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Glutamine increases collagen gene transcription in cultured human fibroblasts

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Abstract

We have previously shown that glutamine stimulates the synthesis of collagen in human dermal confluent fibroblast cultures (Bellon, G. et al. [1987] Biochim. Biophys. Acta, 930, 39-47). In this paper, we examine the effects of glutamine on collagen gene expression. A dose-dependent effect of glutamine on collagen synthesis was demonstrated from 0 to 0.25 mM followed by a plateau up to 10 mM glutamine. Depending on the cell population, collagen synthesis was increased by 1.3-to 2.3-fold. The mean increase in collagen and non-collagen protein synthesis was 63% and 18% respectively. Steady-state levels of α1(I) and α1(III) mRNAs, were measured by hybridizing total RNA to specific cDNA probes at high stringency. Glutamine increased the steady-state level of collagen α1(I) and α1(III) mRNAs in a dose-dependent manner. At 0.15 mM glutamine, collagen mRNAs were increased by 1.7-and 2.3-fold respectively. Nuclear run-off experiments at this concentration of glutamine indicated that the transcriptional activity was increased by 3.4-fold for the pro α1(I) collagen gene. The effect of glutamine on gene transcription was also supported by the measurement of pro α1(I) collagen mRNA half-life since glutamine did not affect its stability. Protein synthesis seemed to be required for the glutamine-dependent induction of collagen gene expression since cycloheximide suppressed the activation. The effect of glutamine appeared specific because analogues and/or derivatives of glutamine, such as acivicin, 6-diazo-5-oxo-L-norleucine, homoglutamine, ammonium chloride and glutamate did not replace glutamine. The influence of amino acid transport systems through plasma membrane was assessed by the use of 2(methylamino)-isobutyric acid and β2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid. The glutamine-dependent induction of collagen gene expression was found to be independent of transport system A but dependent on transport system L whose inhibition induced a decrease in pro α1(I) collagen gene transcription by an unknown mechanism. Thus, glutamine, at physiological concentrations, indirectly regulates collagen gene expression.

Keywords: Glutamine; Gene transcription; Fibroblast; Collagen synthesis

1. Introduction

Culture media used for the growth of fibroblasts are generally supplemented with glutamine (gln) since this amino acid is essential for the survival and growth of mammalian cells [1–3], and has multiple roles in cell metabolism [4–10], particularly in pyrimidine and purine synthesis [9]. Gln is also known to play a role in the regulation of skeletal muscle protein turnover [11,12] and hepatic or renal glucogenesis [8]. A role in wound healing and inflammation has also been reported [10]. In this regard, the ability of fibroblasts to assimilate gln from the extracellular environment through the activity of amino

Abbreviations: MeAIB, 2 (Methylamino)-isobutyric acid; BCH, β2-aminobicyclo-(2.2.1) heptane-2-carboxylic acid; DON, 6-Diazo 5 oxo-L-norleucine; Acivicin, 1-amino-3-chloro-4,5 dihydro-5-isoxazolacetic acid; β-APN, 2-aminopropionitrile fumarate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; Collagenase (E.C.3.4.24.3); Pepsin (E.C.3.4.23.1); Trypsin (E.C.3.4.21.4); GAPDH, glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12); FN, Fibronectin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; OPA, O-phthalaldehyde; FCS, foetal calf serum; DRB, 5,6-dichlororibofuranosyl benzimidazole; kb, kilobase (s); bp, base pair; SSC, standard saline citrate.

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acid transport systems is of vital importance in wound healing [13]. It has been previously demonstrated, by the use of radioisotopic methods measuring [^{14}C] or [^{3}H]proline (pro) incorporation into macromolecules, that gln can exert a stimulating effect on collagen synthesis [14–16]. However, as the concentration of gln in the culture medium also influences the incorporation of pro into collagen, the specific role of gln as an activator of collagen synthesis and its mechanism of action is unknown.

In a previous paper [17] we demonstrated that 10 mM gln in culture medium exerted a stimulating effect on collagen synthesis, compared to the basic level obtained without gln. This effect was evident when the amount of collagen was measured using sensitive methods based on the fluorimetric determination of the pro + hydroxyproline (hyp) content of newly-synthesized collagen. In contrast, when measured by [^{14}C]pro incorporation, an inhibitory effect, that correlated with the decrease of the intracellular pro pool specific radioactivity, was apparent.

It is known that uptake of gln and pro in human fibroblasts is mediated by the same amino acid transport systems [13,18]. In this regard, it has been previously reported that intracellular gln is regulated mainly by an active uptake of extracellular gln [19]. This uptake of gln accounts for 90% of the cellular gln that is turned over, and little gln is derived from exchangeable glutamate (glu). Three transport systems for gln have been demonstrated in cultured human fibroblasts; the Na^+-dependent systems A and ASC and the Na^+-independent system L [13]. System A, and to a lesser extent system ASC, are prominent routes for both gln and pro transport, whereas system L accounts for less than one-fifth of gln uptake. Together, the competitive uptake of gln and pro at the amino acid transport sites and the metabolic transformation of intracellular gln into pro can lead to an isotopic dilution of radioactive pro to be incorporated into collagen, thereby decreasing the specific radioactivity of this molecule and, consequently, the specific radioactivity of newly-synthesized collagen. Thus, measurements of collagen biosynthesis in the presence of gln will be influenced by the effect of gln on amino acid transport. At this time, a gln-dependent activation of protein synthesis has not only been evidenced for collagen but also for heat-shock proteins [20–22], for which a transcriptional effect of gln was demonstrated.

The purpose of this study was to determine the mechanisms of the concentration-dependent effect of gln on the transcriptional and post-transcriptional regulation of collagen and non-collagen protein synthesis. Taking into consideration that an increased availability of gln in the cell environment associated to a transport site-mediated faster uptake of this amino acid by the fibroblasts may play a central role in the metabolism of these cells, we also investigated the role of the amino acid transport systems in the gln-dependent induction of collagen synthesis by the use of specific inhibitors for these systems. The effects of analogues and/or derivatives of gln were also studied in order to assess whether the activation of collagen synthesis is specific for gln.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade and, unless mentioned, were from Prolabo (Paris, France). L-[U-^{14}C]proline (9.25 GBq/mmol), L-[^{35}S]methionine (29.6 TBq/mmol), [α-^{32}P]dCTP and [α-^{32}P]UTP (111 TBq/mmol) were purchased from NEN Research Products (Paris, France). Gln was from Calbiochem (France Biochem, Meudon, France). L-Pro was obtained from Carlo Erba (Paris La Défense, France). L(+) ascorbic acid was from Merck (Darmstadt, FRG). L-Glu, ammonium chloride, 2-mercaptoethanol, 2-aminopropionitrile fumarate (β-APN) and O-phthalaldehyde) (OPA) were obtained from Sigma Chemicals (St. Louis, MO, USA). Bacterial collagenase (CLSPA grade) purchased from Worthington (Freehold, NS, USA) was used after purification by gel filtration chromatography according to the method of Peterkofsky and Diegelman [23]. Fetal calf serum (FCS) and Eagle's minimum essentiel medium (MEM) supplemented with 2 mM gln were from Biopro (Mulhouse, France) and Eagle's minimum essential medium without gln from Institut J. Boy (Reims, France). Plastic culture flasks were from Costar (Dutscher, Strasbourg, France). Pro and hyp were evaluated through the reaction of the free amino acid with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) followed by thin layer chromatography and spectrofluorometric scanning, as already described [24]. The Instagel scintillation cocktail was obtained from Packard (Rungis, France). Molecular biology reagents were from Sigma Chemicals (St. Louis, MO, USA). Ribosomal RNA markers 28S and 18S (calf liver) was from Pharmacia (Uppsala, Sweden). Molecular weight RNA markers ranging from 0.24 to 9.5 kb and random priming kit were bought from Gibco BRL (Paisley, Scotland).

2.2. Cell cultures

Fibroblasts were derived from explants of human foreskins obtained during surgery and grown at 37°C in MEM supplemented with 10% (v/v) FCS, 2 mM gln and antibiotics (penicillin, 200 units/ml; streptomycin 200 μg/ml) in a CO_2/air (1/19) atmosphere. Human embryonic skin fibroblasts from the Flow 7000 strain were also used. All studies were performed on subcultures between passages 3 to 11 for explanted fibroblasts and 20 to 25 for the Flow 7000 strain (split ratio 1/4). Contamination by bacteria or mycoplasma were checked on a routine basis and any contaminated flask was discarded. Cells were counted in a Malassez cell and their viability tested by the Trypan blue test [25] was over 95%.

2.3. Protein synthesis studies

Cells plated into 25-cm^2 plastic dishes were assayed at confluency. One day before the experiments, the culture medium of human dermal fibroblasts was replaced by 5 ml of fresh MEM supplemented with 2 mM gln, 0.5% (v/v) FCS and 50 μg/ml ascorbic acid and cells preincubated for 24 h at 37°C. Then, the medium was discarded, the cells were rinsed twice with 5 ml of MEM and were incubated at 37°C for 24 h in 5 ml of MEM supplemented with 0.2 mM unlabelled pro, 50 μg/ml ascorbic acid, 50 μg/ml β-APN and concentrations of gln appropriate to the type of experiment described below. The labelled amino acids added to the medium were either L-[^{14}C]pro (1, 2 or 3 μCi/ml depending on the purpose of the experiment, or L-[^{35}S]methionine (10 μCi/ml) for characterization of protein by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The dose-dependent effects of gln on collagen and protein synthesis were tested with concentrations of gln ranging from 0 to 10 mM. All the metabolic or competitive factors were studied under the same conditions both without gln and with gln in the medium.

The methods for measuring radiolabelled collagen and non-collagen proteins as well as for the evaluation of hyp and pro collagen content, have been described elsewhere [26]. Briefly, at the end of the incubation period, the medium was collected and the cells were washed with cold phosphate-buffer saline (PBS). The medium and the rinse were pooled and proteinase inhibitors (5 mg/l benzamidine, 2.5 mg/l pepstatin, 1 mg/l leupeptin and 1 mg/l aprotinin) added. The culture medium was first dialyzed at 4°C against running water for 24 h, then against distilled water for another 24 h. Radioactivity incorporated into total medium macromolecules was counted on an aliquot of the dialysate, the remaining solution was lyophilised. The sample was solubilized in 0.1 M ammonium formate buffer (pH 7.8) containing 6 mM calcium acetate and treated with purified bacterial collagenase (4 U/ml) for 24 h at 37°C. At the end of the incubation, non-collagen proteins were precipitated by ethanol (80% v/v) for 18 h at 4°C and then centrifuged at 47000 × g for 30 min. The supernatant containing the collagenase-digestible proteins was evaporated to dryness under a stream of nitrogen and dissolved in distilled water. Radioactivity incorporated into collagen was counted on an aliquot of the solution. The remainder was hydrolyzed in 6 M HCl for 18 h at 110°C. The precipitate containing non-collagen proteins was processed as the supernatant. Hydrolyzates were evaporated to dryness under a stream of nitrogen. Residues were redissolved in distilled water and passed through a Dowex 50 w/x2 column. After elution by NH_4Cl 2 M and evaporation, the amino acids were treated by OPA and NBD-Cl. Concentrations and radioactivities of the two derivatives, NBD-pro and NBD-hyp, were measured after separation by thin layer chromatography on silica gel plates, as

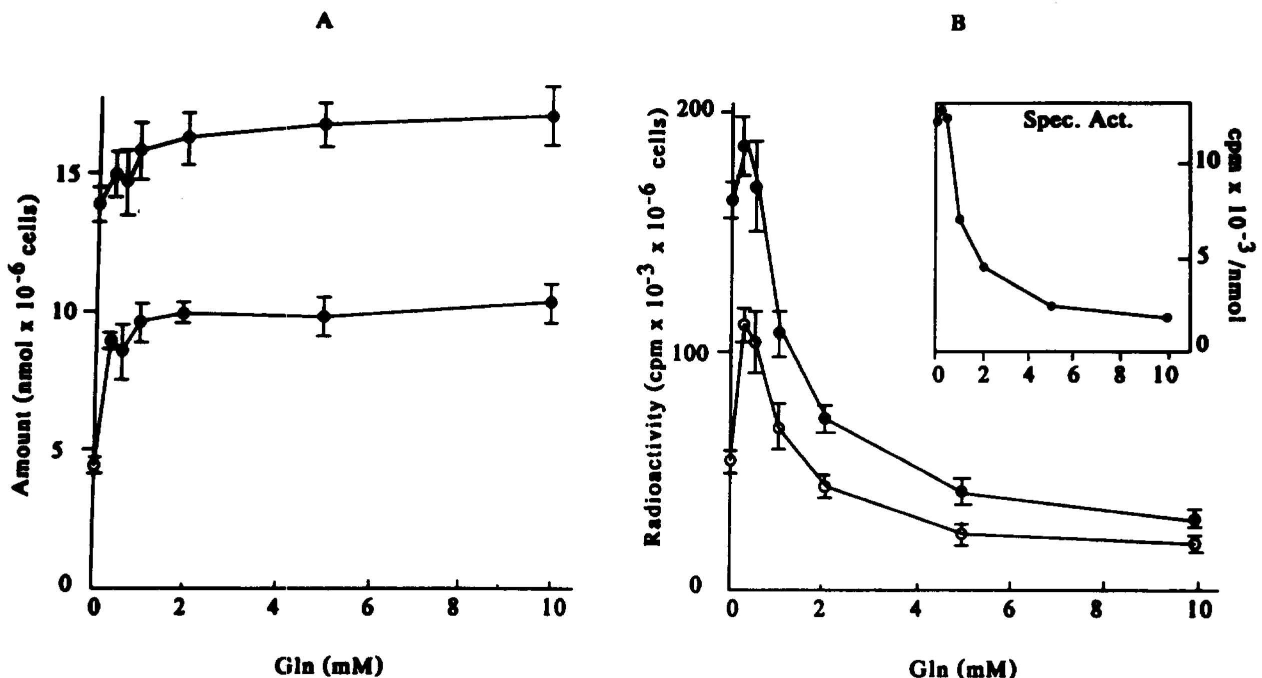


Fig. 1. Effect of varying the concentration of gln in the medium on the synthesis of extracellular non-collagen (●) and collagen proteins (○). Confluent human dermal fibroblasts (strain P18, 11th passage) were incubated in MEM supplemented with [14C]pro (2 μCi/ml), 0.2 mM proline, 50 μg/ml ascorbic acid and 50 μg/ml 2-APN. After 24 h incubation at 37°C, amount (A) and radioactivity (B) of non-collagen and collagen proteins, expressed as Pro and Pro + Hyp respectively, were determined. The specific radioactivity (Spec. Act.) of the hyp precursor is also shown in (B), inset.

Table 1
Comparison between the amounts of collagen and non-collagen proteins synthesized by fibroblasts incubated in the absence or in the presence of 10 mM gln

Experiments (strain of fibroblasts)	Amount of collagen in the medium		Amount of non-collagen proteins in the medium	
	no gln	10 mM gln	no gln	10 mM gln
1-P 18 (11th passage)	4.5 ± 0.3	10.2 ± 0.7 ($P < 0.001$)	13.9 ± 0.6	16.9 ± 1.1 ($P < 0.01$)
2-P 22 (6th passage)	5.9 ± 0.3	9.9 ± 1.1 ($P < 0.01$)	11.6 ± 0.4	16.2 ± 0.9 ($P < 0.001$)
3-P 22 (8th passage)	5.9 ± 0.6	10.0 ± 0.7 ($P < 0.001$)	13.8 ± 1.2	16.8 ± 1.4 ns
4-P 22 (8th passage)	6.7 ± 0.3	10.4 ± 0.9 ($P < 0.01$)	13.1 ± 0.6	15.7 ± 1.0 ($P < 0.02$)
5-P 31 (3rd passage)	9.4 ± 1.0	14.2 ± 0.8 ($P < 0.01$)	16.8 ± 1.3	17.8 ± 0.9 ns
6-P 32 (6th passage)	8.7 ± 0.8	11.3 ± 0.4 ($P < 0.01$)	16.8 ± 1.2	16.8 ± 0.9 ns
7-P 32 (11th passage)	8.8 ± 0.4	12.2 ± 0.9 ($P < 0.01$)	16.4 ± 1.4	19.6 ± 1.9 ns
mean increase in per cent of initial value		+63%		+18%

Human dermal fibroblasts grown to confluence were incubated for 24 h in MEM containing 0.2 mM unlabelled pro, 50 μg/ml ascorbic acid, 50 μg/ml β-APN and 10 mM gln when required. Collagen was determined by fluorometric measurement of its pro + hyp content as described in Section 2. Amount of collagen expressed in nmol (pro + hyp) per 10^6 cells. Amount of non-collagen proteins expressed in nmol of pro per 10^6 cells. Every datum is the mean of 4 evaluations per strain. Many strains were tested in order to account for cell variability. ns, not significant.

already described [24]. All the results were expressed on the basis of 10^6 cells. In some experiments confluent fibroblast cells were incubated with 5 or 10 μg/ml cycloheximide in the absence or in the presence of 0.25 mM gln in the medium for 24 h at 37°C. Total RNA was then isolated and analyzed as below.

2.4. RNA analysis

Total RNA was isolated by the method of Chomczynski and Sacchi [27]. Briefly, the medium was removed and the cells were washed twice with cold PBS and homogenized in 4 M guanidine isothiocyanate solution containing sodium

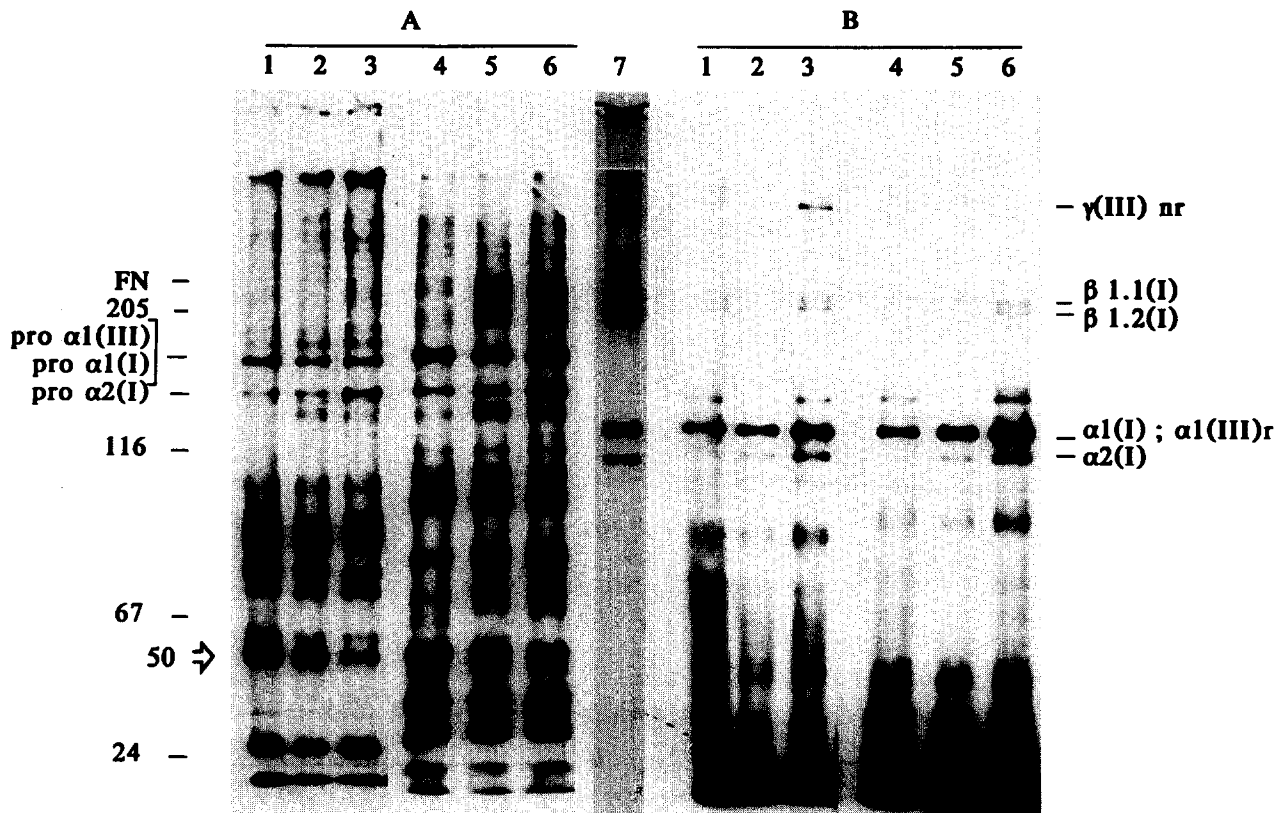


Fig. 2. Electrophoregram of [^{35}S]methionine-labelled polypeptides separated by SDS-PAGE from the medium of human dermal fibroblasts. Non-pepsinized samples (A) and samples treated with 1% (w/v) pepsin for 24 h at 4°C (B). The concentrations of gln in the medium were respectively 0 (lanes 1 and 4), 0.1 mM (lanes 2 and 5) and 0.25 mM (lanes 3 and 6). The samples were analyzed under non-reducing (lanes 1, 2 and 3) and reducing conditions (lanes 4, 5 and 6). Lane 7: type I collagen standard preparation run with the samples and stained with R250 Coomassie blue. The migration positions of fibronectin (FN), α and β chains of type I collagen, pro α chains of types I and III collagen and the apparent molecular mass (kDa) of standard proteins are indicated. Arrow indicates the migration position of the non-collagen protein of Mr 50000.

citrate 25 mM (pH 7.5), 0.5% (w/v) sarcosyl and 0.1 M 2-mercaptoethanol. Total RNA was isolated by phenol/chloroform extraction and precipitated three times running with ethanol. RNA was evaluated by measuring absorbance at 260 nm. For Northern blot analysis, RNA (6 μg of each sample) was denatured in formamide and electrophoresed in 1.0% (w/v) agarose gels containing 2.2 M formaldehyde [28]. RNAs were either stained with ethidium bromide to confirm their integrity or transferred overnight by capillary method in 20 × SSC buffer (stan-

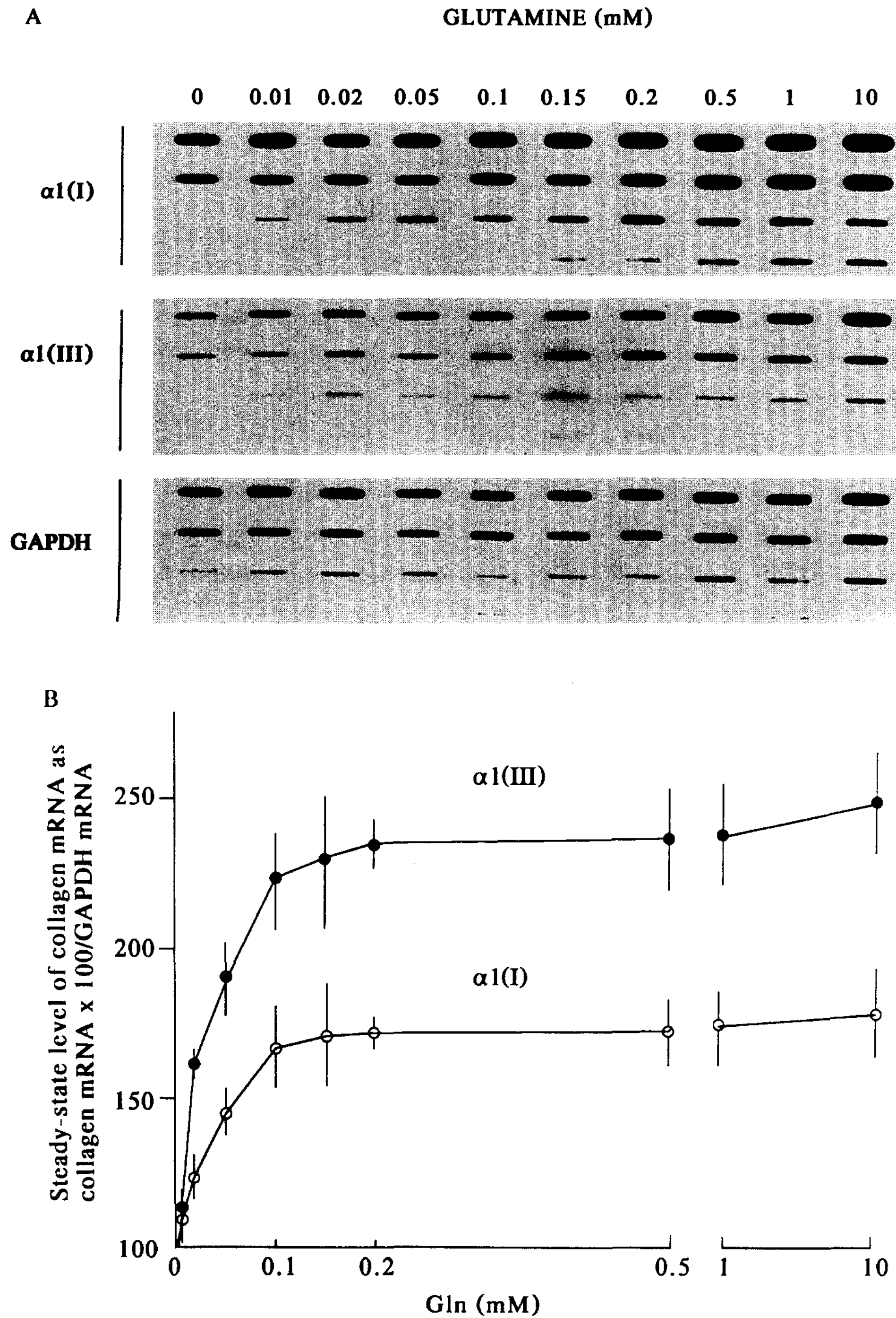
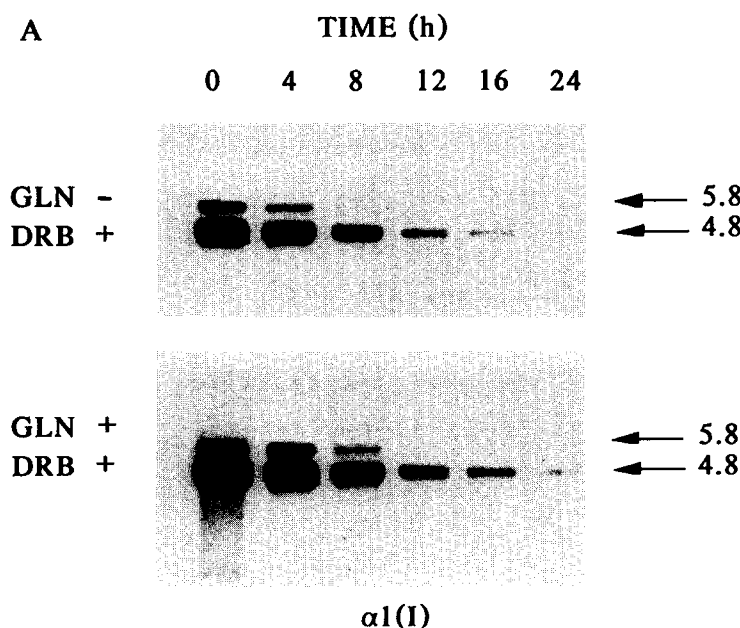


Fig. 3. Effect of gln concentration on Type I and Type III collagen mRNA levels after a 24-h incubation period. A: Slot-blot hybridization of total cellular RNA isolated from cells that were cultured in media containing 0, 0.01, 0.02, 0.05, 0.1, 0.15, 0.2, 0.5, 1 and 10 mM gln for 1 day. Serial dilutions of total RNA (6, 3, 1.5, 0.75 μg) were slotted onto a nylon membrane, baked, and hybridized with cDNA probes of α1(I), α1(III) collagen and GAPDH. B: Influence of gln concentrations in medium on the steady-state mRNA levels determined by densitometric scanning and integration of the peak areas. Values for each collagen chain are given as percent of the results for control cell incubated without gln. All values are normalized to GAPDH signal intensity.

dard saline citrate) to nylon membranes (PALL-Biodyne, Pharmacia). For slot-blot analysis, total RNA in serial dilutions (6, 3, 1.5 and 0.75 μg) was denatured in SSC containing formaldehyde for 15 min at 65°C and applied to nylon membranes (PALL-Biodyne) with a Bio slot SF microfiltration apparatus (Biorad). The membranes were then air-dried and UV-irradiated to allow binding of RNA. Specific hybridization was carried out for 24 h at 42°C with [^{32}P]-labelled cDNA probes as described elsewhere [28]. cDNA probes were labelled to specific activities of $> 10^8$ cpm/μg DNA by random priming-kit and [α-^{32}P]dCTP. The blots were gradually washed under high stringency conditions to avoid cross-hybridization (2 × SSC, 0.1% (w/v) SDS at 25°C for 30 min; 2 × SSC, 0.5% (w/v) SDS at 37°C for 30 min and 2 × SSC, 0.5% (w/v) SDS at 68°C for 30 min), air-dried and exposed to hyperfilm MP (Amersham) at −80°C with a Kodak X-Omatic intensifying screen. Autoradiographic signals were measured using a scanning densitometer Desaga (Merck). Hybridization with other probes was performed as follows:



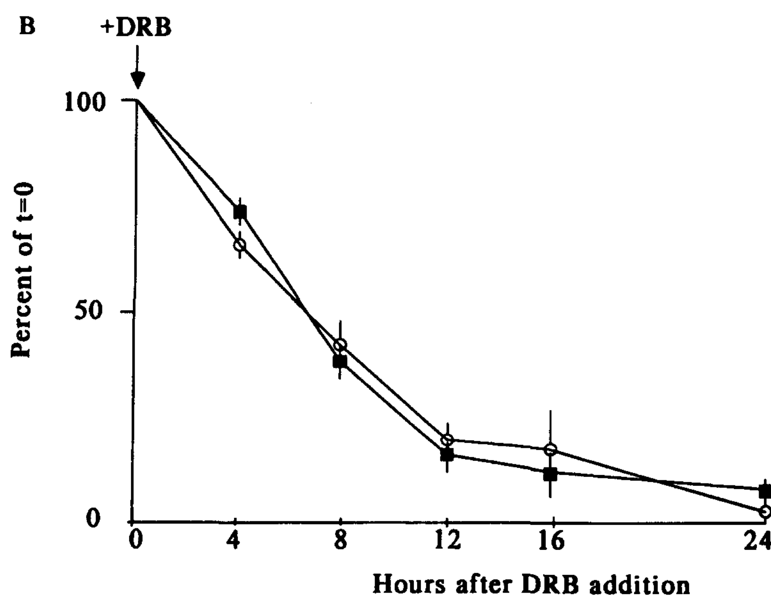


Fig. 4. Effect of gln on the half-life of pro α1(I) collagen mRNA. A: Northern-blot hybridization of total cellular RNA isolated (at different times after DRB treatment) from cells that were cultured in media containing no gln or 0.25 mM gln. The blots were probed with pro α1(I) collagen cDNA. B: Evolution of the pro α1(I) collagen mRNA amounts (autoradiographic signals obtained by densitometry) plotted as percentage of basal values determined without DRB and normalized to the GAPDH signal intensity. The half-life of the collagen mRNA was estimated as the time necessary for its decay from 100% to 50% of its original level. (○-○) control cells, (■-■) gln-treated cells.

nylon blots were washed in phosphate buffer 10 mM (pH 6.5) containing 50% (v/v) formamide for 60 min at 65°C to remove the probe and submitted to another round of hybridization with a new cDNA probe.

2.5. cDNA probes

Plasmid pHCAL1U containing a 670 bp insert specific for the human 1(1) chain [29] and pHFS3 containing a 705 bp insert specific for the C-terminal end of human α1(III) chain [30] were used as the probes for collagen I and III mRNAs, respectively. Plasmid pGDN5 containing a 1.7 kb insert was used as a specific probe for GAPDH mRNA [31].

2.6. Nuclear run-off transcription assay

Confluent human dermal fibroblasts were incubated for 24 h in culture medium containing no gln or 0.25 mM gln. Nuclei were then isolated by the procedure of Greenberg and Ziff [32] with slight modifications [33]. Briefly, trypsinized cells (3×10^7) were washed three times with PBS and treated for 10 min at 0°C with Nonidet P40 lysis buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% (v/v) Nonidet P40. The nuclei were spun down at $500 \times g$, washed three times with 10 mM Tris (pH 8.0), 25% (v/v) glycerol, 5mM $MgCl_2$, 0.1 mM EDTA and suspended in reaction buffer (10 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 2.5 mM dithiothreitol, 1 mM each ATP, CTP, GTP and 250 μCi of [α-^{32}P]UTP in final volume of 0.2 ml. The nuclei were incubated at 30°C for 30 min and lysed in 1 ml of denaturing solution containing guanidine isothiocyanate. One hundred μg of tRNA was added. RNA was then extracted as described above and equal counts of [^{32}P]-labelled RNA were hybridized to each alkali-denatured cDNA(4μg) slotted on nylon membranes. The cDNA for α1(I) collagen, α1(III) collagen and GAPDH were obtained from their respective plasmids by polymerase chain reaction (PCR) amplification with primers derived from polylinker zone. Furthermore, they were purified by gel filtration on chroma spin-100 columns (Clontech.). Plasmid PUC8 DNA was used to determine background activity. Autoradiogramms were scanned by densitometry.

2.7. Collagen mRNA half-life

Confluent fibroblast cells were treated with 20 μg/ml DRB (5,6-dichlororibofuranosyl benzimidazole) for varying periods (0, 4, 8, 12, 16 and 24 h). Total RNA was then isolated and subjected to Northern blot and slot analysis as described above. Values obtained from densitometric scanning of the slot blots were plotted relative to the control (0h time point or without DRB) which was set at 100%. The half-life of collagen mRNA was estimated as the time required to reach 50% of its original level. Slot blots were washed in 50% formamide at 65°C to remove the cDNA probes and reprobed with GAPDH cDNA, to normalize collagen mRNA signal intensity.

2.8. Effects of competitive inhibitors of the glutamine membrane transport systems or of metabolic reactions

In a series of cell culture experiments, we added several competitive inhibitors of gln transport such as 2-(methylamino)-isobutyric acid (MeAIB) (Sigma Chemicals, St. Louis, MO, USA), β2-aminobiclyclo (2.2.1) heptane-2-carboxylic acid (BCH) as well as agents that were known to modify the transport and the metabolism of gln such as 6-diazo-5-oxo-L-norleucine (DON), L-homoglutamine, α-amino-3-chloro-4,5-dihydro-5-isoxazolacetic acid (acivicin), all bought from Sigma.

2.9. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Methionine [L-^{35}S]-labelled polypeptides from the medium of human dermal fibroblasts were separated by SDS-PAGE according to Laemmli [34] with a gel gradient of 5–10% (w/v) acrylamide in 0.025 mM Tris buffer (pH 8.3), 0.1% (w/v) SDS, and visualized by fluorography using Hyperfilm-MP (Amersham, Les Ulis, France), and Kodak X-Omatic cassette C2 with intensifying screens at −80°C.

3. Calculations

The experiments were performed in quadruplicate and expressed as the mean ± S.D. The significance of the results was tested by Student's t-test [35].

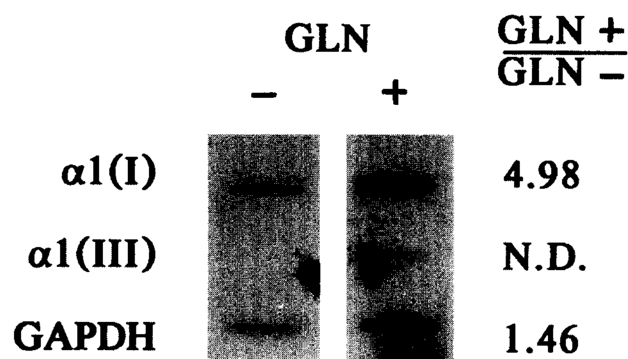


Fig. 5. Effect of gln on the transcription activity of the collagen genes. Nuclear run-off analysis was performed with nuclei isolated from cells cultured in media containing no gln or 0.15 mM gln for 1 day. Equal counts of purified [^{32}P]UTP-labelled RNAs were hybridized to 4 μg each of cDNA probes of α1(I), α1(III) collagen and GAPDH blotted onto nylon membranes. The autoradiograms were obtained by scanning densitometry. N.D. = not determined.

4. Results

4.1. Dose-dependent effect of gln on collagenous and non-collagenous protein synthesis

Increasing the concentration of gln from 0 to 0.25 mM in the medium of confluent human dermal fibroblast cultures, increased the amount of secreted collagen, estimated from the concentration of non-dialyzable collagenase-digested pro + hyp. Then, collagen accumulation reached a plateau from 0.25 to 10 mM gln (Fig. 1A). The amount of non-collagen proteins was also enhanced by increasing the concentration of gln but to a far lesser extent. When the metabolism of collagen was followed by incorporation of [^{14}C]pro into secreted macromolecules, a different pattern was found (Fig. 1B). The collagen radioactivity reached a maximum at 0.25–0.5 mM gln and then quickly decreased.

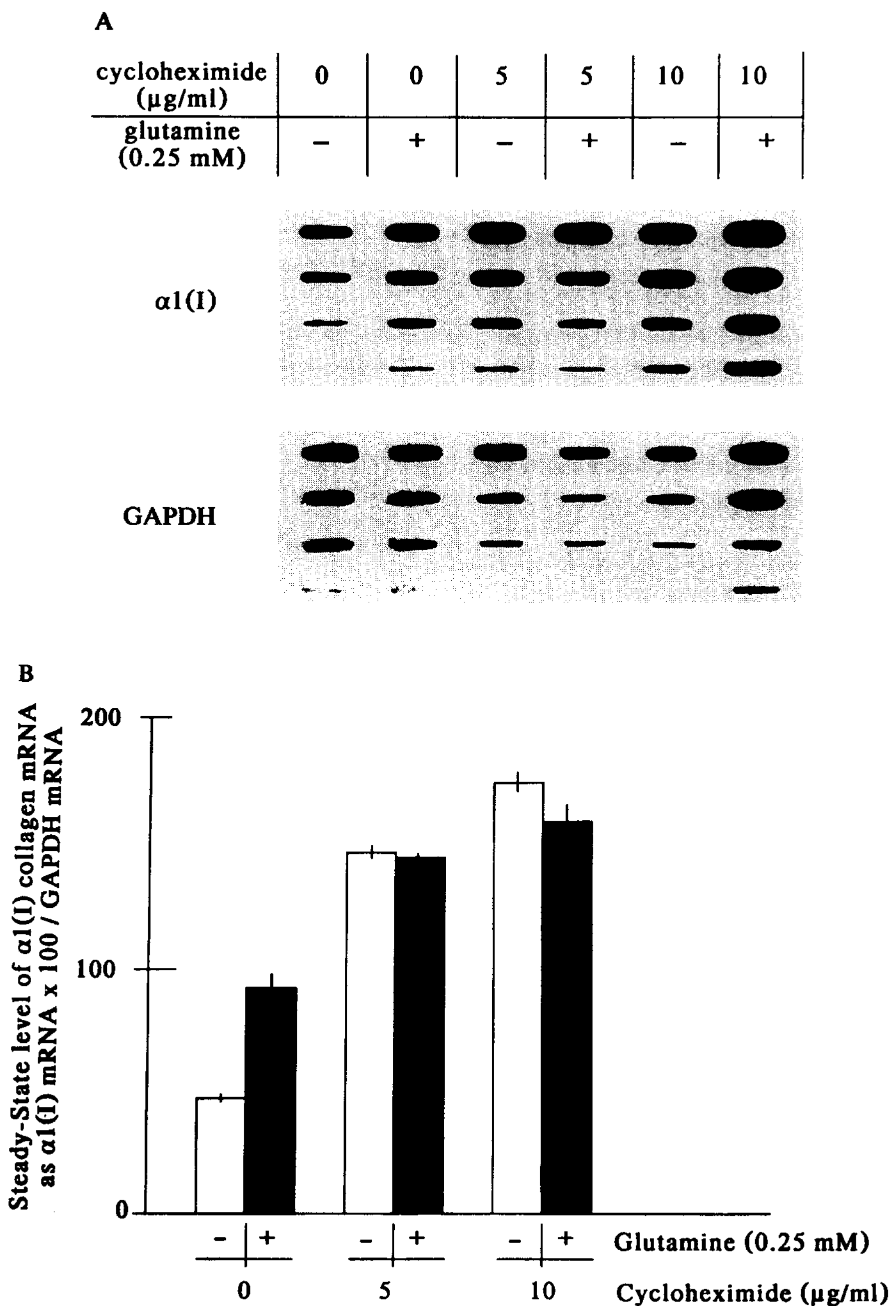


Fig. 6. Protein synthesis is required for the gln-dependent activation of collagen gene expression. A: Slot-blot hybridization of total cellular RNA isolated from cells that were cultured in media containing cycloheximide (0, 5 and 10 µg/ml) and gln (0 and 0.25 mM) for one day. Serial dilutions of RNA (6, 3, 1.5 and 0.75 µg) were slotted onto a nylon membrane. The blots were hybridized with cDNA probes of α1(I) collagen and GAPDH. B: Steady-state mRNA levels as determined by densitometric scanning and integration of peak areas. The values for α1(I) collagen mRNA are normalized as α1(I) mRNA × 100/GAPDH mRNA.

This result was interpreted as an isotopic dilution of the hyp precursor when the concentration of gln in culture medium was increased over 0.5 mM. In all the tested strains, gln induced a significant increase of collagen synthesis despite the existence of a variability from strain to strain (Table 1). The mean increase in per cent of control value was 63% for collagen synthesis and 18% for non-collagen protein synthesis. The degree of collagen hydroxylation [hyp/(pro + hyp)] was not affected by the increase of gln level in the medium. We also verified that no change in cell number and viability occurred over the range of gln concentrations tested.

The SDS-PAGE patterns of [^{35}S]methionine-labelled proteins secreted into the medium are reproduced in Fig. 2. When the concentration of gln in the medium was raised, an increase of types I and III collagens and of a large 200 kDa non-collagen protein was demonstrated together with a decrease of a 50 kDa protein.

4.2. Dose-dependent effect of gln on the steady-state level of collagen mRNA

Confluent dermal fibroblasts were incubated for 24 h with concentrations of gln increasing from 0 to 10 mM in order to determine the influence of this amino acid on the expression of collagen genes. Northern blots of total RNA revealed that the mRNAs of α1(I) (with apparent sizes of 5.8 and 4.8 kb) and α1(III) (with an apparent size of 4.8 kb) collagens were intact and that an increase of these collagen transcripts was paralleled by the increase in gln (data not shown). In comparison, the GAPDH mRNA transcript also seemed to be increased by gln but to a much less extent. The steady-state levels of mRNAs were determined by scanning densitometry of the slot blots and normalized to GAPDH mRNA level (Fig. 3A,B). Gln increased the steady-state level of α1(I) collagen and α1(III) collagen mRNAs in a dose-dependent manner from 0 to 0.15 mM. For the two collagen chains, activation was maximal with 0.15 mM gln and did not change significantly from 0.15 to 10 mM. However, the activation of collagen gene expression by gln was higher in the case of α1(III) than α1(I). The steady-state levels of α1(III) and α1(I) collagen mRNAs were increased by 2.3-and 1.7-fold respectively.

4.3. Collagen mRNA half-life

Values obtained for the decay of collagen mRNA were plotted relative to the control (no DRB) set up at 100% for each series (Fig. 4). The time determining a 50% decrease in signal intensity was taken as the $t_{1/2}$. It was evaluated by plotting the logit of % decrease versus the log of incubation time and calculated by linear regression analysis. The half-life of collagen mRNA was approximately 7 h in control cells and 8 h in gln-treated cells. The difference was not significant. Northern blot analysis of the same RNA samples revealed that the 5.8 and 4.8 kb pro-α1(I) collagen mRNAs decayed with similar kinetics.

4.4. Nuclear run-off transcription assay

In order to test whether the elevation in collagen mRNA levels is due to an increase in the rate of gene transcription, nuclear run-off analysis was performed. The transcription run-off assays using nuclei isolated from control fibroblasts and from gln-treated fibroblasts are shown in Fig. 5. Quantitation of the autoradiogram revealed that gln increased collagen α1(I) premessenger RNA by about 3.4-fold. Collagen α1(III) premessenger RNA was also increased by gln but this increase could not be determined because the signal in the control cells was not detectable.

4.5. Protein synthesis is required for the gln-dependent induction of collagen gene expression

The addition of cycloheximide to confluent cells for 24 h significantly increased the level of transcription of α1(I) collagen mRNA in the absence of gln (Fig. 6). When cells were incubated in the presence of both cycloheximide and 0.25 mM gln, no further increase in collagen transcription was evident.

4.6. Contribution of the transport systems for gln and specificity of glutamine to stimulate collagen synthesis

In order to assess the role of the amino acid transport systems in the gln-dependent induction of collagen synthesis, we used MeAIB to inhibit the membrane amino acid transport system A, and BCH to inhibit the Na^+-independent L transport system. MeAIB had no significant effect on the amount of collagen secreted into the medium. Thus, MeAIB did not counteract the gln-dependent induction of collagen synthesis in the presence of gln. On the other hand, BCH induced a slight non-significant activation of

Table 2
Effect of MeAIB and BCH on secreted collagen in human dermal fibroblast cultures

Addition	Collagen amount (nmol (pro + hyp) per 10^6 cells)	
Me AIB (mM)	no gln	10 mM gln
0	5.9 ± 0.3	9.9 ± 1.1
2	5.7 ± 0.3 ns	9.3 ± 0.8 ns
10	6.2 ± 0.4 ns	9.0 ± 1.2 ns
BCH (mM)		
0	6.6 ± 0.3	10.4 ± 0.9
10	8.1 ± 1.0 ns	7.6 ± 0.9 ($P < 0.02$)

Confluent human dermal fibroblast (strain P 22, 8th passage) were incubated in MEM supplemented with, 0.2 mM pro, 50 μg/ml ascorbic acid 50 μg/ml β-APN, and the factor to be tested. MeAIB and BCH were tested in the presence or in the absence of 10 mM extracellular gln in the medium. The results were obtained after 24 h incubation. ns, not significant.

collagen synthesis and, unlike MeAIB, BCH was able to counteract the stimulatory effect of gln on collagen synthesis (Table 2).

Northern blot and slot blot analyses corroborated the results obtained with BCH (Fig. 7). In the absence of gln in the medium, BCH had little effect on the steady-state level of $\alpha 1$(I) collagen mRNA, whereas it suppressed the gln-dependent induction of collagen gene expression in a dose-dependent manner. The steady-state level of $\alpha 1$(I) mRNA in gln-treated cells was reduced to basal levels when cells were incubated with 10 mM BCH. The effect of MeAIB was not investigated at the transcriptional level since it was not able to inhibit the stimulatory effect of gln on collagen synthesis.

The gln analogues acivicin and DON were individually able to inhibit collagen synthesis but did not prevent the increase in collagen synthesis induced by addition of gln (Table 3). In parallel, these substances did not inhibit the effect of gln on collagen mRNA assessed by Northern blot analysis. Homoglutamine, asparagine, glutamate and am-

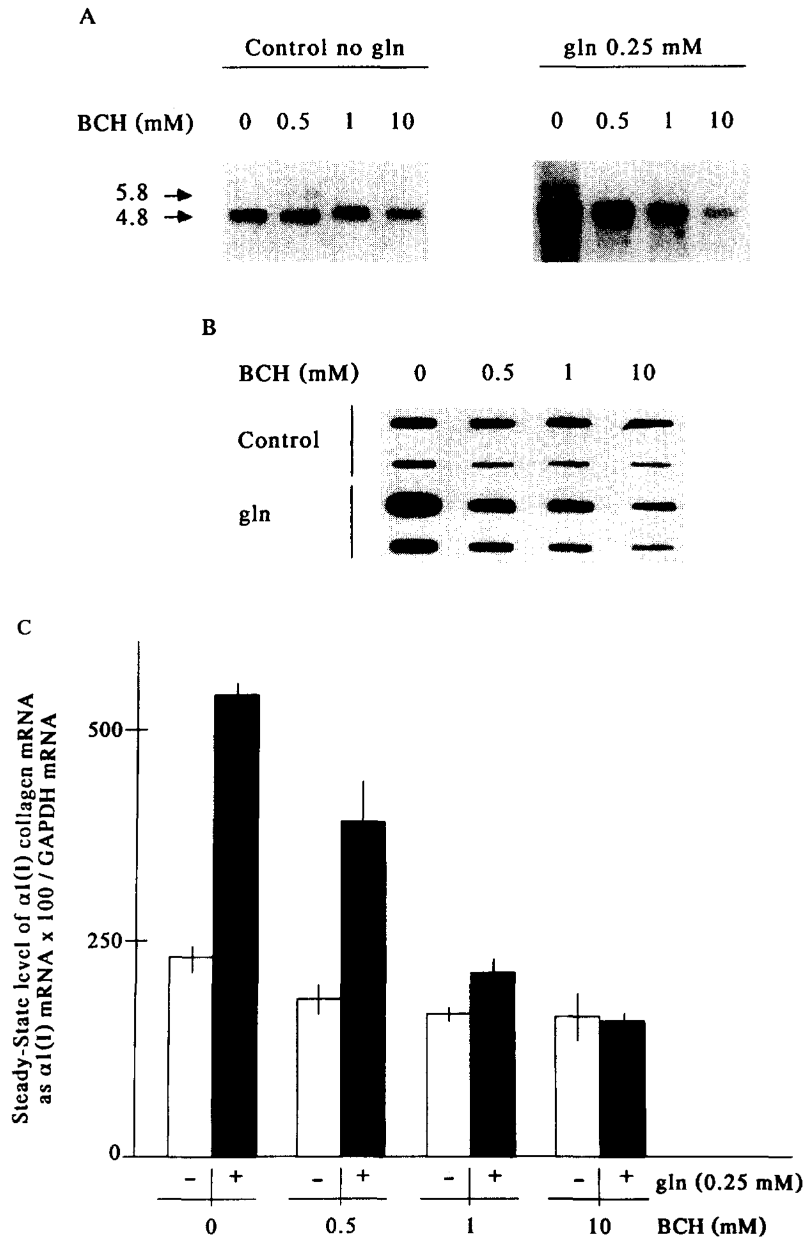


Fig. 7. BCH a specific inhibitor of the Na^+-independent amino acid transport system L inhibits the gln-dependent activation of collagen gene expression. A: Northern-blot analysis of total cellular RNA isolated from cells that were cultured in media containing BCH (0, 0.5, 1 and 10 mM) in the presence or in the absence of 0.25 mM gln for 1 day. The blots were probed with pHCAL1U [collagen $\alpha 1$(I)]. B: Slot-blot hybridization of total cellular RNA. Serial dilutions of total RNA (6 and 3 μg) were slotted onto a nylon membrane, baked and sequentially hybridized with pHCAL1U [$\alpha 1$(I)]; pGDN5. C: Steady-state level of $\alpha 1$(I) collagen mRNA was determined by densitometric scanning and integration of peak areas. The values are normalized to GAPDH signal intensity.

Table 3
Effect of the gln analogues acivicin and DON on the gln-dependent induction of collagen gene expression

Addition	Collagen nmol per 10^6 cells	mRNA α_1(I)×100/GAPDH
None	6.7±0.3	100.0±20.6
gln (10 mM)	10.4±0.9 ($P < 0.001$)	183.9±17.9 ($P < 0.001$)
Acivicin (1 mM)	5.1±0.7	97.6±21.2
Acivicin (1 mM) +gln (10 mM)	7.6±0.8 ($P < 0.001$)	166.7±22.9 ($P < 0.001$)
DON (5 mM)	3.9±0.2	92.9±16.4
DON (5 mM) +gln (10 mM)	5.9±0.3 ($P < 0.001$)	179.2±8.3 ($P < 0.001$)

Confluent human dermal fibroblasts (strain P_{32}, 6th passage) were incubated in MEM supplemented with, 0.2 mM pro, 50 μg/ml ascorbic acid, 50 μg/ml β-APN and the factors to be tested. Acivicin and DON were tested in the presence or in the absence of 10 mM gln in the medium. The steady-state level of α(I) collagen and GAPDH mRNA was determined by slot-blot hybridization of total cellular RNA followed by densitometric scanning and integration of peak areas. The values were normalized to GAPDH signal intensity. The results were obtained after 24 h incubation. The significance of the results is determined on the effect of gln.

monium chloride (NH_4Cl) were individually not able to mimick the effect of gln on collagen synthesis (data not shown).

5. Discussion

In a previous paper [17], we reported that gln was the main metabolic source of a separate proline pool directly involved in protein synthesis by human dermal fibroblasts in confluent monolayer cultures. These results were in agreement with previous studies indicating that intracellular amino acids, particularly pro and gln, are partitioned among several separate pools [19,36–38]. We also demonstrated that an increase of the gln concentrations in the medium influenced not only the incorporation of [^{14}C]pro into proteins, but also the amount of collagen synthesized. The stimulating effect of gln on the collagen synthesis was masked in isotopic studies by the fact that the increase in gln level induces an isotopic dilution of [^{14}C]pro used as a marker for collagen synthesis. Here, we show that gln from 0 to 0.25 mM induces a dose-dependent increase of collagen synthesis followed by a plateau over 0.25 mM and that isotopic dilution is only observed over 0.5 mM. Notably, collagen studies in cell cultures are usually performed at higher concentrations of gln in the medium (2 or 4 mM), whereas the physiological concentration of gln in blood serum, ranging from 0.1 to 0.2 mM, corresponds precisely to that able to modulate the synthesis of collagen. The production of some non-collagen proteins is also activated, whereas the synthesis of others is decreased, indicating that gln exerts its effect not only on collagen synthesis but also selectively on non-collagen protein synthesis. Thus, electrophoregrams of the labelled proteins secreted into the medium demonstrate not only an increase in type I and III collagens and fibronectin, but also the decrease of an unidentified Mr 50 000 protein.

The requirement of gln for fibroblast growth in culture was reported earlier [2]. Although many studies have been devoted to the demonstration that gln is a major nutrient for cells and is also a nitrogen donor [9], few experiments have been undertaken to verify whether gln could exert a regulatory role in protein synthesis. Some papers have shown a relationship between gln supply and protein turnover in skeletal muscle [11,12]. A gln-dependent activation of gene expression has also been demonstrated for heat-shock proteins in proliferating and subconfluent populations of Chinese Hamster ovary cells without heating [20] as well as with heating [21] and more recently in opossum kidney cells [22]. The possible role of heat-shock proteins on the gln-dependent activation of collagen gene expression has not been investigated.

In an attempt to determine by which mechanisms gln could act on collagen synthesis, we investigated the effect of gln on gene transcription. Our results demonstrate that gln exerts its stimulating effect on collagen synthesis indirectly at the transcriptional level. The basis of this statement is as follows: (a) gln increased collagen mRNA level in human dermal fibroblast cultures at concentrations which were also found optimal for collagen protein production; (b) α1(I) and α1(III) hnmRNA were increased in cells incubated in the presence of gln in the medium as demonstrated by nuclear run-off transcription assay; (c) the half-life of collagen α1(I) mRNA was not significantly changed in cells treated by gln; and (e) the steady-state level of collagen α1(I) mRNA was not changed in cells incubated in the presence of both cycloheximide and gln as compared to the level obtained without gln. This latter result suggests that de novo synthesis of protein is required for the gln-dependent induction of collagen gene expression. Cycloheximide by itself exerts a stimulating effect on the transcription, as compared to the basic level obtained without cycloheximide, by an unknown mechanism. However, unless the increase in transcription can be directly correlated with the increase in mRNA, nuclear post-transcriptional regulation cannot be ruled out as a contributor to the gln effects. The mechanisms by which gln activates collagen gene expression are yet unknown but from a recent report [39] showing that simple homopolymeric gln stretches, existing predominantly in transcriptional regulatory proteins, can activate in vitro transcription when fused to the DNA binding domain of the yeast transcription factor GAL4, we postulate that gln-rich transcriptional factors may be implicated in the activation of collagen gene expression by gln. Therefore, the synthesis of these factors should be greatly diminished in gln-depleted cells.

Taking into consideration that de novo synthesis of protein seemed to be required for the gln-dependent induction of collagen gene expression, we studied the effect of inhibiting the membrane pro and gln transport systems on collagen gene expression. A recent report indicates that

L-gln enters the cultured human fibroblasts through all three transport systems for neutral amino acids (see Section 1). We found that MeAIB, a specific inhibitor of the Na^+-dependent system A [40], inhibited the uptake of [^{14}C]pro by 90% and 48% respectively in the absence and in the presence of 10 mM gln in the medium, whereas BCH, an inhibitor of the Na^+-independent system L [41], stimulated uptake by 33% and 67% respectively (data not shown). Regarding the intracellular concentration of gln and glu, when the medium was not supplemented with gln, BCH caused a decrease of glu from 24.6 ± 1.7 to 7.4 ± 1.2 nmol per 10^6 cells ($P < 0.001$) and a decrease of gln from 5.4 ± 0.1 to 4.0 ± 0.6 nmol ($P < 0.002$). When the medium was supplemented with 10 mM gln, BCH caused a decrease of glu from 44.6 ± 1.8 to 21.7 ± 3.8 nmol per 10^6 cells ($P < 0.001$) and a decrease of gln from 56.0 ± 2.8 to 41.6 ± 6.6 nmol ($P < 0.005$). From these additional results, we believe that by inhibiting system L, and consequently the gln-uptake, the intracellular conversion of gln to pro via glu is diminished. This decrease promotes the uptake of [^{14}C]pro by the system A which was shown to be the prominent route for pro transport in this study (see above the effect of MeAIB on the pro-transport system A).

As regards protein synthesis, both MeAIB and BCH caused a decrease in the incorporation of radioactivity into collagen. However, in the absence of gln in the medium, neither MeAIB nor BCH had any effect on the amount of synthesized collagen. In the presence of gln, BCH inhibited the stimulatory effect of this molecule on collagen synthesis at both the transcriptional and protein production level. These results suggest that BCH, by inhibiting the gln-transport system L, may cause a decrease in the availability of gln for the synthesis of some proteins involved in the gln-dependent induction of collagen gene expression.

A number of papers have reported that intracellular amino acids including pro and gln are partitioned among several pools and that aminoacylation of transfer RNA occurs near or on the plasma membrane, in relation with the amino acid transport system [19,36–38,42]. Here, it is proposed that selection of gln for protein synthesis takes place in close conjunction with the amino acid transport system L. In addition, the inability of analogues and/or derivatives of gln such as acivicin, DON, homoglutamine, NH_4Cl and glutamate to mimick the effect of gln on collagen gene expression, support the assumption for a specific role of gln in this regulatory process. Thus, activation of type I collagen transcription by an increase of gln in the range of physiological blood concentrations may reflect an adaptation of collagen synthesis to external conditions and possibly to the diet supply of amino acids.

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References

[1] Holley, R.W. (1975) Nature 258, 487–490.
[2] Eagle, H., Oyama, V.I., Levy, M., Horton, C.L. and Fleischman, R. (1956) J. Biol. Chem. 218, 607–616.
[3] Raivio, K.O. and Seigmiller, J.E. (1973) Biochim. Biophys. Acta 299, 273 282.
[4] Tate, S.S. and Meister, A. (1978) The Enzymes of glutamine metabolism, pp. 77–125 Academic Press, New York.
[5] Haussinger, D. (1989) Metabolism 38, suppl. 1, 14–17.
[6] Windmueller, H.G. and Spaeth, A.E (1974) J. Biol. Chem. 249, 5070–5079.
[7] Kovacevic, Z. and Mc Giran, J.D. (1983) Physiol. Res. 63, 547–605.
[8] Lavoinne, A., Baquet, A. and Hue, L. (1987) Biochem. J. 248, 429–437.
[9] Engström, W. and Zetterberg, A. (1984) J. Cell. Physiol. 120, 233–241.
[10] Bulus, N., Cersosimo, E., Ghishan, F. and Abumrad, N.N. (1989) Metabolism 38, suppl. 1, 1–5.
[11] Mac Lennan, P.A., Brown, R.A. and Rennie, M.J. (1987) FEBS Lett. 215, 187–191.
[12] Mac Lennan, P.A., Smith, K., Weryk, B., Watt, P.W. and Rennie, M.J. (1988) FEBS Lett. 237, 133–136.
[13] Dall'Asta, V., Rossi, P.A., Bussolati, O., Guidotti, G.G. and Gazzola, G.C. (1990) Biochim. Biophys. Acta 1052, 106–112.
[14] Blumenkrantz, N. and Asboe-Hansen, G. (1973) In Vitro 8, 343–346.
[15] Rönnemaa, T., Vihersaari, T. and Ranta, H. (1977) Scand. J. Clin. Lab. Invest. 37, 351–356.
[16] Lehtinen, P., Takala, I. and Kulonen, E. (1978) Connect. Tiss. Res. 6, 155–159.
[17] Bellon, G., Monboisse, J.C., Randoux, A. and Borel, J.P. (1987) Biochim. Biophys. Acta 930, 39–47.
[18] Gazzola, G.C., Dall'Asta, V. and Guidotti, G.G. (1980) J. Biol. Chem. 255, 929–936.
[19] Darmaun, D., Matthews, D.E., Desjeux, J.F. and Bier, D.M. (1988) J. Cell Physiol. 134, 143–148.
[20] Cai, J.W., Hughes, C.S., Shen, J.W. and Subjeck, J.R. (1991) FEBS Lett. 237, 133–136.
[21] Sanders, M.M. and Kon, C. (1991) J. Cell. Physiol. 146, 180–190.
[22] Nissim, I., States, B., Hardy, M., Pleasure, J. and Nissim, I. (1993) J. Cell. Physiol. 157, 313–318.
[23] Peterkofsky, B. and Diegelman, R. (1971) Biochemistry 10, 988–994.
[24] Bisker, A., Pailler, V., Randoux, A. and Borel, J.P. (1982) Anal. Biochem. 122, 52–57.
[25] Schen, H.F. and Strecker, H.J. (1975) Biochem. J. 150, 453–461.
[26] Bellon, G., Randoux, A. and Borel, J.P. (1985) Collagen Relat. Res. 5, 423–435.
[27] Chomczynski, P. and Sacchi, N. (1987) Analyt. Biochem. 162, 156–159.
[28] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in Molecular Cloning: A laboratory Manual, Cold laboratory, Cold Spring Harbor, New York.
[29] Vuorio, T., Mäkelä, J.K., V.M. and Vuorio, E. (1987) Arch. Dermatol. Res. 279, 154–160.
[30] Sandberg, M. and Vuorio, E. (1987) J. Cell. Biol. 104, 1077–1084.
[31] Fort, P., Marty, L., Piechaczyk, M., El Sabrouty, S., Dani, C., Jeanteur, P. and Blanchard, J.M. (1985) Nucleic Acids Res. 13, 1431–1442.
[32] Greenberg, M.E. and Ziff, E.B., (1984) Nature, 311, 433–438.
[33] Ausubel, F.M., Brent, R., Kingstton, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Strukl, K. (1989) Current Protocols in Molecular Biology, Vol. 1, pp. 4101–4108. Wiley, New York.
[34] Laemmli, U.K., (1970) Nature 227, 680–685.
[35] Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, Iowa State University Press, Ames.

[36] Hildebran, J.N., Airhart, J., Stirewalt, W.S. and Low, R.B. (1981) Biochem. J. 198, 249–258.
[37] Opsahl, W.P. and Ehrhart, L.A. (1987) Biochem. J. 243, 137–144.
[38] Oshita, T., Kawada, T., Endo, Y. and Natori, Y. (1984) J. Biochem. 96, 651–657.
[39] Gerber, H.P., Seipel, K., Georgiev, O., Höfferer, M., Hug, M., Rusconi, S. and Schaffner, W. (1994) Science 263, 808–811.
[40] Christensen, H.N., Oxender, D.L., Liang, M. and Vatz, K.A. (1965) J. Biol. Chem. 240, 3609–3616.
[41] Christensen, H.N., handlegten, M.E., Lam, I., Tager, M.S. and Zand, R. (1969) J. Biol. Chem. 244, 1510–1520.
[42] Van Venrooij, W.J., Moonen, H. and Van Loon-Klaassen, L. (1974) Eur. J. Biochem. 50, 297–304.