The predominant nutrient source in embryonic birds is the lipid-rich yolk. After hatch, a rapid transfer to the utilization of exogenous feed high in carbohydrate and protein must occur. This change is concomitant with intense intestinal growth in which the small intestine develops rapidly and preferentially in the immediate posthatch period (1). At hatch, crypts are not detectable in the chick small intestine; these become defined within hours after hatch and proceed to grow and proliferate rapidly for ~4 d (2,3). All epithelial cells in the chick intestine are proliferating at hatch, and, as crypts develop, proliferation becomes localized mainly to the crypts within 5–8 d, although a percentage of villus enterocytes continue to proliferate after this period (2,3).

Because poultry eggs do not hatch simultaneously, but show a Gaussian curve of hatching with time, logistics generally determines that birds are removed from the hatchery only after the maximal number have hatched. This means that some birds may be without food and water for >48 h before initial access to feed. It has been previously reported that lack of access to feed for 48 h depresses intestinal development and growth through the time of marketing in chicks and poults (4). However, the effect of lack of access to food on the transcriptional control of intestinal development has not been examined.

Members of the homeobox (hox) gene family share a conserved sequence of 180 nucleotides known as the homeodo-minal. Homeodomain-bearing proteins serve as transcription factors, which play important roles in pattern formation and in determining the positional identity of cells during development (5). These transcription factors bind DNA regulatory sequences through the conserved homeodomain, a helix-turn-helix motif rich in basic amino acids (6). The homeobox genes are expressed in embryos where they participate in axial patterning and in the definition of cell identity (7). Although their expression is generally reduced at birth, some homeobox genes remain active in organs that display active cell renewal such as the intestine (8). The mammalian homeobox genes, cdx1, cdx2 and cdx4 have been described in the murine embryo (9–12) and in human intestine (13–17); two caudal homeobox genes have been described in chickens, i.e., cdxA (18) and cdxB (19). There is 95% similarity between the chick cdxA and the rat, mouse and human cdx1 and cdx2 in the amino acid sequence of the homeobox domain. The cdxB also has 95% similarity to the mouse cdx4 in the homeobox domain (19). The cdxA protein appears at ~stage 3 (20) when the primitive streak in the chicken embryo assumes its elongated form (21). Gut closure in the chicken embryo takes place over a period of 3.5–4 d from the formation of the head fold (stage 6) to ~d 5 of incubation (22). CdxA plays a major role in gut development during embryogenesis and posthatch and is expressed exclusively in the chick small intestine (22). However, quantitative aspects of cdxA mRNA or protein expression have not been addressed. Cloning and sequencing of cdxB (previously known as cHox-cad2) was reported by Serrano et al. (23), and the highest expression of chick cdxB...
mRNA was during early neurulation. The murine homologue cdx4 exhibits a graded expression pattern with greater concentration in the posterior regions and is present at high levels in all three germ layers (10). This homeobox gene is expressed from 7 d postconception (gastrulation) until 10.5 d postconception (late neurulation). CdxB mRNA was found in the mesoderm and endoderm, and is found primarily in the proliferative posterior region of the embryo (19).

Because cdx genes have been described as playing a major role in regulation of gut formation and intestinal gene control (5,11,22), cdxA and cdxB expression were determined during embryonic and posthatch intestinal development in chicks. In addition, the effects of lack of access to feed on cdx expression were examined.

MATERIALS AND METHODS

Embryos and chicks. Embryos and chicks (Ross × Ross) from the age of 5 d of incubation to 7 d posthatch were obtained from a commercial hatchery from a maternal flock between 40 and 55 wk in lay (Kvuzat Yavne, Yavne, Israel). Thirty embryos or chicks were sampled at every age and treatment group. Time of hatch was defined by removing birds from the hatchery trays as they cleared the shell. Whole embryos were taken from 5 d of incubation for analysis; 2 cm jejunal segments were taken for further analysis from 15-d-old embryos to 7-d-old chicks. In the starvation experiment, the control group had immediate free access on hatching to water and to a diet (24) formulated to meet or exceed NRC (25) requirements for the entire experimental period (control). The starved group had no access to feed from 7 d postconception (gastrulation) until 10.5 d postconception (late neurulation). The cdxA mRNA coding region of the cdxA gene (18):

5'-GAGGACAAAGGACAAGTACCGGG-3' (sense primer corresponding to coding nucleotides 397–419) and 5'-CCTTCTCCTCTTTTCCGCTTCCG-3' (complement primer corresponding to coding nucleotides 566–584).

The following primers were used in a reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify a 189-bp sequence of the mRNA coding region of the cdxA gene (18):

5'-GAACCAGAGTCAC-3' (complement primer corresponding to the original sequence, fragments were sequenced by automated sequencing using an Applied Biosystem 373A DNA sequencer (Applied Biosystem, Foster City, CA). Nucleic acid sequences were analyzed using the GCG suite programs (28).

Northern Blot. For Northern blot analysis, 30 μg of total RNA was denatured and separated by electrophoresis on 1.5% agarose/1.1 mol/L formaldehyde gel. After electrophoresis, RNA was transferred overnight by capillary transfer onto a nylon membrane, Hybond-N (Amer sham Pharmacia Biotech, Bucks, UK) and then fixed on the membrane by UV at 340 nm for 2 min. Hybridization. Three probes were used for hybridization: 1) the isolated 189-bp cDNA fragment of chicken cdxA; 2) the isolated 237-bp cDNA fragment of cdxB; and 3) the β-actin cDNA, which was used to normalize variations in the total RNA loading. The probes were labeled with 32P-dCTP by random priming (Biological Industries). Prehybridization was at 42°C for 4 h, hybridization was conducted at 42°C overnight and a high stringency wash [saline sodium citrate/0.1% SDS at 65°C] was conducted according to the procedures recommended by Amersham for Hybrid N membranes (Amer sham). Blots were exposed for 16 h at −80°C to Kodak XAR-5 film in the presence of an intensifying screen. The 2.6-, 2.6- and 2.2-kb bands were visualized using cdxA, cdxB and β-actin probes, respectively.

Western blot. In the analysis of cdxA expression in the distinct endoenterocyte populations, only 5 × 106 separated cells were taken for analysis. In all other experiments, whole tissue was homogenized for analysis. Intestinal cells were lysed in NP-40 lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% NP-40 with 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride, leupeptin and pepstatin (10 mg/L each), aprotinin (20 μg/L]). Tissues and cell extracts were sonicated using an ultrasonic cell disintegrator (Micoson, Farmingdale, NY), clarified by centrifugation at 13,000 × g for 5 min at 4°C, and frozen at −80°C. Tissues and cells were normalized to a protein content of 20 μg/lane measured with a protein assay kit (Bio Rad, Hercules, CA). 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 was performed after blocking the membrane in TBS-Tween, 30 g/l bovine serum albumin (BSA) for 4 h. The primary antibody, 6A4a (anti-cdxA from ascites (22) was diluted 1:5000 in TBS-Tween, 30 g/L BSA and was incubated with the nitrocellulose membrane overnight at 4°C. The membrane was washed three times for 15 min each with TBS-Tween; the secondary antibody, antimouse antibody coupled to horseradish peroxidase (Amersham) which was diluted 1:10000 in TBS-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) as recommended by the manufacturer.

Differential isolation of intestinal epithelial cells. Differential isolation of villi and crypt region cells was carried out by a modification of the method of Weiser (29), described by Brown and Sepulveda (30) and Ferraris et al. (31). After removal, a jejunal segment was rinsed thoroughly with ice-cold PBS and flushed twice with ice-cold PBS containing 1 mmol/L DTT. Then the segment was everted, placed on a polyethylene transfer pipette and incubated at 37°C for 12 min in a citrate buffer containing (mmol/L): 96 NaCl, 1.5 KCl, 27 Na citrate, 8 KH2PO4 and 5.6 Na2HPO4, pH 7.3. The segment was then transferred into a plastic tube at 37°C containing 0.025% BSA, 1.5 EDTA, 0.5 DTT and 1g/L BSA. The plastic tube was then placed in a shaking-water bath (37°C) for 35 min. The cells detached during the incubation were collected in the plastic tube and centrifuged at 1200 × g for 3 min. The pellet was then mixed with NP-40 lysis buffer. The remains of the mucosa were scraped, washed with PBS and mixed with NP-40 lysis buffer. The nature of the isolated cells was determined under a light microscope. Of the cells detached during shaking, 94% had distinct morphological features of mature enterocytes. Of the cells scraped from the mucosa remains, 85% were nonpolar crypt epithelial cells and the remaining cells were mainly fibroblasts.

Denstometric analysis. The cdxA, cdxB and β-actin mRNA films and the cdxA protein film were scanned with a high resolution scanner, and the gel-pro densitometer software (Version 3.0, Media Cybernetics, Silver Spring, MD) was applied to determine the amount of mRNA or protein in each band. The amount of mRNA or protein is given in arbitrary units (AU).

2 Abbreviations used: BSA, bovine serum albumin; DTT, dithiothreitol; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcriptase-polymerase chain reaction.
Immunohistochemical analysis. For immunohistochemistry, a mid-intestinal segment was taken from the 15-d incubation group, and 0.8-cm jejunal segments were removed, washed with saline and fixed overnight at 4°C in 4% paraformaldehyde in all groups. After dehydration, the tissues were paraffin embedded, and 5-μm serial sections were prepared. The sections were then incubated in a temperature-controlled microwave at 92°C to enhance epitope recognition. The anti-cdxA monoclonal antibody used was made by the 6A4 clone as described by Frumkin et al. (21). The antibody used for immunohistochemistry was obtained from ascites fluid and was diluted 1:250. After the primary antibody, we used the EnVision + system (Dako, Glostrup, Denmark) in which a polymeric conjugate consists of a large number of peroxidase and secondary antibody molecules bound directly to an activated dextran backbone (32).

For proliferating cell nuclear antigen (PCNA) staining, the slides were incubated in 3% hydrogen peroxide in methanol for 10 min to quench endogenous peroxidase. PCNA-positive cells were measured by use of monoclonal anti-PCNA antibody followed by the use of peroxidase-ABC (Zymed PCNA staining kit, Zymed Laboratories, San Francisco, CA) according to the manufacturer's directions. Counterstaining was with hematoxylin and slides were dehydrated and mounted in Histomount (Zymed).

Statistical analysis. Least-squares means of results are presented after factorial ANOVA with treatment and time as main effects using the General Linear Models procedures of SAS (33). Differences between means were tested using t tests between each pair and significance was P < 0.05 unless otherwise stated.

**Table 1**

<table>
<thead>
<tr>
<th>Age, d</th>
<th>Villus length, μm</th>
<th>Villus width, μm</th>
<th>Surface area, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Starved</td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>320 ± 6.9d</td>
<td>325 ± 7.1bc</td>
<td>59.2 ± 2.6b</td>
</tr>
<tr>
<td>2</td>
<td>335 ± 4.9d</td>
<td>322 ± 3.8c</td>
<td>60.0 ± 6.1b</td>
</tr>
<tr>
<td>3</td>
<td>382 ± 2.9c</td>
<td>317 ± 4.3bc</td>
<td>63.3 ± 6.1b</td>
</tr>
<tr>
<td>4</td>
<td>477 ± 6.8b</td>
<td>329 ± 3.6bc</td>
<td>74.1 ± 5.1ab</td>
</tr>
<tr>
<td>5</td>
<td>507 ± 12.1ab</td>
<td>351 ± 9.3bc</td>
<td>95.8 ± 6.1a</td>
</tr>
<tr>
<td>6</td>
<td>507 ± 11.0ab</td>
<td>501 ± 9.3a</td>
<td>97.8 ± 7.1a</td>
</tr>
<tr>
<td>7</td>
<td>508 ± 6.3a</td>
<td>507.8 ± 5.9a</td>
<td>96.4 ± 4.2a</td>
</tr>
</tbody>
</table>

1. Results are means ± SEM, n = 6. * Different from control, P < 0.05; a–d values in columns without a common superscript differ, P < 0.05.
2. No access to feed from 0–2 d posthatch, normal access to feed beginning at 3 d.
posthatch, 91.5% of crypt cells were PCNA positive. In chicks starved for 48 h posthatch, a smaller proportion of proliferating cells in the jejunal crypt was observed (Fig. 2A), and 24 h after feeding, this increased to >70% before reaching a plateau with ~50% of crypt cells proliferating after 5 d. Along the villus, the proportion of proliferating cells decreased rapidly posthatch in fed chicks, with proportions of proliferating cells decreasing to <40% 4 d posthatch. Starving for 48 h induced a more rapid decrease in the proportion of proliferating cells along the villus before feeding; however, after access to feed, the proportion of proliferating cells along the villus increased to 50% at d 4 before decreasing to ~10% at d 7.

Levels of cdxA mRNA and protein expression in embryos and chicks. The jejunal RT-PCR products were used as probes to identify cdxA mRNA. Northern blot analysis of intestinal total mRNA revealed that cdxA is expressed differentially at various stages of development (Fig. 3A). At 5 d of incubation, the level of mRNA was 249 (AU) and increased mRNA transcription was observed at 15 d, with no further change until hatch. However, posthatch, further increases occurred until d 2, which did not change through 7 d posthatch. Western blot analysis (Fig. 3B and C) showed a gradual increase in the level of the cdxA protein from 15 d of incubation to 2 d posthatch after which time values reached a plateau. The concentration of the cdxA protein and mRNA were correlated (r = 0.88, P < 0.01). The expression of cdxA mRNA was not significantly affected by access to feed. However, Western blot analysis (Fig. 4) showed less cdxA protein in starved chicks than in controls at 24 and 48 h posthatch. After access to feed beginning at 48 h posthatch, cdxA protein concentration was increased and was greater than at 96 h posthatch.

Localization of cdxA protein in the jejunal mucosa. At hatch, cdxA was observed in a distinctive, uniformly stained monolayer of cells adjacent to the muscularis mucosa on the luminal side [intervillous epithelium (33,34), Figure 5A]. At 2 d posthatch, the cdxA protein became uniform from the base to the villous tip (Fig. 5B). This pattern was similar at 5 d (Fig. 5C) and 7 d posthatch (data not shown). At hatch, the lower portions of the villi were more strongly stained compared with the villous tip (Fig. 5D); however, from 2 d posthatch, the expression of cdxA protein became uniform from the base to the villous tip (Fig. 5E) and this pattern was maintained at 5 d (Fig. 5F) and 7 d posthatch (data not shown). In starved chicks at 48 and 72 h posthatch, staining was less dense than in the control group (data not shown). However, at 96 h posthatch, the villi from starved chicks were more intensely stained compared with control chicks.

Expression of cdxA protein in villous enterocytes and crypt cells. To further elucidate the distribution of cdxA along the crypt-villus axis, enterocytes were differentially isolated from the villus and crypts at 2 (Fig. 6A) and 7 (Fig. 6B) d posthatch. Western blots indicated that the amount of cdxA/cell expressed along the villus was greater than in the crypts at both time points. The ratios of villus/crypt cdxA on d 2 and 7 were 2.79 ± 0.09 and 2.64 ± 0.10, respectively, P < 0.05.

Levels of cdxB mRNA expression. The jejunal RT-PCR products were used as probes to identify cdxB mRNA. Expression of cdxB mRNA was determined by Northern blot analysis (Fig. 7). A steady increase in mRNA levels was observed from 5 d of incubation until hatch after which concentrations reached a plateau. Lack of access to feed decreased cdxB expression in chicks starved for 48 h at 48 and 72 h posthatch (Fig. 7). However, in these chicks at 96 h posthatch, expression of cdxB mRNA had returned to levels in fed chicks.

Correlations. CdxB mRNA concentrations were correlated (P < 0.001) with both villus cell number (r = 0.72) and villus surface area (r = 0.76). CdxA was not significantly correlated with villus cell numbers or surface area, but a positive correlation was observed between cdxA protein and the nonproliferating cells in the villus in posthatch chicks (r = 0.73, P < 0.001).

**DISCUSSION**

Small intestinal expressions of the cdxA and cdxB homeobox genes increased during embryonic and posthatch development but were depressed by lack of access to feed, which decreased intestinal growth in the chick.

In the immediate posthatch period, dramatic changes occur
in the metabolism and morphology of the chick small intestine. In the initial hours posthatch, the small intestines grow faster than the rest of the body with maximal small intestine size/body weight at 2–5 d (4). In chicks, intestinal developmental status at hatch is intermediate between altricial species in which intestinal development lasts more than a month after birth (mice and rats) (34) and precocial species in which intestinal development occurs earlier in utero (sheep, pigs and humans) (35,36). Hence, in chicks, as in neonatal rodents, all intestinal epithelial cells are proliferating at hatch. Within a short period, proliferation becomes localized mainly to the crypts, which develop in the posthatch hours (2,37,38). Thus, during the posthatch period of intestinal development, homeobox genes are expected to play an active role. Previous studies in chicks have reported the presence of cdxA and cdxB during embryonic development (19,22) but did not examine possible roles of cdx genes in posthatch intestinal development.

In this study, expression of cdxA mRNA relative to β-actin increased with embryonic age until 2 d posthatch after which concentrations changed little. However, changes in cdxA localization differed pre- and posthatch. During late embryonic development and hatch, when crypts have not yet developed, cdxA protein was uniformly distributed in the proliferative intervillous epithelium monolayer, which developed into cdxA-positive crypts by 2 d posthatch. At these stages, cdxA protein was expressed along the villus with a gradient decreasing toward the tip [see also 22]. However, after 4 d posthatch,
cdxA protein in the chick small intestinal epithelium was uniformly distributed over the villus.

The immediate posthatch period in chicks was characterized by extensive enteroocyte maturation, localization of proliferation to the crypts, and hypertrophy and gain of polarity along the villus (3). Fetal nascent enteroocytes that line the pig and chick small intestine differ structurally and functionally from enteroocytes produced postnatally and posthatch, respectively (3,39). In chicks, we found that posthatch, cdxA mRNA concentrations were correlated with nonproliferating enteroocytes. Comparison of the quantitative expression of cdxA protein in proliferating crypt cells with differentiated villi enteroocytes at 2 and 7 d posthatch indicated that higher cdxA protein/cell was present in the villous enteroocytes compared with crypt enteroocytes. Thus, although cdxA protein was found in all intestinal epithelial cells, higher cdxA protein concentrations were found in mature cells. This may be due to the requirement for cdxA protein as a transcription factor (22).

CdxA was assumed to act similarly to murine cdx1 in the reports of Frumkin et al. (18,21,22). However, the pattern of cdxA localization observed in this study differs in some respects from reports describing the mouse homologue cdx1, in particular in the posthatch period. In the mouse small intestine, crypt formation begins just before parturition and villi are separated by a proliferating intervillous epithelium (40). This intervillus epithelium undergoes reshaping during the first two postnatal weeks to form the crypts (37), similar to what was observed here in chicks. However, the murine cdx1 is restricted mainly to the proliferative crypt compartment in the postnatal and adult intestine (12,17,41) compared with distribution along the villi in chicks. Furthermore, cdxA is highly expressed during gastrulation (18), whereas cdx1 only begins to appear during this period (42). During embryonic development, the murine cdx1 is expressed in the endoderm with no detectable levels in the mesenchyme, whereas cdxA is expressed in the mesenchyme (41). In adult mice, cdx2 is expressed in all epithelial cells irrespective of their degree of differentiation, with higher concentrations in differentiated enterocytes (43,44). In addition, cdx2 activates intestinal genes that define the differentiated enterocyte (44–48). It therefore appears that the pattern of cdxA expression resembles more closely that of cdx2, although it also has similar homology to cdx1 (18).

It has been reported that both cdx4 and its chicken homologue cdxB are transiently expressed for a short period during embryogenesis (10,19). CdxB has been reported to regulate the axial patterning during development (19); however, a role for cdxB has not yet been described. In this study, we observed that cdxB is expressed at later stages of development than previously reported (19). A steady increase in cdxB mRNA expression accompanied intestinal growth from 15 d of incubation to 5 d posthatch. Expression of cdxB mRNA was correlated with both villous cell number and villous surface area; thus, cdxB is expressed at constant concentrations in chick epithelial cells.

After examining the embryonic and posthatch expression of cdxA and cdxB under normal physiologic conditions, we examined jejunal cellular dynamics and the cdxA and cdxB transcription factors after starvation, which considerably retarded body weight increase and intestinal growth. Malnutrition induced by dietary restriction and severe starvation produced a series of metabolic changes that lead to reduction in body weight, depression of immunocompetence and altered function of the digestive system, particularly of the liver and small intestine. Furthermore, the intestine is the first organ system that is affected directly by changes in nutrient intake, and it also displays the most rapid and dramatic changes in response to nutrient deprivation (49). During starvation, a decrease in cell proliferation in both villi and crypts was observed, which led to fewer cells per villus and a smaller surface area. Similar results have been reported in starved adult rats and mice in which starving induced quiescence within the crypt compartment and decreased villous size, and refeeding stimulated the cells to renew the cell cycle (50,51).

In the present study, lack of access to food downregulated cdxA protein and cdxB mRNA. However, after feeding, proliferation and production of differentiated enteroocytes were enhanced. Expression of cdxA and cdxB increased after feeding, but this response lagged behind the initial proliferative response to feeding in both the crypts and the villi. This lag may be associated with the role of cdxA in enterocyte maturation (71).

We conclude that the chicken homeobox genes cdxA and cdxB are expressed in all enteroocytes during embryonic and posthatch development; however, cdxA may have a role in enterocyte maturation posthatch. Starvation depressed the expression of both the cdxA and cdxB transcription factors, which appear to be involved in intestinal development and maintenance. Further research is required to elucidate the specific role of these transcription factors in chicks.

LITERATURE CITED


