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Ontogeny of brush border carbohydrate digestion and uptake in the chick

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Ingestion of carbohydrates from the small intestine is the major route of energy supply in animals. In mammals these functions develop both pre- and postnatally and are coordinated for the sucking period. In birds, the physiological requirements are different and hatchlings ingest diets rich in complex carbohydrates soon after hatching. The present study examined the ontogeny of intestinal carbohydrate uptake in the chicken. The expression of mRNA for a brush border enzyme, sucrase–isomaltase (SI), which is critical in disaccharide digestion, was determined, together with that of the Na–glucose transporter (SGLT)-1, which is the major apical glucose transporter, In addition, the homeobox gene cdx, which is involved in inducing SI expression in mammals was examined. It was found that the expression of cdxA mRNA and cdxA protein increased from day 15 of incubation until hatch, after which further changes were small. CdxA protein was shown to bind to the promoter region of SI in the chick indicating that cdxA is similar to the mammalian cdx2. The mRNA of SI was observed at 15 d incubation, increased from 17 d of incubation to a peak on day 19, decreased at hatch and had a further peak of expression 2 d post-hatch. In contrast, the mRNA of SGLT-1 was not detected until 19 d of incubation when a major peak of expression was observed followed by a decrease to low levels at hatch and small increases post-hatch. It appears that both SI and SGLT-1 mRNA are expressed before hatch in the chick, but the ontogeny of expression is controlled by different mechanisms.

Small intestine: Ontogeny: Carbohydrate: Chick

Small intestinal assimilation of carbohydrate is fundamental for energy supply in animals. This process is affected by luminal digestion of polysaccharides, brush border digestion of tri- and disaccharides and uptake of glucose to the enterocyte, predominantly by the brush border Na–glucose transporter (SGLT)-1. However, these mechanisms are either not operating or act at very low rates prenatally and develop with different timetables postnatally in different species (Ferraris, 2001).

The adult intestinal phenotype is established following a series of developmental transitions defined by the expression of various specific sets of genes (transcriptosomes) in the individual cells (Traber, 1997). Regulatory transcription relationships in the small intestine have been examined for the brush border carbohydrate-assimilating enzyme sucrase–isomaltase (SI) (Wu *et al.* 1992; Tung *et al.* 1997) and lactase–phlorizin hydrolase (Traber, 1997; Fang *et al.* 2000) in mammals. SI mRNA is first expressed in the mouse at low levels until the sucking–weaning transition (Tung *et al.* 1997). Transgenic mouse studies have indicated that the patterns of SI expression are regulated by multiple functional *cis* acting DNA elements (Markowitz *et al.* 1995; Tung *et al.* 1997) contained in a 201-nucleotide in the 5′-flanking region of the gene. DNA regulatory elements and associated DNA-binding proteins have been examined in the promotor region (Suh *et al.* 1994; Wu *et al.* 1994) and at least three groups of transcriptional proteins have been identified, including caudal-related cdx, hepatocyte nuclear factors and nuclear proteins that interact with a GATA-binding site (Traber, 1997). Studies have indicated that hepatocyte nuclear factor-1 and cdx2 (caudal homeobox gene) bind to the SIF3 element of the SI promoter and can partially explain the spatial and temporal regulation of SI expression in the neonatal mouse (Boudreau *et al.* 2001; Krasinski *et al.* 2001). Transcription of the lactase–phlorizin hydrolase gene is also activated by cdx2 binding to the promoter region during enterocyte differentiation (Fang *et al.* 2000); however, the spatial distribution of this and the SI genes along the mature villus differ (Goda *et al.* 1999).

Abbreviations: cdx, homeobox gene of the caudal family; EMSA, electrophoretic mobility shift assay; SGLT, Na–glucose transporter; SI, sucrase–isomaltase.

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The fetal intestine in mammals expresses SGLT1 mRNA (Ferraris, 2001) and in the rat jejunum lactase activity and SGLT1 activity increased with the lactose load during the sucking period (O'Connor & Diamond, 1999), but SGLT1 mRNA appears to be little affected by diet and modulation of activity appears to be translational or post-translational (Ferraris, 2001).

Homeobox genes encode nuclear transcription factors involved in patterning and cell differentiation in development of metazoans. These genes regulate molecules involved in cellular interactions, such as cell adhesion molecules and extracellular matrix components (Edelman & Jones, 1993) and are regulated by other homeobox genes and growth factors (Lorentz *et al*. 1997). Homeobox genes of the caudal family (cdx) are expressed in early stages of development and generally only remain active in the gastrointestinal tract. In mammals, cdx1 was the first homeobox gene discovered in endodermal tissues: it exhibits an increasing gradient expression along the longitudinal axis of the gut and is mainly expressed in undifferentiated crypt cells, whereas cdx2 was found mainly in the villus (Silberg *et al.* 2000). It appears that cdx2 provokes pleiotropic effects triggering cells towards the phenotype of differentiating enterocytes (Lorentz *et al*. 1997).

In the chicken, these processes have not been intensively studied. The cdx genes in the chick differ from mammals and two homeobox genes have been described and termed cdxA (Frumkin *et al.* 1991) and cdxB (Morales *et al.* 1996); cdxA has 95 % homology to the mouse cdx1 and cdx2 in the amino acid sequence of the homeobox domain and cdxB has 95 % homology to the amino acid composition of the mouse cdx4. It was assumed that cdxA acted similarly to murine cdx1 (Frumkin *et al.* 1994) and cdxB to cdx4; however, we have recently shown that cdxA may have a role in enterocyte maturation and that cdxB is expressed at much later developmental stages than cdx4 (Geyra *et al.* 2002). In the chicken following hatching, induction of carbohydrate digestion from exogenous complex carbohydrates occurs over several days (Noy & Sklan, 2001) and lactase–phlorizin hydrolase is not found, or needed, since lactose is not usually fed. However, the ontogeny of the mechanisms of carbohydrate digestion and uptake have not yet been examined in this species.

The present study examines the expression and activity of SI, SGLT1 and cdxA as related to the ontogeny of carbohydrate ingestion in the jejunum of the chick before and after hatch and in addition indicates that cdxA binds to the promoter region of SI in the chick.

Methods

Embryos and chicks

Embryos (Ross × Ross) from 15 d of incubation to 7 d post-hatch were obtained from a commercial hatchery (Kvuzat Yavne, Yavne, Israel) from a maternal flock between 40 and 55 weeks in lay, and chicks were obtained at hatch. Twenty embryos or five chicks were sampled at each age. Time of hatch was defined by removing birds from the hatchery trays as they cleared the shell and 20 mm jejunal segments were taken for further analysis. On hatching, chicks had immediate free access to water and to a commercial starter diet containing 230 g crude protein (N × 6·25) and 56 g fat/kg based on soyabean meal, wheat and maize (Matmor Feedmill, D. N. Evtach, Israel), meeting or exceeding National Research Council (1994) recommendations. All chicks were maintained in temperature-controlled brooders. Chicks were monitored daily for body weight. All procedures were approved by the Animal Care and Welfare Committee of our Institute.

RNA extraction

Total RNA was isolated (Chomczynski & Sacchi, 1987) from the jejunal segment using TRI reagent (10 ml/g tissue) according to the manufacturer's protocol (MRC Molecular Research Center, Cincinnati, OH, USA).

Preparation of cDNA probes for sucrase–isomaltase, sodium–glucose transporter-1, cdxA and β-actin

Two primers were used in a reverse transcriptase–polymerase chain reaction to amplify a 786 bp sequence of the mRNA coding region of the chicken SI gene (Uni, 1998): (forward) 5′-ATGACATCAACAGTGTTCTCTTCAC-3′ and (reverse) 5′-TCTGTCCATGGTCATGCAAATCTTG-3′.

Two primers were used in a reverse transcriptase–polymerase chain reaction to amplify a 970 bp sequence of the mRNA coding region of the chicken SGLT-1 gene (Gal-Garber *et al.* 2000): (forward) 5′-TGGCGGGCTTCTACCGCAGCGAG-3′ and (reverse) 5′- CCCGGTAGGTCACCAGTCCCCAG-3′.

Two primers were used in a reverse transcriptase–polymerase chain reaction to amplify a 189 bp sequence of the mRNA coding region of the cdxA gene (Frumkin *et al.* 1991): (forward) 5′-GAGGACAAAGGACAAGTACCGGG-3′ and (reverse) 5′-CCTTCCTCTCTTTCGCCCTCCG-3′.

Two primers were used in a reverse transcriptase–polymerase chain reaction to amplify a 241 bp sequence of the mRNA coding region of the β-actin gene (Huber *et al.* 1998): (forward) 5′- AACCCTAAGGCCAACCGTGAAAAG-3′ and (reverse) 5′-TCATGAGGTAGTCTGTCAGGT-3′.

The reverse transcriptase–polymerase chain reaction products were visualized on an agarose (1·5 %) gel, stained with ethidium bromide, excised from the gel and purified with DNA isolation system (DNA Isolation Kit; Biological Industries, Kibbutz Beit Haemek, Israel). To confirm that the fragments obtained corresponded to the original sequences, fragments were sequenced by automated sequencing using an Applied Biosystem 373A DNA sequencer (Applied Biosystem, Foster City, CA, USA). Nucleic acid sequences were analysed using the GCG suite programs (Devereux *et al.* 1984).

Northern blot analysis

For Northern blot analysis, 30 μg total RNA, from different ages, was denatured and separated by electrophoresis on agarose (1·5 %)–1·1 M-formaldehyde gel. After

electrophoresis, RNA was transferred overnight by capillary transfer onto a nylon membrane (Hybond-N; Amersham Pharmacia Biotech, Amersham, Bucks., UK), and then fixed on the membrane by u.v. at 340 nm for 2 min. The four probes isolated (see earlier) were used for hybridization. The isolated β-actin cDNA was used to normalize variations in the total RNA loading. The probes were labelled with [^{32}P]dCTP by random priming (Biological Industries). Pre-hybridization was done at 42°C for 4 h, hybridization was conducted at 42°C overnight and a high-stringency wash (0·1 % SDS in 0·015M sodium citrate and 0·15M sodium chloride (1 ml/l) at 60°C) was conducted according to the procedures recommended by Amersham for Hybond N membranes (Amersham Pharmacia Biotech). Blots were exposed for 16 h at −80°C to Kodak XAR 5 film (Kodak, Rochester, NY, USA) in the presence of an intensifying screen.

Western blot analysis

Intestinal tissues were sonicated using an ultrasonic cell disrupter (Microson, Farmingdale, NY, USA), clarified by centrifugation and frozen at −80°C. Tissues were normalized to protein content. Embryonic or intestinal proteins were subjected to electrophoresis on SDS–polyacrylamide gels and transferred onto nitrocellulose (Schleicher and Schuell, Dassel, Germany). Detection of the cdxA protein was performed after blocking the membrane in 5 % bovine serum albumin in 0·05 % Tween (30 ml/l) for 4 h. The primary antibody, 6A4αcdxA from ascites (Frumkin *et al.* 1994) was diluted 1:5000 in 5 % bovine serum albumin in 0·05 % Tween (30 ml/l) and was incubated with the nitrocellulose membrane overnight at 4°C. The membrane was washed for three times for 15 min each with 5 % bovine serum albumin in 0·05 % Tween, the secondary antibody, antimouse antibody coupled to horseradish (*Armoracia rusticana*) peroxidase (Amersham Life Sciences, Amersham, Bucks., UK) which was diluted 1:10 000 in 5 % bovine serum albumin in 0·05 % Tween, was added and incubated for 50 min at room temperature. After washing as previously described, the peroxidase reaction was performed with the ECL kit (Amersham Life Sciences) as recommended by the manufacturer. The films were scanned with a high-resolution scanner and Gel-pro densitometer software (version 3.0; Media Cybernetics, Silver Spring, MD, USA) was applied to determine the amount of mRNA or protein in each band. The amount of mRNA or protein is given in arbitrary units.

Electrophoretic mobility shift assay

In order to elucidate the specific role of cdxA in intestinal gene activation a modification of the electrophoretic mobility shift assay (EMSA) was performed. EMSA is used to detect the interaction of DNA binding proteins with their DNA recognition sequences. The crude nuclear extracts are incubated with a radiolabelled DNA probe. The complexes are separated from the free probe by migration through a non-denaturing polyacrylamide gel, with the complexes migrating more slowly.

Using standard procedures, 250 ng double-stranded oligo was end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labelled oligo was purified from unincorporated [γ-^{32}P]ATP in a final volume of 100 μl. The oligos were stored at −20°C. The oligonucleotide probes used for the SIF1 region were all end-labelled with ^{32}P: GTGCAATAAAACTTTATGAGTA, CCACGTTATTTTGAAATACTCAT (Suh *et al.* 1994). The non-specific probes were (negative control): GGTACTACTTCTAGCTTCGGAA, CCATGATGAAGATCGAAGCCTT.

Equal volumes of each complementary oligo dissolved in water (300 μM) were mixed together and heated to 95°C for 5 min. The annealed duplex oligo was then diluted further with water to a final EMSA stock concentration of 3 μM. The oligo stocks were stored at −20°C.

Nuclear extract preparation

Extracts were prepared from 4 d old chick jejunum. The tissue samples were homogenized. Cells were collected by centrifugation (500 ***g***, 5 min) and resuspended in 1 ml ice-cold PBS, then transferred to a microfuge tube and pelleted briefly in a bench-top microfuge for 30 s at maximum speed (10 000 ***g***, 4°C). Cell pellets were resuspended in 400 μl ice-cold buffer A (10 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid pH 7·9, 10 mM-KCl, 0·1 mM-EDTA, 0·1 mM-ethylene glycol-*O*,-*O*′-bis(2-amino-ethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM-dithiothreitol, 0·5 mM-phenylmethylsulfonylfluoride, 30 mM-diisopropyl-fluorophosphate) and left on ice for 20 min. NP40 solution was added (25 μl, 100 ml/l) and the sample was vortexed vigorously for 1 min at 4°C. The nuclei were pelleted with a 30 s spin and then transferred to a fresh tube containing 150 μl ice-cold buffer C (20 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid pH 7·9, 0·4 M-NaCl, 1 mM-EDTA, 1 mM-ethylene glycol-*O*,-*O*′-bis(2-amino-ethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM-dithiothreitol, 1 mM-phenylmethylsulfonylfluoride) and vortexed vigorously at 4°C for 15 min. The nuclear extracts were cleared by 10 min centrifugation and the supernatant fractions containing the transcription factors were resuspended after desalting in EMSA buffer. A typical EMSA extract gave a protein concentration of 2 mg/ml.

Electrophoretic mobility shift assay super shift

In a total volume of 9 μl, containing a fixed amount of protein, the nuclear extract and specific antibodies to cdxA were combined and incubated for 30 min at room temperature. To this, 1 μl loading buffer was added (90 mM-EDTA, glycerol (300 ml/l), xylene cyanol (0·1 g/l)). The samples were run on an acrylamide (5 %) gel with 0·25 M-Tris–Borate–EDTA buffer at 300 V at 4°C. The radioactive gels were stored at −80°C and exposed to a storage phosphor screen.

Statistics

Least squares means of results are presented from five replicates of four embryos or five chicks after ANOVA as a split plot with time using the general linear models

procedures of SAS (1986; SAS Institute Inc., Cary, NC, USA). Differences between means were tested using Duncan's multiple range test and significance was $P<0{\cdot}05$ unless otherwise stated.

Results

Expression of cdxA mRNA and protein

Expression of cdxA mRNA relative to β-actin was low in the embryo at 15 d of incubation (Fig. 1(A)) and increases in expression were observed from 17 d of incubation until hatching; post-hatch little further increases in expression were observed. Changes in the concentrations of the cdxA protein with embryonic age are shown in Fig. 1(B) and these increased in parallel with changes in mRNA expression.

Expression of sucrase–isomaltase and sodium–glucose transporter-1 mRNA

The expression of mRNA for SI and of SGLT1 relative to β-actin was also determined (Fig. 2). The SI gene was expressed at 15 d of incubation and relative expression changed little by 17 d of incubation; however, at 19 d of incubation a 3-fold increase in expression was observed, and this was followed by a decrease at hatch with a 40 % increase on day 2 post-hatch followed by a decrease to day 3 with little further change by day 7. Expression of mRNA SGLT1 was not detectable at 15 and 17 d of incubation, but high levels of expression were observed at 19 d. Expression decreased by approximately 10-fold at hatch, after which small increases occurred until 7 d post-hatch.

Gel-shift assay

In order to examine whether the SI gene response elements formed specific DNA–protein and DNA–protein–antibody complexes, a gel-shift assay was carried out with chick jejunal nuclear extract (lanes B, C, E and F respectively). The resulting gel is shown in Fig. 3. The SI promoter, jejunal nuclear extract and anti-cdxA antibodies showed a shift in mobility as compared with SI promoter and the nuclear extract, suggesting that cdxA protein interacts with the response element.

Discussion

The newly hatched chick must make the transition from metabolic dependence on the yolk sac to utilization of

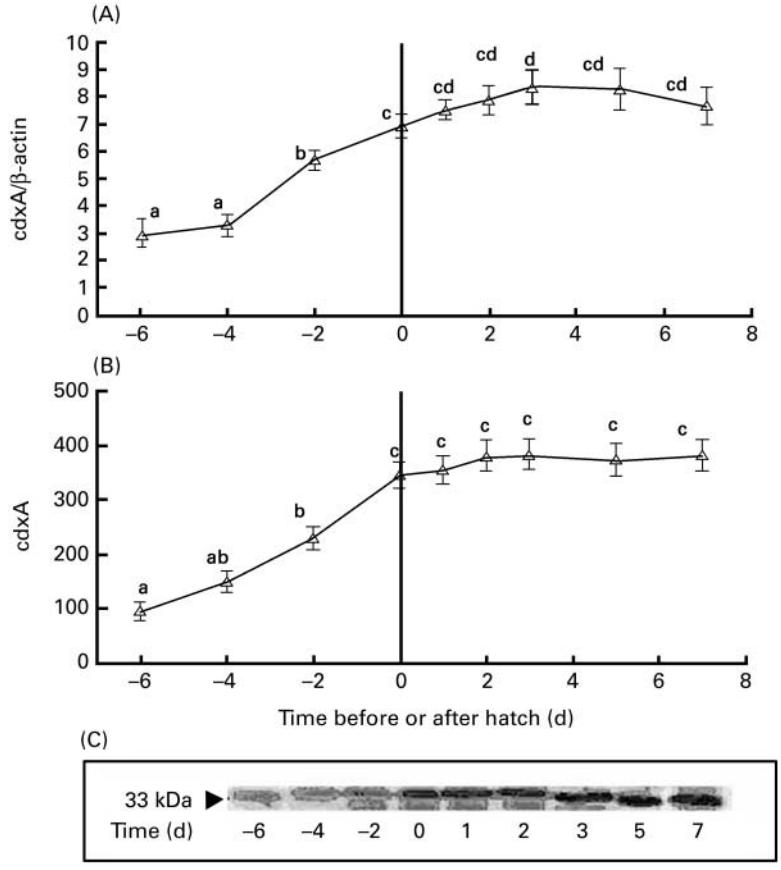

Fig. 1. Expression of cdxA (homeobox of the caudal family) mRNA (A) and cdx protein (B) and a representative Western blot of the 33 kDa protein (C) from 15 d of incubation until 7 d post-hatch. Concentrations of cdx mRNA are normalized to β-actin mRNA concentrations. For details of procedures, see p. 748. Values are means with their standard errors (n 5). [a,b,c,d]Mean values with unlike superscript letters were significantly different ($P<0{\cdot}05$).

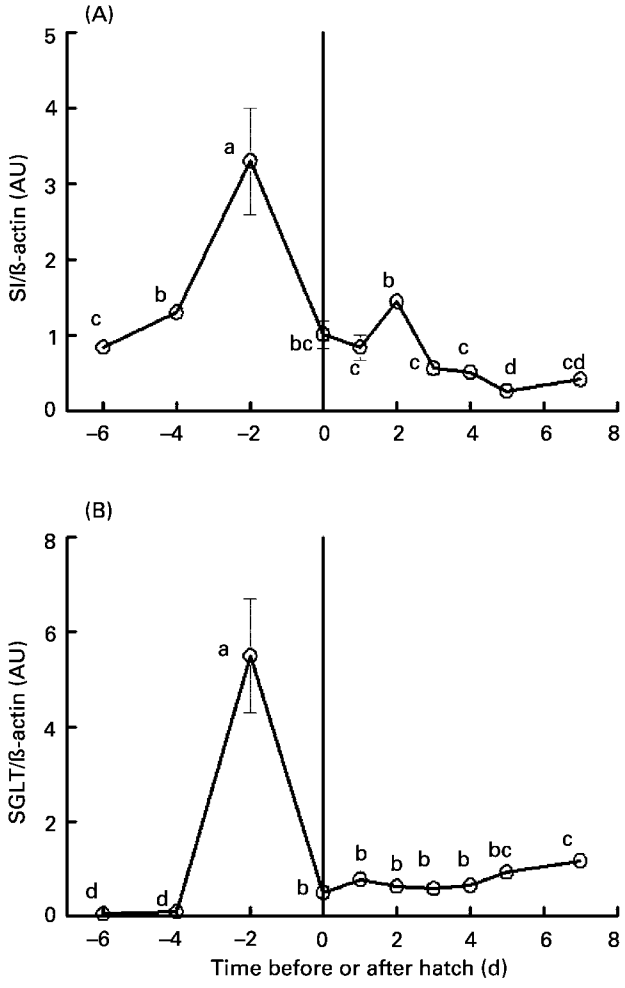

Fig. 2. Expression of sucrase–isomaltase (SI) mRNA (A) and Na–glucose transporter (SGLT)-1 mRNA (B) from 15 d incubation until 7 d post-hatch. Concentrations are normalized to β-actin concentrations. For details of procedures, see p. 748. Values are means with their standard errors (n 5). ^{a,b,c,d}Mean values with unlike superscript letters were significantly different ($P<0{\cdot}05$).

exogenous feed rich in carbohydrates. To make this change, the necessary digestive and absorptive mechanisms must be initiated. The present study examined temporal changes in expression of mRNA of two of the major factors involved: SI, an apical membrane-anchored enzyme involved in disaccharide digestion, and SGLT1, the apical membrane-anchored glucose transporter. While mRNA expression is not necessarily correlated with protein activity, for SGLT1 activity this appears to be the case (Ferraris & Diamond, 1997).

In mammals, SI activity is induced by both cdx2 and other transcription factors binding to the SI promotor region. Lactase–phlorizin hydrolase, which is also involved in intestinal carbohydrate assimilation in mammals, is activated temporally at a similar stage, but differentially by transcription factors (Krasinski *et al.* 2001). The present study in chickens indicates that cdxA binds to the SI promotor region in a manner similar to the mammalian cdx2. We have recently suggested on the basis of temporal and spatial data that chicken cdxA is similar to mammalian cdx2 (Geyra *et al.* 2002), rather than cdx1 as originally suggested (Frumkin *et al.* 1994); the present study indicates similar promoter binding activity. In previous studies expression of cdxA in the chick was found from early embryonic stages, where it participates in axial determination during gastulation (Frumkin *et al.* 1994; Geyra *et al.* 2002), and, as shown previously and in the present study, both mRNA and protein concentrations increased in the jejunum with developmental stage, reaching a plateau after hatch. In the mouse intestine, SI is expressed at low levels late in fetal development when the stratified endoderm cells transform into columnar epithelium with nascent villi (Tung *et al.* 1997). This low level of SI is maintained until 16–17 d postnatally, when rapid increases in expression occur pre-weaning which, together with many other changes in gene expression, result in the phenotype of the adult intestine (Boudreau *et al.* 2001). In the chicken, development will obviously be different since there is no sucking phase, and complex carbohydrates must be digested and taken up soon after hatch. In the chicken embryo the temporal pattern of SI expression shows activity before hatch and before any carbohydrate is ingested, with a major increase in expression at 19 d incubation. The present study suggests that the initiation of this transcription is regulated, at least in part, by cdxA, which binds to the SI promoter region and was expressed in increasing quantities during this period. In pre- and postnatal mammals concentrations of mRNA of SI and cdx2 were not correlated (Boudreau *et al.* 2001), as was also apparent in the present study.

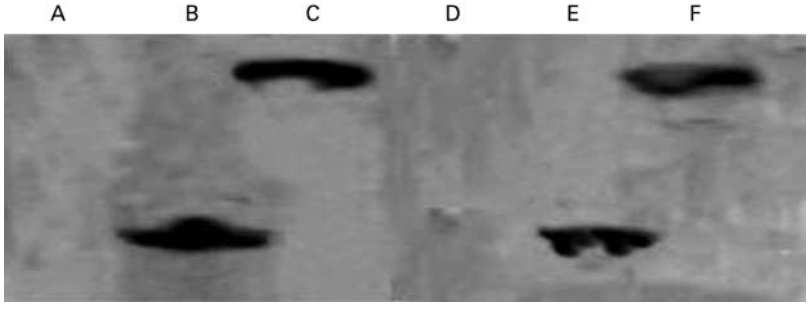

Fig. 3. Gel super-shift mobility assay with anti-cdxA (homeobox of the caudal family) monoclonal antibodies. Lane A, non-specific sequence including nuclear protein extract; lanes B and E, (sucrase–isomaltase (SI) F1 element from SI promoter including nuclear protein extract; lanes C and F, SIF1 element from SI promoter including nuclear protein extract and anti-cdxA monoclonal antibodies; lane D, non-specific sequence including nuclear protein extract and anti-cdxA monoclonal antibodies. For details of procedures, see p. 748.

In the small intestine of mammals and human subjects, active transport of glucose has been observed in the fetus with brush border glucose transport gradually increasing with gestational age, with a spurt in activity observed in the final stages of gestation (Ferraris, 2001). Expression of SGLT1 in sucking rats is not up-regulated by dietary carbohydrate intake (Jiang & Ferraris, 2001), although it has been speculated that this lack of regulation may be restricted to enterocytes synthesized neonatally (Ferraris, 2001). Typically, enterocytes containing SGLT1 are observed in the upper portion of the villus and the distribution along the crypt–villus axis may be influenced by the carbohydrate composition of the diet (Ferraris, 2001). SGLT1 expression has not been found to be influenced by cdx in mammals. The major transport of glucose in mammals is by SGLT1; however, GLUT5 is capable of some glucose transport (Ferraris, 2001), whereas in some birds passive transport has been reported to be a major route of transport (Chediack *et al.* 2001). Expression of SGLT1 mRNA in the developing chick embryo was not detected until 19 d incubation when a dramatic transient surge in expression was observed, which decreased by hatch, and following ingestion of carbohydrates post-hatch there was a small constant increase in expression until 7 d. This low expression of SGLT1 mRNA close to hatch is consistent with recent studies that have indicated that uptake of glucose was relatively low in hatching chicks and increased gradually post-hatch (Sulistiyanto *et al*. 1999; Sklan & Noy, 2000; Noy & Sklan, 2001). This was attributed in those studies to a possible lack of Na close to hatch (this Na is co-transported with glucose by SGLT1) and also to the presence of hydrophobic yolk in the lumen. The present study, however, indicates that low transporter production may limit uptake in the apical membrane in the chick jejunum immediately post-hatch. The pattern of expression of SGLT1 with development is, however, somewhat different from that of SI. Thus the chick, which must assimilate exogenous carbohydrates soon after hatch, expresses both SI and SGLT1 mRNA during the late embryonic stages, but the ontogeny of this process appears to be controlled by different mechanisms. The ability to absorb exogenous carbohydrates changes rapidly post-hatch, but the capacity to absorb glucose may be limited in the immediate post-hatch period by SGLT1 availability.

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