Isoprenaline-induced transcription of 4 β -galactosyltransferase is inhibited by both cycloheximide and actinomycin D in rat parotid acinar cells

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A cDNA clone for the Golgi enzyme 4 β -galactosyltransferase (EC 2.4.1.38) was used to determine the steady-state mRNA content in cultured rat parotid acinar cells. Isoprenaline, a β -adrenergic-receptor agonist, caused an increase in steady-state amounts of mRNA for 4 β -galactosyltransferase in cultured acinar cells as well as in specific activity of the enzyme. The amount of 4 β -galactosyltransferase-specific mRNA was dependent on transcription of the gene, as determined by incubation of cells with the RNA polymerase inhibitor actinomycin D, concomitant with the time of isoprenaline treatment. Transcription of the 4 β -galactosyltransferase gene also required the active biosynthesis of additional cellular factors, since isoprenaline-induced increases in mRNA amounts were not observed on co-incubation with the protein-synthesis inhibitor cycloheximide.

INTRODUCTION

Glycoproteins are present in essentially all biological sources from viruses to higher animals, and fulfil a variety of biological functions. Certain cellular functions require the oligosaccharide portion of glycoproteins, including receptor-mediated pinocytosis by the galactose receptor in liver, in which recognition of the carbohydrate moiety of circulating glycoproteins determines their removal from the circulation (Struck & Lennarz, 1980). The proper intracellular compartmentalization of lysosomal hydrolases also depends on the structure of the oligosaccharide moiety of these glycoproteins (for review, see von Figura & Hasilik, 1986). In comparisons of transformed cells with normal cells, there appear to be alterations on the outer chains of oligosaccharide structures of cell-surface glycoproteins and also changes in the activities of the enzymes responsible for catalysing the transfer of these sugar moieties (Chatterjee & Kim, 1977; Patt *et al.*, 1975).

4 β -Galactosyltransferase (EC 2.4.1.38) is the most studied of the galactosyltransferases involved in glycoprotein biosynthesis. This enzyme is involved in the addition of galactose to *N*-acetylglucosamine (in a β 1,4-anomeric configuration) of glycolipids, proteoglycans and *O*- and *N*-linked glycoprotein oligosaccharides. 4 β -Galactosyltransferase is usually membrane-associated, although soluble forms of the enzyme have been found in milk (Brew *et al.*, 1968), serum (Fujita-Yamaguchi & Yoshida, 1981) and saliva (Humphreys-Beher *et al.*, 1984). Membrane-associated galactosyltransferase has classically been localized to the Golgi apparatus (Schachter & Roseman, 1980; Smith & Brew, 1977), but a portion of the cellular activity has been found at the cell surface (Roth *et al.*, 1971; Pierce *et al.*, 1980). Numerous reports have shown that alteration of cell-surface galactosyltransferase activity might be involved in cell adhesion (Roth *et al.*, 1971; Roseman, 1970), recognition (Pierce *et al.*, 1980), differentiation (Weiser, 1973) and

embryogenesis (Shur, 1982). The correlation of altered galactosyltransferase activities is particularly intriguing, because of their possible role in transformation via alterations in cell-surface recognition (Roth *et al.*, 1977; Higgins & Anderson, 1981; Klohs *et al.*, 1982).

The chronic administration of isoprenaline (a β -adrenergic-receptor agonist) results in physiological changes, which include parotid-gland cell hypertrophy and hyperplasia. At the molecular level, DNA, RNA and protein syntheses are all increased after isoprenaline administration, probably due in part to the mediation of cyclic nucleotides (Barka, 1965; Grand & Schay, 1978; Ludford & Talamo, 1983), since the addition of a β -antagonist blocks these changes. The introduction of high doses of phosphodiesterase inhibitors such as theophylline and caffeine (Wells & Humphreys-Beher, 1985) as well as certain hormones (Medina *et al.*, 1984; Imai *et al.*, 1982) will also cause parotid-gland hypertrophy and changes in parotid-gland gene expression. Such agents cause an increase in enzyme activity for *N*-acetylglucosamine 4 β -galactosyltransferase, even though a second enzyme activity, *N*-acetylgalactosamine 3 β -galactosyltransferase (or mucin transferase), remains unchanged (Humphreys-Beher *et al.*, 1984).

We have previously shown that isoprenaline causes increased synthesis of the mRNA for the 4 β -galactosyltransferase as well as causing topographical re-arrangements of the enzyme and the secretion of active enzyme into saliva (Humphreys-Beher *et al.*, 1984, 1986, 1987). In the present report I have examined the effects of inhibitors of protein and RNA synthesis on steady-state amounts of mRNA for 4 β -galactosyltransferase in tissue cultures of rat parotid acinar cells *in vitro*. As has been previously shown *in vivo* with isoprenaline treatment, an increase in both enzyme specific activity and mRNA amounts for 4 β -galactosyltransferase results from the incubation of acinar cells with this drug. The increase in steady-state amounts of 4 β -galactosyltransferase mRNA appears to be dependent not only on transcription by

cellular polymerases but also on the presence of protein factors that positively influence mRNA transcription rates in acinar cells in response to isoprenaline.

EXPERIMENTAL

Materials

($\pm$)-Isoprenaline, cycloheximide, actinomycin D, bovine submaxillary mucin, UDP-galactose, ovalbumin, collagenase and hyaluronidase were purchased from Sigma Chemical Co. UDP-[1- 14 C]galactose (300 mCi/mmol) was purchased from New England Nuclear. [α - 32 P]dCTP and dTTP (800 Ci/mmol) were purchased from Amersham. All other culture reagents were from Gibco. Matrigel biomatrix was purchased through Collaborative Research. All reagents for agarose-gel electrophoresis were of ultrapure quality from BRL. Wistar-strain rats were purchased from Charles River Breeding Farms. All other chemicals were of reagent-grade quality and purchased from commercial sources. The tubulin cDNA probe was generously given by Dr. Vincent Kidd, Department of Cell Biology and Anatomy, University of Alabama at Birmingham.

Acinar-cell culture

Intact parotid acinar cells from rats were freed from connective tissue by using collagenase and hyaluronidase as described by Oliver *et al.* (1987). Approx. 10^6 cells were cultured per 30 mm well coated with the Matrigel polymer, by the methods of Oliver *et al.* (1987), including the use of 10% (v/v) heat-inactivated rat serum. Cells were allowed to attach to the gel matrix for 48 h before application of agents involved in promoting or inhibiting 4 β -galactosyltransferase gene expression. At zero time (48 h), cells were given fresh media containing 1 μ M-isoprenaline. In addition, other groups of cells received 20 μ g of cycloheximide/ml or 4 μ g of actinomycin D/ml (with or without the addition of isoprenaline) to the culture media (Linquest, 1980; Greenberg & Perry, 1971). Finally, a third group of cells received actinomycin D or cycloheximide for 3 h, were washed free of the inhibitors, and were then incubated for an additional 3 h in fresh media containing isoprenaline to re-initiate 4 β -galactosyltransferase transcription.

In a separate series of experiments, the degradation of 4 β -galactosyltransferase mRNA was measured over a 3 h period after the induction of active isoprenaline-induced transcription by a prior 3 h incubation in media containing 1 μ M-isoprenaline. After the incubation periods described above, cells were lysed into a guanidine isothiocyanate mixture for RNA isolation (Chirgwin *et al.*, 1979). For galactosyltransferase enzyme assays, the cells were rinsed of media with phosphate-buffered saline (Oliver *et al.*, 1987). The cells were then liberated from the surface of plastic dishes with 1.0 ml of phosphate-buffered saline containing 0.5% Triton X-100. The resulting slurry was removed by Pasteur pipette, and a protein assay performed by the modified Lowry assay of Schacterle & Pollack (1983). Bovine serum albumin was used as a protein standard.

Galactosyltransferase assays

The activity of 4 β -galactosyltransferase was measured as previously described by Humphreys-Beher *et al.* (1984). In brief, cell lysates were obtained as described

above and resuspended to give a final protein concentration of 200 μ g/ml. The assay mixture (total volume 50 μ l) contained 0.1 M-Mes (pH 6.3), 25 mM-MnCl₂, 0.5% Triton X-100, 1 mM-UDP[1- 14 C]galactose (2 mCi/mmol), 10 mM-ovalbumin (for 4 β -transferase) or asialo-(bovine submaxillary mucin) (for 3 β -transferase) as acceptor, and 0–0.2 mg of the cell lysate. After incubation for 1 h, the reaction was terminated by the addition of 1 ml of ice-cold 10% (v/v) trichloroacetic acid, and the precipitate was recovered on glass-fibre filters.

Northern and dot-blot analysis of 4 β -galactosyltransferase RNA

Parotid-gland total RNA was recovered by ultracentrifugation in a Beckman SW50.1 rotor as described previously (Humphreys-Beher *et al.*, 1984). Poly(A)-containing RNA was recovered after oligo(dT)-cellulose chromatography (Aviv & Leder, 1972). The 10 μ g of denatured poly(A)-containing RNA was electrophoresed on a formaldehyde denaturing gel, and the RNA was transferred to nitrocellulose filters as described by White & Bancroft (1980). Hybridization was performed at 37 °C with 2.5×10^7 c.p.m. of nick-translated 32 P-labelled 4 β -galactosyltransferase (Humphreys-Beher *et al.*, 1986) or β -tubulin (Cowan *et al.*, 1981; Cleveland *et al.*, 1980) cDNA probes.

For RNA dot-blot analysis, 30 μ g portions of formaldehyde-denatured total RNA from control and experimental parotid-gland acinar-cell cultures were spotted on nitrocellulose filters with a Schleicher and Schuell mini-fold apparatus as described by White & Bancroft (1980). The filters were then probed with the 32 P-labelled nick-translated human 4 β -galactosyltransferase cDNA (Humphreys-Beher *et al.*, 1986; Bunnell *et al.*, 1987). Conditions of the hybridization and autoradiography were as described previously (Chandra *et al.*, 1983; Humphreys-Beher *et al.*, 1986).

RESULTS

Chronic administration of the β -adrenergic-receptor agonist isoprenaline in rats causes a 6–10-fold increase in both the specific activity and steady-state amounts of mRNA for the Golgi-membrane marker enzyme 4 β -galactosyltransferase (Humphreys-Beher *et al.*, 1984, 1986). To investigate further the isoprenaline-mediated regulation of gene expression for this enzyme, parotid-gland acinar cells were isolated from rats and cultured *in vitro* by the method of Oliver *et al.* (1987). The cells were cultured at 37 °C for 48 h to allow attachment to the gel matrix. After this time fresh medium containing 1 μ M-isoprenaline was applied to the cells, and incubation was continued for 3 h before harvesting. As shown in Table 1, the addition of isoprenaline to the culture media resulted in a 6-fold increase in specific activity for the 4 β -galactosyltransferase enzyme. A second galactosyltransferase enzyme activity, the 3 β -galactosyltransferase (no EC number), remained unchanged in the presence of isoprenaline. This same result has previously been shown from whole-animal studies with chronic isoprenaline treatment of parotid glands (Humphreys-Beher, 1985). The introduction of the transcriptional inhibitor actinomycin D or the protein translation inhibitor cycloheximide along with isoprenaline prevented the increase in 4 β -galactosyltransferase activity (Table 1). The concentration of inhibitor used in each case was determined

Table 1. 4 β - and 3 β -Galactosyltransferase activities in isoprenaline-treated rat acinar cells *in vitro*

Specific activity is expressed as nmol of galactose incorporated/h per mg of protein (mean $\pm$ S.D.) for 5×10^6 cells. Four wells of cells were assayed for each experimental conditioning. The 4 β -transferase was determined with ovalbumin as macromolecular acceptor, and the 3 β -transferase with asialo-(bovine submaxillary mucin) as macromolecular acceptor.

Treatment	Specific activity	
	4 β -Galactosyl-transferase	3 β -Galactosyl-transferase
Control	0.13 $\pm$ 0.02	1.73 $\pm$ 0.16
IPR*	0.87 $\pm$ 0.09	1.81 $\pm$ 0.15
Control + cyc†	0.11 $\pm$ 0.01	1.37 $\pm$ 0.14
Control + act D†	0.16 $\pm$ 0.02	1.62 $\pm$ 0.15
IPR + cyc‡	0.10 $\pm$ 0.02	1.70 $\pm$ 0.17
IPR + act D‡	0.10 $\pm$ 0.03	1.75 $\pm$ 0.10
IPR§	0.98 $\pm$ 0.12	1.83 $\pm$ 0.13
IPR + cyc/IPR§	0.75 $\pm$ 0.09	1.80 $\pm$ 0.14
IPR + act D/IPR§	0.97 $\pm$ 0.10	1.78 $\pm$ 0.10

* Cells were incubated for 3 h in the presence of 1 μ M-isoprenaline (IPR) before harvest.

† Cells were incubated in media containing either 20 μ g of cycloheximide (cyc)/ml or 4 μ g/ml of actinomycin D (act D)/ml for 3 h before harvest.

‡ Cells were incubated in media containing either 20 μ g of cycloheximide/ml or 4 μ g of actinomycin D (act D)/ml in addition to isoprenaline for 3 h before harvest.

§ Cells were incubated in isoprenaline or isoprenaline with the addition of the above inhibitors. After 3 h fresh medium containing isoprenaline was introduced for all three sets of cells, and incubation at 37 °C was continued for an additional 3 h before harvesting as described in the Experimental section.

by separate dose-response experiments. The concentrations therefore used in these studies reflect those which provided maximal inhibition of RNA or protein synthesis, but were not toxic to rat acinar cells for the 3 h incubation period (results not shown). The addition of either cycloheximide or actinomycin D to control cells had little effect on either galactosyltransferase activity after 3 h. This is most likely the result of the slow rate of turnover for these enzymes by the cell. Strous & Berger (1982) have previously shown the half-time for [35 S]methionine-labelled 4 β -galactosyltransferase disappearance from HeLa cells to be 19 h.

The removal of cycloheximide or actinomycin D from the culture media and replacement with media containing only isoprenaline caused the restoration of the increase in 4 β -galactosyltransferase after an additional 3 h incubation. There was no additional increase in specific activity for 4 β -galactosyltransferase in acinar cells which had been cultured in the presence of isoprenaline for a total of 6 h compared with cells assayed after 3 h in the presence of isoprenaline.

Confirmation that the increased synthesis of 4 β -galactosyltransferase was due to an isoprenaline-mediated increase in gene expression was obtained by Northern hybridization of parotid acinar-cell poly(A)-containing RNA with the cDNA clone for 4 β -galactosyltransferase. When 5 μ g of poly(A)-containing RNA

Fig. 1. Northern-blot analysis of poly(A)-containing RNA from control and isoprenaline-treated rat parotid acinar cells in culture

Lane C, control RNA; lane I, 3 h-isoprenaline-treated acinar cells. For the isolation of poly(A)-containing RNA, 10^9 cells were isolated from culture after 48 h. (a) Hybridization of the cDNA probe to 4 β -galactosyltransferase; (b) hybridization pattern of the β -tubulin cDNA probe used as a control.

from control and isoprenaline-treated acinar cells in culture was probed with a human 4 β -galactosyltransferase cDNA (Humphreys-Beher *et al.*, 1986), a 4-fold increase was observed in the steady-state amount of a single 4 β -galactosyltransferase mRNA species approx. 1.7 kb in size. The increase in the amount of 1.7 kb species was confirmed by densitometer scanning (Fig. 1). This result is similar to the 6-fold increase observed by direct enzyme assay. The autoradiograph for galactosyltransferase cDNA hybridization was overexposed in an attempt to identify the existence of any other mRNA species that may have been induced by isoprenaline. The same RNAs were also probed with a β -tubulin cDNA probe as a control (Cowan *et al.*, 1981; Cleveland *et al.*, 1980), and equivalent hybridization signals were seen in both samples (Fig. 1). The identification of a single induced mRNA species hybridizing with the 4 β -galactosyltransferase cDNA would indicate that the increased enzyme activity for this transferase is the result of increased steady-state amounts of the mRNA from a single gene.

To evaluate further the response of 4 β -galactosyltransferase gene expression as a consequence of exposure of acinar cells to isoprenaline, steady-state amounts of mRNA for the transferase were examined separately in the presence of the protein-synthesis inhibitor cycloheximide and the transcription inhibitor actinomycin D. The results of these studies are presented in Fig. 2. When acinar cells were cultured in media containing actinomycin D concomitant with the addition of isoprenaline, no increase in steady-state amounts of specific 4 β -galactosyltransferase was observed. After 3 h there was a noted decline in hybridization as compared with the control cells (Fig. 2). This was most likely due to

Fig. 2. RNA dot-blot analysis of isoprenaline-treated rat parotid acinar cells

(a) Hybridization of the 4 β -galactosyltransferase cDNA probe to 30 μ g of total RNA isolated from: (1) control cells; (2) cells cultured in the presence of isoprenaline for 3 h; (3) cells cultured in the presence of isoprenaline/cycloheximide for 3 h; (4) cells cultured in the presence of isoprenaline/actinomycin D for 3 h; (5) cells harvested after 6 h with isoprenaline; (6) cells cultured as in (3), washed free of inhibitor, and incubated for an additional 3 h in media with isoprenaline; (7) cells cultured as in (4), washed free of inhibitor, and incubated in media containing isoprenaline for an additional 3 h. (b) Time course of decay in the hybridization of the isoprenaline-induced 4 β -galactosyltransferase mRNA in the presence of cycloheximide or actinomycin D. Lane A, cells cultured in the presence of isoprenaline for 3 h, the media was then removed and cells cultured in medium without isoprenaline. The RNA was isolated from cells at the following intervals after removal of isoprenaline: (1) zero time; (2) 30 min; (3) 1 h; (4) 2 h; (5) 3 h. Lane B, introduction of the protein-synthesis inhibitor cycloheximide after 3 h with isoprenaline in the culture media. Times of sample hybridization assay were the same as for lane A. Lane C, cells cultured in the presence of the transcriptional inhibitor actinomycin D, after a 3 h preincubation with isoprenaline in the culture media. Time course of sample preparation for the hybridization assay was the same as those used in lanes A and B.

RNA degradation during the incubation period. Removal of actinomycin D and replacement with fresh media containing isoprenaline restored transcription of 4 β -galactosyltransferase mRNA. A similar decrease in steady-state 4 β -galactosyltransferase RNA was observed when cells were treated with cycloheximide in conjunction with isoprenaline. Again, the removal of the protein-synthesis inhibitor and incubation of the cells with media containing only isoprenaline resulted in increased steady-state amounts of the transferase mRNA (Fig. 2).

The rate of decline in steady-state amounts of 4 β -galactosyltransferase RNA in acinar cells induced with isoprenaline for 3 h was measured after the cells were transferred to media containing either cycloheximide or actinomycin D. As shown in Fig. 2, 4 β -galactosyltransferase mRNA was decreased as early as 30 min after the addition of the above compounds (lanes B and C). A higher rate of turnover of transferase RNA was evident for cycloheximide-treated cells compared with actino-

Fig. 3. RNA dot-blot analysis of control rat parotid acinar cells

Lane A, control cells cultured over 3 h without isoprenaline added to the growth media; lane B, incubation of acinar cells in the presence of actinomycin D; lane C, incubation of acinar cells in the presence of cycloheximide. RNA was isolated from cell lysates after the following intervals in the presence of inhibitor: (1) zero time; (2) 30 min; (3) 1 h; (4) 2 h; (5) 3 h.

mycin D-treated cells, as determined from densitometer scans (results not shown) at the 30 min time point. Confirmation of this observation was further provided by assaying the amount of label bound to the RNA at the 30 min time point. The RNA from the 30 min treatment with cycloheximide bound 4500 ± 50 c.p.m. of the 32 P-labelled probe, and the RNA isolated from the actinomycin D-treated cells bound 5250 ± 50 c.p.m. of the probe. The quantity of probe bound in control and 3 h-isoprenaline-treated cells was 5000 ± 125 c.p.m. and 22050 ± 200 c.p.m. respectively. At later times (2 or 3 h) the higher rate of mRNA turnover for 4 β -galactosyltransferase in the presence of cycloheximide was clearly evident. When neither inhibitor was included, the steady-state amount of galactosyltransferase mRNA was quite stable over 2 h, but showed a decrease in hybridization at 3 h.

In a further attempt to delineate the mechanism of regulation of 4 β -galactosyltransferase gene expression in tissue culture, cells were incubated in either actinomycin D or cycloheximide without prior treatment with isoprenaline. As shown in Fig. 3, when acinar cells were incubated in the presence of the transcriptional inhibitor actinomycin D, there was a rapid decrease in mRNA that was hybridized to the 4 β -galactosyltransferase cDNA probe after 30 min. By 1 h, and at later times, the extent of hybridization with the probe was very low. In contrast, when cells were cultured with the protein-synthesis inhibitor cycloheximide, the amount of RNA hybridizing to the cDNA probe remained constant over 3 h in the presence of inhibitor. Since the mRNA amount did not increase, it appears that the increase in steady-state mRNA amounts observed after isoprenaline treatment was not due to an increase in mRNA stability, which would reflect a decrease in the rate of mRNA turnover.

DISCUSSION

In the above experiments the protein-synthesis inhibitor cycloheximide was shown to block increased steady-state amounts of mRNA for 4 β -galactosyltransferase

when added to acinar cells at the time of isoprenaline treatment. This result suggests that increased RNA production from this gene requires the positive influence of protein factors other than RNA polymerase that are affected by isoprenaline. The decline in steady-state amounts of 4 β -galactosyltransferase mRNA in the presence of cycloheximide after isoprenaline induction indicates the continuous need for the protein modifier to allow for the accumulation of galactosyltransferase mRNA.

Isoprenaline imparts its influence on acinar-cell function through the second messenger cyclic AMP. The activation of adenylate cyclase by isoprenaline results in a large number of cellular changes that are affected by the corresponding increase in cyclic AMP. Therefore, it may be postulated that the increased steady-state amounts of 4 β -galactosyltransferase mRNA after isoprenaline treatment result from alterations in the rate of mRNA turnover. The introduction of the protein-synthesis inhibitor cycloheximide may prevent the synthesis of a factor involved in mRNA degradation. The addition of isoprenaline to acinar cells results in the biosynthesis, or increases the half-life, of a cellular factor that regulates the rate of 4 β -galactosyltransferase mRNA turnover. Cycloheximide would interfere with the synthesis of the protein which would need to be present continuously to affect mRNA stability. Since the presence of cycloheximide in control cell cultures did not result in an increase in steady-state mRNA amounts, this interpretation does not appear to be valid.

Cyclic AMP has been shown to cause transcriptional changes in a number of cellular genes, which in parotid-gland acinar cells include the proline-rich-protein gene family (Ann & Carlson, 1985). Unlike the activation of transcription by steroid hormones (Yamamoto, 1985), β -agonists do not traverse the plasma membrane coupled to β -adrenergic receptors. The activation of RNA transcription may reside in the direct interaction of either cyclic AMP or cyclic AMP-dependent DNA-binding proteins with DNA to promote gene expression in response to the agonist. For 4 β -galactosyltransferase-gene expression to occur, isoprenaline may initiate the synthesis of a tissue-specific factor that causes increased transcription from the transferase-gene promoter. There is accumulating evidence that transcriptional activity of various genes requires the presence of tissue-specific factors that bind to specific DNA sequences to exert their influence (Mercola *et al.*, 1985; Scholer & Gruss, 1984). Cell-specific DNA-binding proteins essential for enhanced gene expression may also be important in modulating other aspects of gene expression. It is interesting to note the high steady-state amount of 4 β -galactosyltransferase mRNA in the rat submandibular gland, whereas HeLa cells show a low steady-state amount of mRNA. Both cell types possess β -adrenergic receptors on the cell surface that respond to isoprenaline. However, neither cell demonstrates a change in steady-state 4 β -galactosyltransferase mRNA as a consequence of β -agonist exposure. From our results with parotid acinar cells, these observations suggest that HeLa cells may be unable to synthesize the 4 β -galactosyltransferase protein transcriptional factor while it is constitutively present in the submandibular gland. The administration of the protein-synthesis inhibitor to rats has been shown to result in a decrease in the steady-state amounts of 4 β -galactosyltransferase mRNA in the submandibular

gland after 6 h (M. G. Humphreys-Beher, unpublished work). It is clear that the characterization of DNA sequences determining cell-specific expression requires the isolation and characterization of their putative *trans*-acting protein factors.

I thank Ms. Gloria Robinson for technical assistance. Additional recognition is given to Ms. Carolyn Harris for the preparation of this manuscript. Funding for this project was from the National Cystic Fibrosis Foundation, grant DE 08439 from the National Institutes of Dental Research, and the Department of Microbiology at the University of Alabama at Birmingham.

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Received 11 March 1987/17 August 1987; accepted 17 September 1987