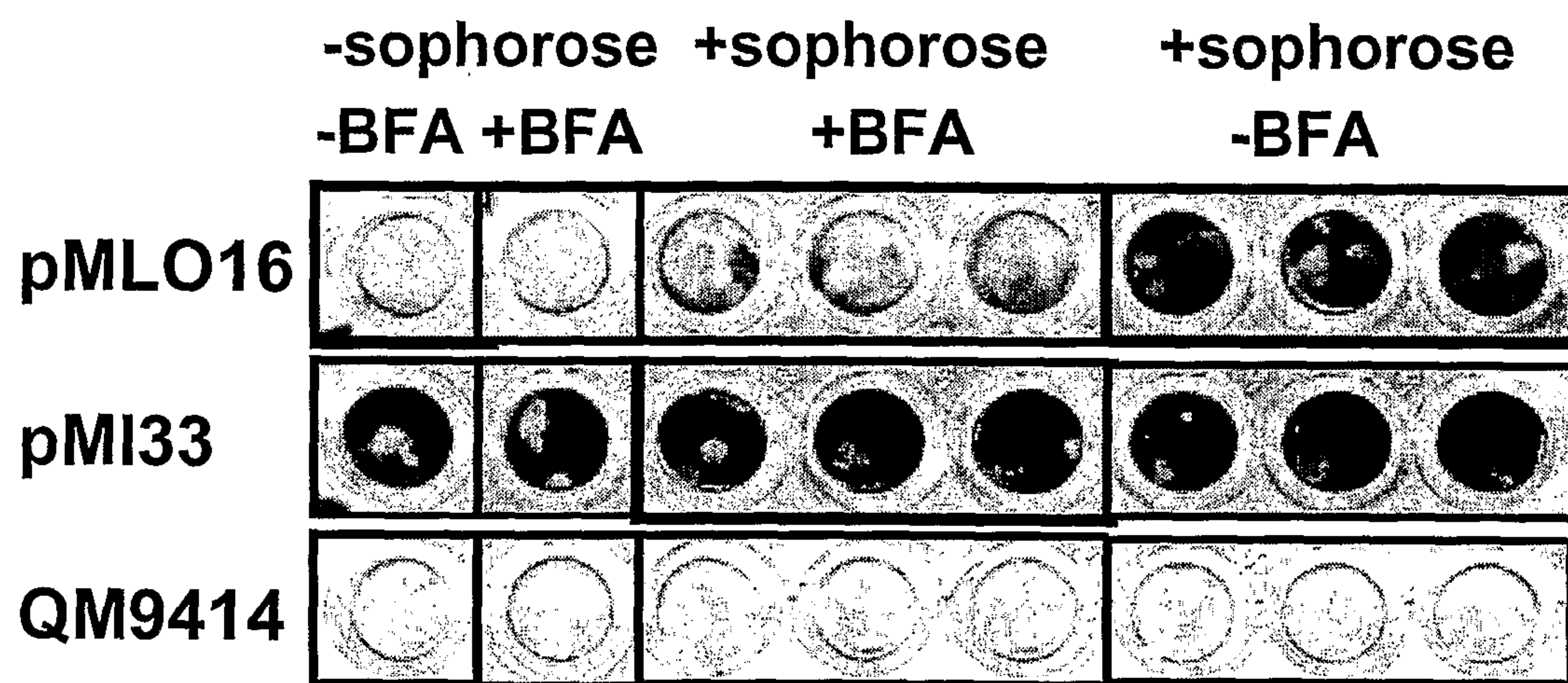


Fig. 18B

