Insulin-like growth factor-I (IGF-I) mRNA in the small intestine of suckling and adult rats

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Abstract The presence and cellular localization of insulin-like growth factor-I (IGF-I) mRNA in the small intestine of suckling and adult rats was studied. A sensitive reverse transcription (RT) competitive-polymerase chain reaction (PCR) revealed IGF-I gene expression in both age groups. Adult tissue contained 3-fold higher levels of IGF-I mRNA in comparison with sucklings. Using an in situ hybridization technique, IGF-I transcripts were localized mainly in enterocytes and goblet cells in the intestinal crypts of adult rats. By using this technique, IGF-I mRNA was not detected in jejunum of 12-day-old rats.

Key words: Insulin-like growth factor; Gastrointestinal tract; Messenger RNA; In situ hybridization; Competitive polymerase chain reaction

1. Introduction

Mucosa of the gastrointestinal (GI) tract exhibits one of the most rapid cell turnover rates of any tissue in the body. Rapidly proliferating enterocytes migrate from crypt to villus tip, losing proliferative capacity and increasing differentiation capability [1]. The mechanisms regulating gastrointestinal growth are complex and are influenced by nutritional, hormonal, pharmacological, and luminal factors [2]. A growing body of evidence suggests that peptide growth factors are essential in the regulation of gastrointestinal growth and function.

Insulin-like growth factor-I (IGF-I) is a peptide that induces cell division and differentiation in a wide range of tissue and cell types [3–6]. A major site for the synthesis of circulating IGF-I is the liver, but IGF-I peptide is also produced locally in a number of tissues. Therefore, IGF-I likely has both paracrine and autocrine functions. Most actions of IGF-I are thought to be mediated by binding to two IGF receptor types, called type 1 and type 2 receptors [7]. These receptors are distributed throughout the GI tract of several mammalian species, including the rat [8–16].

The expression of IGF-I mRNA in rat small intestine was first detected by Lund et al. [17] and was later reported in mammalian gastrointestinal tissues of numerous other species [9,18–24]. Nevertheless, intestinal levels of IGF-I mRNA during the suckling period, when IGF-I peptide is delivered via milk, are not well described. In order to evaluate IGF-I gene expression in the developing small intestine, we have established a very sensitive reverse transcription (RT) competitive-polymerase chain reaction (PCR) assay, and by using this technique, we have quantified intestinal levels of IGF-I mRNA in suckling and adult rats.

Exact cellular localization of IGF-I mRNA transcripts in the small intestine is not defined. We have recently reported localization of epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) mRNA in the small intestine of rats using a sensitive non-radioactive in situ hybridization technique [25]. Based on our previous experience with this method, we used this technique to clarify the localization of IGF-I transcripts in the small intestine of suckling and adult rats.

2. Materials and methods

2.1. Tissue preparation

Sprague-Dawley male rats bred in our colony were used in this experiment. Adult rats were fed a standard laboratory diet (Lab Blox, Tech-Lab, Indianapolis, IN). Fifteen adult 65- to 75-day-old male rats (450–520 g) and thirty suckling 12-day-old male and female rats (31–35 g), originally from six different litters, were used in this study. Rats were anesthetized and killed by decapitation. Jejunum was quickly removed and snap-frozen in liquid nitrogen and stored at −75°C until processed for RNA extraction. Small parts of tissues were oriented for sectioning by embedding in OCT compound (Tissue Tek II, Miles, Elkhart, IN), frozen in isopentane (cooled in liquid nitrogen) and stored at −75°C for in situ hybridization.

2.2. RNA extraction

Total RNA was isolated from rat tissues using a modification of the single-step RNA isolation method [26]. Briefly, 1 ml of TRIzol Reagent (Gibco BRL, Gaithersburg, MD) was homogenized with about 100 mg of frozen tissue. Total RNA was recovered from the aqueous phase by precipitation with isopropyl alcohol, the concentration was calculated from the absorbance at 260 nm (A_{260}) and the purity was determined by A_{260}/A_{280} ratio (Spectronic 1000, Milton Roy Company, Rochester, NY). The integrity of RNA samples was verified by electrophoresis on 1.2% agarose gels (SeaKem GTG Agarose, FMC BioProducts, Rockland, ME) containing formaldehyde (2.2 M) and ethidium bromide in 1× MOPS [40 mM MOPS (pH 7.0), 10 mM sodium acetate and 1 mM EDTA (pH 8.0)].

2.3. Synthesis of IGF-I competitor DNA

A 314 base pair (bp) long fragment of DNA containing rat IGF-I primer sequences was constructed using the PCR MIMIC Construction Kit (Clontech, Palo Alto, CA). In order to be able to separate and quantify PCR products from competitive PCR on agarose gel, the size of competitor (standard) DNA was designed 167 bp shorter than the size of PCR product amplified from rat IGF-I mRNA. The competitive DNA was prepared in a two-step PCR reaction according to the standard manufacturer's protocol (Clontech). The competitor DNA was purified on a CHROMA SPIN column (Clontech), quantified by spectrophotometry, and stored in aliquots at −75°C.

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2.4. Reverse transcription (RT)

Single-stranded complementary DNA (cDNA) was reverse-transcribed from 750 ng of total RNA in a 10 μl reaction mixture containing 25 U of murine leukemia virus reverse transcriptase, 2.5 μM random hexamers, 10 U of RNase inhibitor, 1 mM of each dNTP, 5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl (pH 8.3). All chemicals used were from Perkin Elmer, Norwalk, CT. The reaction mixture was incubated for 20 min at room temperature, 25 min at 42°C, and then the enzyme was heat inactivated for 5 min at 99°C. The reaction mixture was kept at 4°C until the start of PCR amplification. The amounts of total RNA used in the RT reactions were calculated from the absorbance at 260 nm, and verified by densitometry of the 28S ribosomal RNA band separated on denaturating agarose gels (by Gel Doc 1000 Documentation System with Molecular Analyst/PC software, BIO-RAD, Hercules, CA).

2.5. Competitive PCR amplification

The reverse transcriptase product (10 μl) was mixed with a 40 μl PCR reaction mixture containing 0.2 μM of each of the upstream and downstream rat IGF-I primer, 1 U of Taq DNA polymerase (Perkin Elmer), 1 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl (pH 8.3). Two μl of a defined concentration of competitor DNA was added to each tube. Rat IGF-I primers were synthesized by Biosource International, Menlo Park, CA. The IGF-I sense primer was 5′-ACATCTCCCATCTCTCTGGA-3′ (nucleotides 99–118); the IGF-I antisense primer was 5′-TCTGAGTCTTGGGCATGTCA-3′ (nucleotides 561–580) [27]. The mixture was subjected to 30–35 cycles of PCR amplification on a Perkin Elmer DNA Thermal Cycler 480. The PCR cycle conditions included: melting for 1 min at 94°C, annealing for 1 min at 63°C and primer extension for 2 min at 72°C.

Twenty μl of the amplification products were separated on 2% agarose gel in 40 mM Tris-acetate, 1 mM EDTA (pH 8.0) buffer, stained with ethidium bromide. The band densities were evaluated by using Gel Doc 1000 system with Molecular Analyst/PC software (BIO-RAD). In order to assess relative amounts of natural IGF-I PCR product, the density ratios between the competitor and the target bands were calculated. The PCR products were size-verified using a DNA ladder and the identity of natural IGF-I PCR band was confirmed by Southern blot analysis using a digoxigenin-labeled (DIG-labeled) antisense IGF-I RNA probe. Control reactions without reverse transcriptase or without total RNA yielded no product bands.

2.6. In situ hybridization and probes

In situ hybridization was performed using a technique previously described in detail [25]. A 786 bp *Eco*RI fragment of rat IGF-I cDNA, P2 [27], cloned into plasmid pGEM 4Z (Promega, Madison, WI) was kindly provided by Dr. P.K. Lund, University of North Carolina, Chapel Hill, NC. Antisense and sense RNA probes were generated by in vitro transcription using the RNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) according to the standard manufacturer's protocol. Size of the RNA probes was verified by electrophoresis. DIG incorporation was measured by dot blot analysis of serial dilutions of the probes. Cryostat sections of rat jejunum (6–8 μm) were treated with proteinase K at 37°C for 10 min, then prehybridized with hybridization buffer (HB) at 37°C for 2 h. Hybridization buffer contained: 50% formamide, 0.75 M NaCl, 25 mM EDTA, 1× Denhardt's, 0.1 M dithiothreitol (DTT), 0.2% sodium dodecyl sulfate (SDS), 100 μg/ml yeast transfer RNA, and 300 μg/ml heat denatured herring sperm DNA. After prehybridization, 20 μl of HB (without herring sperm DNA) containing DIG-labeled antisense or sense (for control sections) RNA probes were added to each section, and the slides were incubated in a moist chamber at 55°C overnight. The signal was detected by using the Nucleic Acid Detection Kit (Boehringer Mannheim) according to the standard manufacturer's protocol. The sections were lightly counterstained with hematoxylin and stored in the dark.

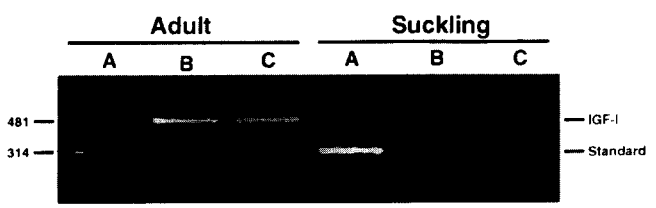

Fig. 1. IGF-I mRNA intestinal levels quantified by RT competitive-PCR. Total RNA extracted from jejunum of adult and suckling rats was reverse-transcribed into cDNA. A constant amount of intestinal cDNA was co-amplified with two-fold serial dilutions of the competitor DNA. The natural 481- and competitor (standard) 314-bp-long PCR products after amplification for 30 cycles were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide. The following amounts of the competitor DNA were used: lanes A, 12.8×10^{-2} amol; lanes B, 6.4×10^{-2} amol; lanes C, 3.2×10^{-2} amol.

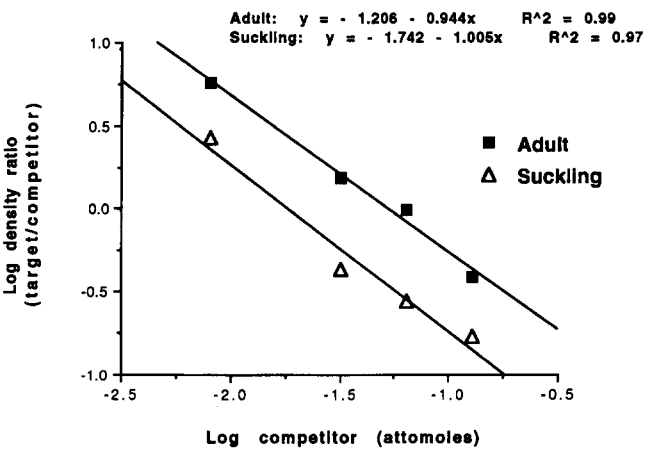

Fig. 2. Quantification of IGF-I mRNA by using RT competitive-PCR. Competition between natural IGF-I PCR product and competitor PCR product (used in four different concentrations) was determined by computer imaging the intensity of 481 and 314 bands on agarose gel. Mean values of the logarithm of the density ratio of natural (target) to competitor (after correction for the difference in size) were plotted as a function of the logarithm of the amount of competitor added to the reaction. The total amounts of IGF-I mRNA in the samples were calculated from the competitor values at the equivalence points (log density ratio = 0).

3. Results

3.1. Quantification of IGF-I mRNA

The sensitive RT competitive-PCR was used for quantification of IGF-I mRNA levels in the small intestine. Fig. 1 shows a typical picture resulting from RT competitive-PCR used for the quantification of IGF-I mRNA in the jejunum of adult and suckling rats. To correct for differences in nucleotide length, the band density of the competitor was multiplied by 1.5 (i.e. 481/314). The logarithm of the density ratio of the target band to the competitor band was then plotted versus the logarithm of the starting concentration of competitor DNA (Fig. 2). At the competition equivalence point (log density ratio = 0), the original concentration of IGF-I cDNA in the PCR reaction corresponds to the initial concentration of competitor DNA. The values obtained for the equivalence point were multiplied by a factor of 2, because the competitor DNA was double-stranded and the target cDNA was single-stranded at the beginning of the PCR reaction.

The total amounts of IGF-I mRNA (mean ± S.E.M.) calculated in rat jejunum were 0.141 ± 0.013 and 0.049 ± 0.004 amol of IGF-I mRNA per μg of total RNA in adults (n = 10) and in 12-day-old sucklings (n = 12), respectively. However, it is necessary to assume that the reverse transcription of mRNA into cDNA is performed with high efficiency. Since all samples were assayed under the same reaction conditions, we consider

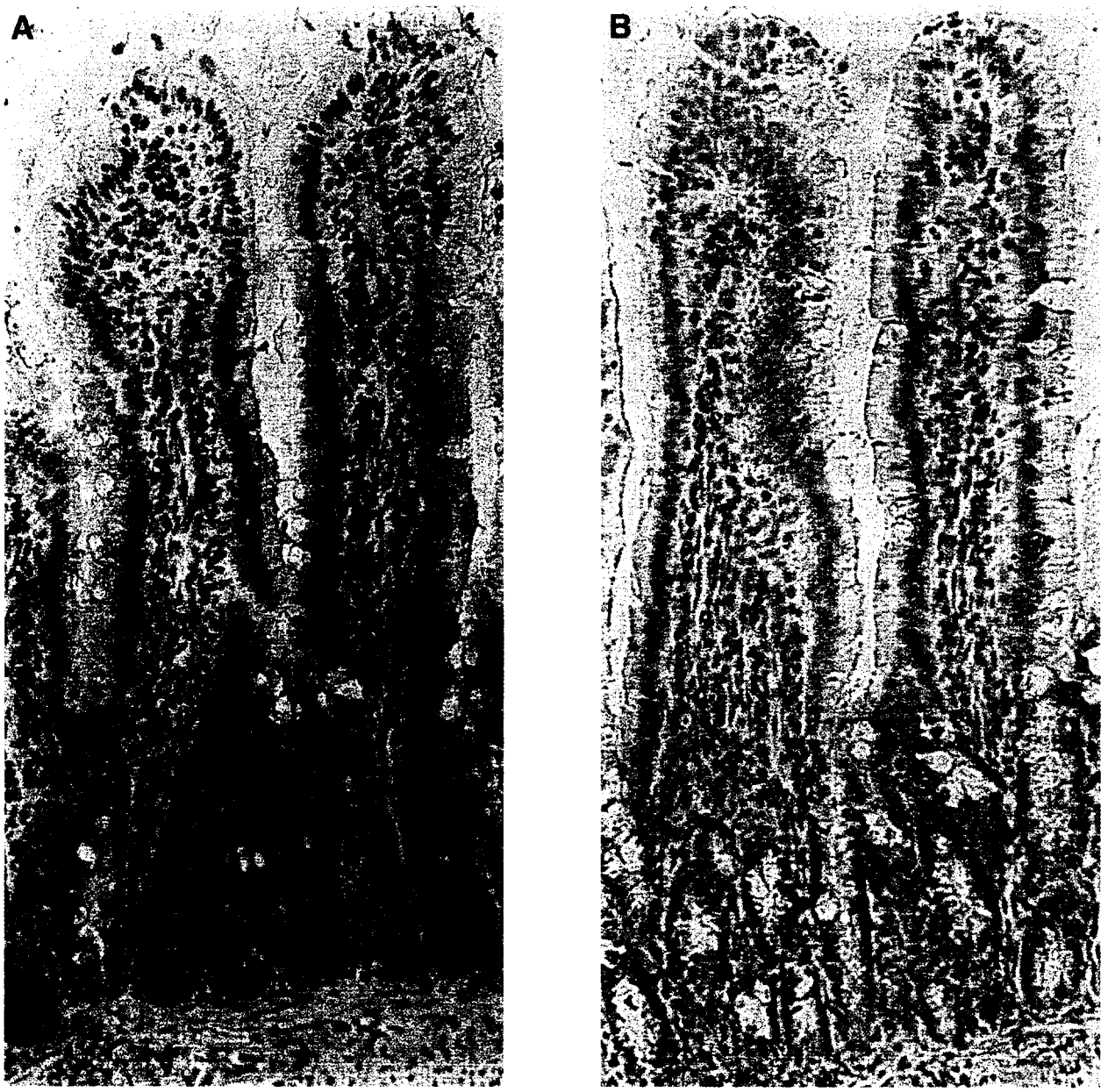

Fig. 3. In situ hybridization of IGF-I mRNA with DIG-labeled RNA probes in sections of adult male rat jejunum. A strong positive signal for the presence of IGF-I mRNA is noted in the crypt cells (A). Hybridization with control IGF-I sense-RNA probe (B). Original magnification ×200.

it more appropriate to present our results as a relative difference between adult and suckling intestinal tissues. In summary, the expression of IGF-I mRNA in rat jejunum was about 3-fold higher in adults than in sucklings.

3.2. *Localization of IGF-I mRNA*

To determine the histological localization of IGF-I mRNA in the rat GI tract, in situ hybridization was performed.

In duodenum and jejunum of adult male rats, an intense positive staining was evident in the intestinal crypt cells (Fig. 3A). The enterocytes of the lower half of the villus either exhibited faint, or in most cases, no staining for the presence of IGF-I mRNA. No staining was observed in the upper half of the villus. After hybridization with the sense IGF-I probe, no signal was detected (Fig. 3B). Greater magnification of intestinal crypts suggested localization of IGF-I mRNA mainly to the enterocytes from the upper part of the crypt and in the goblet cells (Fig. 4). One out of four animals exhibited a very light 'positive' staining in a few lamina propria cells. However, the same intensity of staining was also observed in serial sections after hybridization with the sense control probe.

In suckling male rats (12 days old), no positive staining for the presence of IGF-I mRNA was detected either in the crypt cells or in the lamina propria after hybridization with the antisense probe (results not shown).

4. Discussion

Although the presence of IGF-I mRNA in rat intestinal

Fig. 4. Detail of the section from rat jejunum. A strong positive signal is detected in the enterocytes from the upper part of the crypts and in the goblet cells. Original magnification ×450.

tissue has been previously reported, there is no detailed information regarding the IGF-I mRNA levels and the cellular site of IGF-I gene expression in developing rat small intestine. In this study, we have used a very sensitive RT competitive-PCR to measure the IGF-I mRNA levels in the rat small intestine. The relative abundance of IGF-I mRNA was approximately 3 times higher in adult rats, in comparison with suckling rats. An in situ hybridization technique with non-radioactive DIG-labeled RNA probes was used for the cellular localization of IGF-I transcripts in the rat small intestine. IGF-I mRNA was clearly detected in the crypt cells of adult male rats. A signal for the presence of IGF-I mRNA was also apparent in the enterocytes from the lower part of the villus. In contrast to adults, IGF-I transcripts were not detected in the small intestine of suckling rats.

The biological functions of IGF-I within the small intestine are not completely known. However, a number of studies support the speculation that proliferative growth in the crypt epithelium can be stimulated by IGF-I. The expression of IGF-I mRNA in the GI tract has been studied mainly in rats. Lund et al. [17] detected IGF-I mRNA in fetal and adult rat intestine by using Northern blot analysis, and these findings first suggested the rat intestine as a site of IGF-I synthesis. The expression of IGF-I mRNA in the rat stomach during development (from late fetal to 50-days postnatal) was studied by using solution hybridization/RNase protection

assays [18]. Stomach IGF-I gene expression dramatically declined during the first postnatal week. Albiston et al. [20] compared the abundance of IGF-I mRNA throughout the adult rat GI tract. Stomach and colon exhibited significantly higher IGF-I expression, in comparison with the small intestine. Recently, Winesett et al. [23] presented a detailed study concerning the regulation of IGF-I system in the rat small intestine during altered nutritional status. The changes in jejunal mass in this study (during fasting and after refeeding) correlate with changes in levels of serum IGF-I and jejunal IGF-I mRNA. Fasting significantly reduced the abundance of IGF-I mRNA in the jejunum. The authors suggest that circulating and locally expressed IGF-I contribute to changes in jejunal mass induced by fasting and refeeding.

Large amounts of biologically active peptides are present in mammalian milk, including IGF-I [28–30], and play important roles during development. Rat milk [31,32] contains significant quantities of IGF-I peptide (16.0 ± 6.9 ng/ml) and it is likely that the GI tract is a target tissue for IGF-peptide [33,34]. A recent study from our laboratory showed that radiolabeled IGF-I and -II delivered orogastrically with milk to the newborn rat were recovered from the gastrointestinal lumen and tissue [35].

Previously, we have shown differences in the gene expression and peptide levels of EGF and TGF-α in the developing rat small intestine [25,36]. Based on these observations, we decided to quantify and localize IGF-I mRNA in the developing small intestine. IGF-I transcripts were not detected in adult or suckling intestinal samples by the Northern blot technique, probably because of the low sensitivity of this method. A very sensitive RT-PCR detected IGF-I gene expression in intestinal samples from both age groups, but was unable to distinguish the quantitative differences between suckling and adult rats (results not shown). Reverse transcription competitive-PCR was established in order to measure small differences in the relative amounts of IGF-I mRNA with reasonable accuracy and reproducibility. As little as a 2-fold change was discernible, making the method quantitatively comparable to Northern blot or RNase protection assay, and having the sensitivity and speed possible only with PCR. Results from RT competitive-PCR revealed about 3-fold higher levels of IGF-I mRNA transcripts in the rat small intestine of adults than in sucklings.

Cellular localization of IGF-I peptide and mRNA in the human fetal GI tract was described by Han et al. [24,37]. Interestingly, IGF-I immunoreactivity was only observed in the epithelial cells of the mucosa with a higher intensity of staining in the villus than in the crypt cells. In contrast, in situ hybridization localized IGF-I transcripts to lamina propria and submucosa. Winesett et al. [23] observed a low signal for IGF-I expression in the cells of the lamina propria not only after hybridization with antisense probe, but also with sense (control) probe. The authors speculate that their difficulties in IGF-I localization were due to the low abundance and/or widespread, low-level expression of IGF-I mRNA in multiple cell types.

In our study, we localized the expression of IGF-I mRNA to the crypt cells of adult rat duodenum and jejunum. A strong signal in the crypt enterocytes and goblet cells was elucidated upon higher magnification. The presence of IGF-I receptors preferentially in crypt cells [8,12], together with our results from in situ hybridization, suggest an autocrine/paracrine role for IGF-I in the rat small intestine. Moreover, the decrease in detection of IGF-I transcripts from the crypt to the villus tip corresponds with the decreasing density of IGF-I receptors in the same area. These results invite speculation that IGF-I participates more in the proliferation of crypt cells than in the differentiation of mucosal cells, migrating from crypt to villus tip. Steeb et al. [38] and Zhang et al. [22] recently published studies demonstrating that the enhancement of intestinal mucosal crypt depth, villus height, and villus surface area by IGF-I treatment is associated with a significant increase in epithelial cell proliferation.

We were unable to localize IGF-I mRNA in the developing small intestine using the in situ hybridization technique. Since RT competitive-PCR revealed significant differences in the abundance of IGF-I mRNA in suckling and adult rats, we speculate that intestinal IGF-I mRNA levels in suckling rats were probably under the detectability level of the in situ hybridization technique. It seems reasonable to infer that exogenous IGF-I peptide as is present in milk and bile [39], may down-regulate IGF-I mRNA expression in the developing rat GI tract.

These findings support studies suggesting autocrine/paracrine functions of IGF-I in the GI tract, as well as providing a rationale for further investigation of the role and fate of milk-borne IGF-I during intestinal development. The limitation of endogenous GI tract IGF-I synthesis observed in suckling rats may relate to significant enteral IGF-I intake via milk and bile.

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