Coordinate Developmental Regulation of Purine Catabolic Enzyme Expression in Gastrointestinal and Postimplantation Reproductive Tracts

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Abstract. Using histochemical detection, we have visualized in situ the complete metabolic pathway for the degradation of purine nucleotides. From the tongue to the ileum, diverse epithelial cell types lining the lumen of the mouse gastrointestinal (GI) tract strongly coexpress each of the five key purine catabolic enzymes. Dramatic increases in the expression of each enzyme occurred during postnatal maturation of the GI tract. Using in situ hybridization, an intense accumulation of adenosine deaminase (ADA) mRNA was detected only within GI epithelial cells undergoing postmitotic differentiation. In a similar manner, at the developing maternal-fetal interface, high level expression of the purine catabolic pathway also occurred in a unique subset of maternal decidual cells previously known to express high levels of alkaline phosphatase and ADA. This induction occurred almost immediately after implantation in the periembryonic maternal decidual cells, shortly thereafter in antimesometrial decidual cells, and later in cells of the placental decidua basalis: all of which contain cell types thought to be undergoing programmed cell death. The expression of the pathway at the site of embryo implantation appears to be critical because its pharmacologic inhibition during pregnancy has been found to be embryolethal or teratogenic. Purine destruction at these nutritional interfaces (placenta and gastrointestinal tract) seem to override any potential economy of purine salvage, and may represent biochemical adaptation to nucleic acid breakdown occurring in the context of dietary digestion or extensive programmed cell death.

PURINE nucleotide synthesis and degradation are essential for life. Genetic defects in the purine metabolic pathway can cause devastating human disease, such as Lesch-Nyhan syndrome or severe immunodeficiency (Giblett, 1972; Carrera and Carson, 1987; Kredich and Hershfield, 1989). The importance of the purine pathway is also illustrated by the wide variety of potent and clinically beneficial drugs such as allopurinol, 6-mercaptopurine, methotrexate, and trimethoprim that have resulted from pharmacological targeting of purine pathway enzymes (for review, see Elion, 1989). The purine ring, large quantities of which are required for cell growth and proliferation, requires for its synthesis more than a dozen enzyme activities, a spectrum of metabolic substrates, and a substantial amount of chemical energy.

Given the metabolic expense of purine biosynthesis, it might be considered surprising that mice and other mammals do not reuse dietary purine nucleotides for DNA and RNA synthesis (Sonoda and Tatibana, 1978; Ho et al., 1979; Savaiano et al., 1980; Uauy, 1989). Consistent with this, rat duodenal mucosa has long been shown to produce the nonreusable purines, uric acid and allantoin, from AMP (Getler et al., 1949; Wilson and Wilson, 1962; Berlin and Hawkins, 1968). However, although the enzymatic properties of individual purine catabolic enzymes have been well characterized, and the localization of several of them has been described (Sackler, 1966; Chechik et al., 1983; Chinsky et al., 1990), the ability of the enzymes to function together in a tissue- and cell type-specific manner has not been studied.

During previous studies of human adenosine deaminase (ADA)[1] gene regulatory elements in transgenic mice (Aronow et al., 1989) we noted the profound degree of postnatal upregulation of the endogenous mouse ADA gene (Lee, 1973; Chinsky et al., 1990), and among several tissues in the postimplantation reproductive tract (Knudsen et al., 1988). Some of these tissues are not sites of high-level ADA expression in humans and this led us to question the role of ADA expression in these locations. Specifically, humans express ADA at high levels in the duodenum and thymus, but at much lower levels in most other tissues including tongue

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1. *Abbreviations used in this paper*: ADA, adenosine deaminase; AP, alkaline phosphatase; dCF, 2′ deoxycoformycin; CAT, chloramphenicol acetyltransferase; 5′NT; 5′-nucleotidase; GI, gastrointestinal; GUAase, guanase; NBT, nitroblue tetrazolium; p.c., post-coitus; PMS, phenazine methane sulphonate; PNP, purine nucleoside phosphorylase; UA, uric acid; XO, xanthine oxidase.

and esophagus (Aronow et al., 1989). In mice, ADA expression occurs in a somewhat similar pattern but very high-level expression also occurs in tongue, esophagus, forestomach, and placenta. The level of ADA expression in human placenta is not known to be high, but the placentas of rat, cat, cow, guinea pig, and rabbit have been reported to contain very high levels of ADA at some stages of gestation (Brady and O'Donovan, 1965; Sim and Maguire, 1970).

Developmental studies have also provided clues as to why ADA is expressed at high level in some locations. Lee (1973) has shown that the activities of ADA, xanthine oxidase (XO), and uricase increase dramatically during the postnatal maturation of the stomach and duodenum in mice (Lee, 1973). However, the cell types involved were neither identified nor were the three other enzymes measured that would be necessary to complete the metabolic pathway (5'-nucleotidase [5' NT], purine nucleoside phosphorylase (PNP), and guanase [GUAase]). In the present study, we demonstrate that the entire series of purine catabolic pathway enzymes are expressed in the same cell types and undergo developmental coregulation in the proximal gastrointestinal tract. Moreover, we have shown in situ that the enzymes function together in a linked fashion. We also observe a similar coexpression of purine catabolic enzymes in a distinct population of cells of the postimplantation maternal decidua long been known to express high levels of 5'-nucleotidase alkaline phosphatase (Finn, 1971; Hall, 1971) and more recently ADA (Knudsen et al., 1988, 1991). Like the mature gastrointestinal tract, a substantial subset of cells in the reproductive tract also appears committed to the degradation of purine nucleotides.

Materials and Methods

Animals, Tissues, and Materials

(C57Bl/6 × C3H) F1 mice were fed Purina 5001 chow (23.5% protein, 6.5% fat; Ralston Purina Laboratories, St. Louis, MO) ad lib. Noon on the day of vaginal plug appearance was designated day 0.5 post-coitus (p.c.). Mice were killed by cervical dislocation immediately before collecting tissue samples. Human tissue samples were culled from discarded adult and pediatric biopsy specimens in accord with Institutional Review Board guidelines. Enzymes and purine compounds were the highest quality available from Sigma Chemical Co. (St. Louis, MO). Purine compounds were analyzed by means of HPLC (see below) with the effluent monitored at 254 nm. All were found to be >99% pure.

Enzyme and mRNA Quantitation

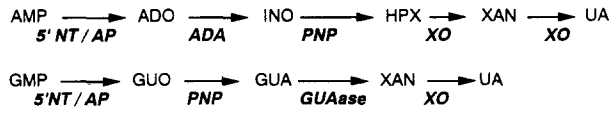
The specific activity of ADA was determined using total soluble extracts (Wiginton, 1981). Homogenates were made in ~20 vol of 0.1 M $KHPO_4$, sonicated, and centrifuged for 20 min at 15,000 *g*. The absolute quantities of ADA mRNA in total cellular RNA (Chomczynski and Sacchi, 1987) were determined by RNaseA protection, PAGE, and surface radioactivity measurement (model 603 analyzer; Betagen, Waltham, MA) using internal standardization with known quantities of purified sense strand RNA, and normalized per microgram of total RNA as previously described (Aronow et al., 1989) using a transcription vector made from the cDNA clone pADA 5-29 (Yeung et al., 1985).

Histochemical Detection of Purine Catabolic Enzymes

Tissues for histochemistry were fixed for 4 h at 4°C in PBS containing 30% sucrose and 0.5% glutaraldehyde. This degree of fixation gave improved histology compared to previous methods (Spencer et al., 1968; Knudsen et al., 1988) without discernible effects upon the distribution or intensity of observed stains. Histochemical staining for the purine catabolic enzymes was performed based on modifications of the procedure originally devised for the detection of ADA (Spencer et al., 1968; Knudsen et al., 1988). The terminal step for each histochemical reaction is the oxidation of hypoxanthine or xanthine by XO leading to the reduction of nitroblue tetrazolium (NBT) to an insoluble blue formazan reaction product. The composition of each cocktail is described in the legend to Fig. 1, where the individual components (when included) are present at the following concentrations: NBT, 0.8 mM; AMP, 1 mM; inosine, 1 mM; guanine, 1 mM; PNP, 10 μg/ml; phenazine methane sulphonate (PMS), 0.15 mM; adenosine, 1 mM; hypoxanthine, 1 mM; ADA, 5 μg/ml; and XO, 0.07 U/ml.

All incubations were performed in 50 mM $NaHPO_4$ buffer pH 7.4 at 37°C for varying lengths of time as indicated (15 min to 1 h in the dark). Doing this provided a rough quantitative index of enzymatic activity as indicated by the intensity of formazan deposition. Sections were rinsed in water and cover-slips were applied using Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA). Photographs were taken within 48 h to avoid a prominent nuclear staining artifact that occurred upon prolonged storage. For control experiments, to specifically inactivate ADA enzyme activity, adjacent sections were incubated in 1 μM 2' deoxycoformycin for 30 min at room temperature. To specifically inactivate XO activity, sections were preincubated with 1 mM oxypurinol for 30 min. To observe the inhibitory effect of oxypurinol, PMS had to be omitted which led to only a slight diminution in the intensity and brilliance of the blue formazan reaction product. The 30-min preincubation in the absence of stain did not impair the intensity of the subsequent staining reaction (not shown).

A. Purine Nucleotide Degradation Pathways:



B. Cocktails for the Detection of Purine Catabolic Enzymes:

Enzyme(s) to Detect	Added Substrate	Added Enzyme(s)
Individual Enzymes		
5'NT, AP:	AMP	ADA + PNP + XO
ADA:	ADO	PNP + XO
PNP:	INO	XO
GUAase:	GUA	XO
XO:	HPX	-
In Situ Coupled Enzymes		
5'NT → ADA:	AMP	PNP + XO
5'NT → PNP:	AMP	XO
5'NT → XO:	AMP	-
ADA → PNP:	ADO	XO
ADA → XO:	ADO	-
PNP → XO:	INO	-

localized enzyme(s)? → blue formazan ppt.

Figure 1. Purine nucleotide catabolism and histochemistry. (*A*) The metabolic routes for the degradation of purine nucleotides are summarized. When DNA and RNA are degraded by DNase and RNaseA, 5' deoxynucleoside monophosphates and a mixture of 2' and 3' nucleoside monophosphates are produced, respectively. Intestinal AP and NT hydrolyze the phosphoryl esters, and the resulting nucleosides can be transported across plasma membranes and acted upon by subsequent enzymes. In primates, XO is the terminal step for purine catabolism, whereas most other mammals oxidize UA to allantoin via uricase. XO activity renders the purine ring non-reutilizable for purine salvage. (*B*) Biochemical basis for the histochemical localization of purine catabolic enzyme activities. To localize any enzyme in the catabolic pathway (*A*), cocktails were formulated in which the substrate for the desired enzyme is provided, then enzymes for the subsequent steps were supplemented in the mix. Reducing potential generated by the oxidation of xanthine to uric acid causes in situ reduction of NBT to form a dark blue formazan reaction product. To test for the connectivity of all of the catabolic enzymes in situ (5'NT → XO), AMP is the substrate and no exogenous enzymes are added.

In Situ Hybridization

For in situ hybridization, unfixed tissue was embedded in OCT compound (Miles Laboratories, West Haven, CT), snap frozen, cut at a thickness of

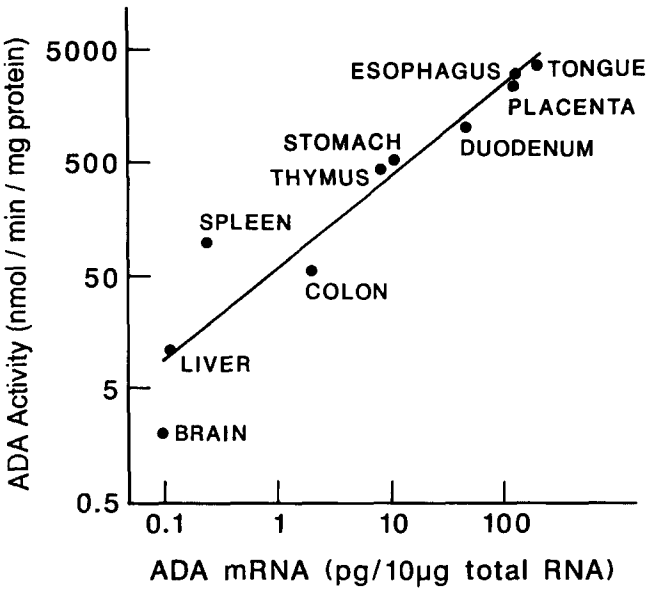


Figure 2. Tissue-specific expression pattern of ADA mRNA and enzyme in adult mouse tissues. The specific activity of ADA was determined using total soluble extracts, mRNA was quantitated by RNase protection using ^{32}P-labeled probes made antisense to mouse ADA mRNA and standardization to known quantities of purified sense strand RNA and normalized per μg of total RNA, both as previously described (Aronow et al., 1989). Placental RNA and enzyme extracts were obtained from the placenta of a day-16 pregnancy. Note that the graph is a double-log plot and that between some tissues there are differences >1,000-fold. Data points from a single set of experiments are shown, duplicate analyses of the extracts and RNAs have shown variations of 10–50% of the values that are shown.

6 μm, and fixed in 4% paraformaldehyde in PBS for 1 min. Prehybridization was carried out at room temperature for 0.25 h as previously described (Aronow et al., 1989), except that prehybridization and hybridization solutions were supplemented with 10 mM DTT and 0.1 mM alpha-thio-UTP. The addition of these agents appeared to virtually eliminate the occurrence of background and artifactual labeling by unrelated probes (not shown).

HPLC Analysis of Purine Metabolites

Tissue portions (~50 mg) were incubated at 37°C in 1 ml of a solution that contained 25 mM $NaPO_4$, pH 7.2, and 1 mM AMP. At the indicated times, 100 μl-aliquots were removed and added to an equal volume of ice-cold 10% TCA, incubated at 4°C for ~1 h, and centrifuged at 15,000 *g* for 3 min. Supernatants were neutralized by extraction with an equal volume of tri-*N*-octylamine/chloroform (22:78) and stored at −80°C. Purine compounds were separated by HPLC on a Delta-Pak C-18 reverse-phase column (Millipore, Inc., Milford, MA) using 50 mM ammonium formate, pH 4.35 (0.5 M stock prepared by neutralization of 0.5 M formic acid with concentrated ammonium hydroxide) as the initial mobile phase for 10 min, with a gradient to 100% methanol at 20 min. Uric acid (UA) (3.24 min), xanthine (4.21 min), hypoxanthine (3.73 min), inosine (11.58 min), adenosine (15.75 min), and AMP (4.88 min) were quantitated by means of comparison of peak areas (absorbance monitored at 254 nm) to that of known standards.

Results

Tissue and Cell Type-specific ADA Regulation

To ascertain the extent to which variation in ADA enzyme activities among different mouse tissues is attributable to variations in ADA mRNA accumulation, quantitative RNAase protection assays were performed. As depicted in Fig. 2, there is a strong correlation of ADA enzyme levels with mRNA levels over a 4-log range of tissue-specific ADA expression. Since the proximal GI tract and placenta are considerably higher in ADA expression than other mouse tissues, and there are striking species differences with respect to expression from some of these sites, we reasoned that the identification of the expressing cell types in these tissues could provide clues to explain this interesting expression pattern.

The cell types responsible for generating the high level of ADA mRNA present in total RNA from the proximal GI and reproductive tracts were identified by in situ hybridization. As shown in Fig. 3, within the GI tract there was an intense accumulation of ADA mRNA in a highly localized fashion within a diverse set of maturing epithelial structures. In the tongue, esophagus, and forestomach, ADA mRNA accumulation was confined to the superficial layers of the squamous epithelium with little or no signal present in the more immature basal layers (Fig. 3, *A–G*). The duodenal mucosa showed a gradient of signal that was highest in the villous tips, lower around the villous base, and virtually absent in the crypts. This pattern of expression was completely recapitulated by histochemical staining specific for ADA activity (Chinsky et al., 1990; ourselves, not shown). Because villous epithelial cells are derived from mitoses that occur in the crypts, and squamous epithelial cells are derived by maturation from basal cell mitoses (for review see Potten and Hendry, 1983; Gordon, 1989), ADA gene expression to form ADA mRNA must be subject to stringent regulation during the postmitotic maturation of these cells. From duodenum to terminal ileum, each of the small bowel segments that we have examined exhibited similar crypt to villous gradients of ADA mRNA. However, the distal small bowel exhibited less total signal, consistent with results of RNAase protection assays showing ADA mRNA to be present in total RNA from the distal ileum at 25–50% of its relative abundance in total RNA isolated from duodenum (not shown).

We have also observed high-level ADA mRNA and enzyme accumulation at several stages of placental development within decidual and cytotrophoblastic tissue (Fig. 3, *H–J*), in general agreement with immunohistochemical results of Knudsen et al. (1988, 1991). The specific cell types that are responsible will be described below. A variety of other adult and fetal mouse tissues have also been evaluated. Histochemical staining and in situ hybridization of lung, uterus, cervix, colon, skin, brain, pancreas, lymph nodes, and spleen failed to reveal any restricted cell types that accumulate a high level of enzyme or mRNA (not shown). Thus, since ADA expression does not occur at high level in epithelial or placental cell types that are morphologically quite similar to the expressing cell types, there appears to be strong regional specificity to ADA's expression in the GI tract and placenta.

To consider the potential function of high-level ADA expression in the GI tract and placenta, it is important to note that ADA performs a central step in purine catabolism (Fig. 1 *A*), allowing for either purine salvage via hypoxanthine phosphoribosyl transferase or purine degradation via XO. Salvage and degradation of GMP uses several of the same enzymes, with the additional requirement for GUAase activity in degradation. If the deamination of adenosine or deoxyadenosine by ADA was essential for regulating local concen-

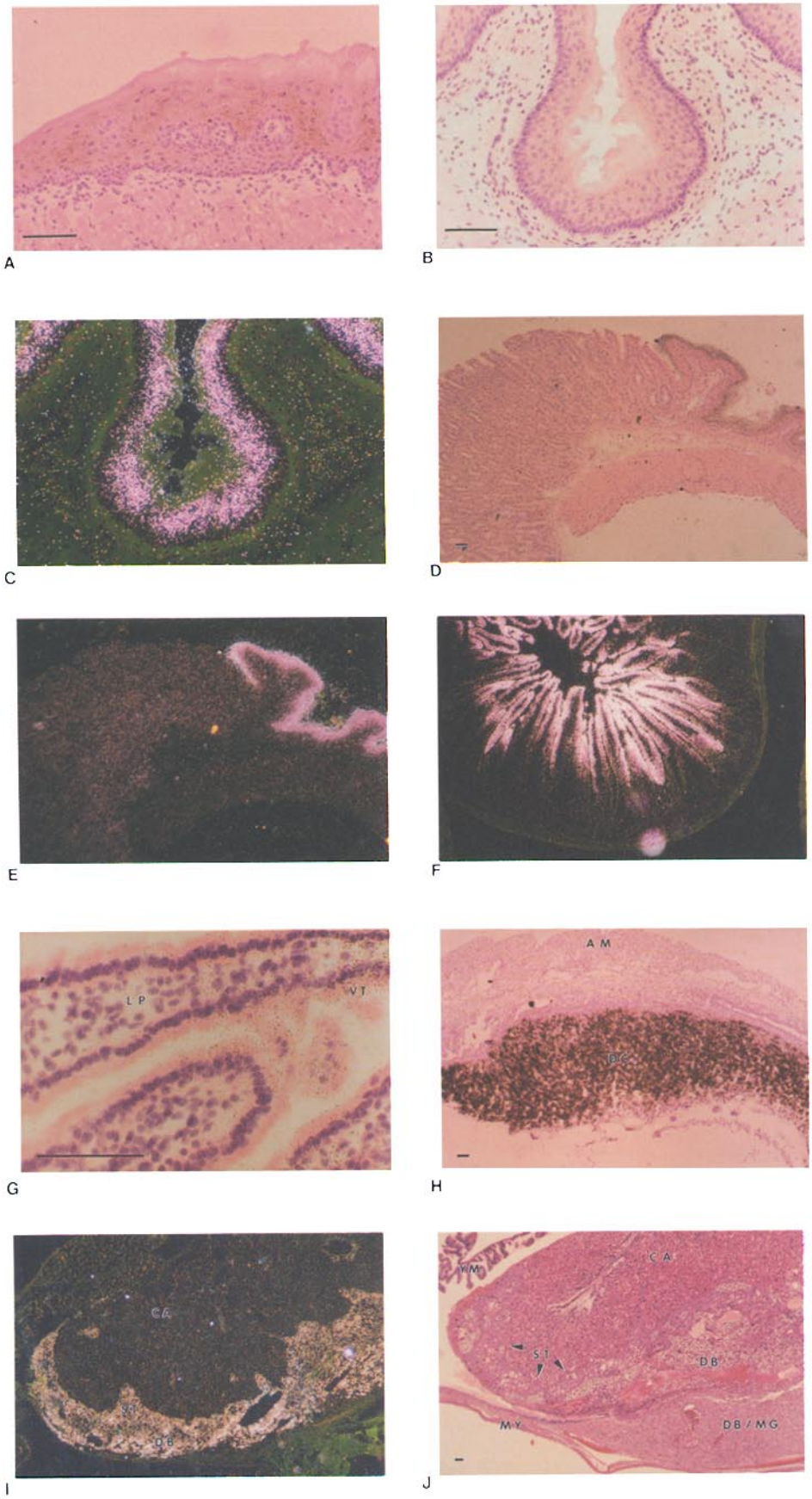


Figure 3. In situ hybridization for the localization of ADA mRNA in adult mouse tissues. ^{35}S-labeled antisense RNA probes were hybridized as previously described (Aronow et al., 1989) to cryostat sections of mouse tongue, esophagus, stomach, duodenum, and placenta. Silver grains appear black in the brightfield (*A*, *B*, *D*, *G*, and *H*) and white in the darkfield (*C*, *E*, *F*, and *I*) views. Highly localized signal is evident in the squamous epithelium lining the tongue (*A*), esophagus (*B* and *C*), forestomach (*D* and *E*), and in the epithelial cells located at the tips of the villi in the duodenum (*F* and *G*). In the stomach there is a strong signal in the squamous epithelium of the forestomach but a much lower signal in the glandular mucosa (*D* and *E*). Note the absence of signal over germinal epithelial cells in the tongue, esophagus and forestomach (*A–D*). The duodenum shows intense signal in the epithelial cells lining the villous tips (*VT*) and no signal in the lamina propria (*LP*) or the epithelial cells towards the base of the villi or in the crypts. Strong signal is evident in decidualized reproductive tract cells at distinct stages of gestation (*H–J*). At day 9 (*H*), there is a massive accumulation of ADA mRNA in the decidual capsularis (*DC*) cells, present along the antimesometrial (*Am*) side of the uterus. In the day 16 placenta (*I* and *J*) strong signal is present in the spongiotrophoblastic layer (*ST*) and decidua basalis (*DB*), but not in the chorioallantoic plate (*CA*). *J* is a section of a 16-d placenta fixed in Bouin's solution and embedded in paraffin to show better detail of these layers and additional components (yolk-sac membrane, *Ym*, myometrium, *my*; decidua basalis/metrial gland; (*DB/MG*). The plane of *I* is parasagital to the centrally localized metrial gland. In separate experiments, no signal was detected in mouse skin or in respiratory, colonic, or cervical mucosa with ADA probe, and no signal was detected in any of the above tissues when probed with the sense strand of mouse ADA mRNA, a control probe antisense to bacterial CAT mRNA, or to the sense strands of a variety of other cDNA probes. Slides were exposed for 48–72 h. Bars, 50 μm.

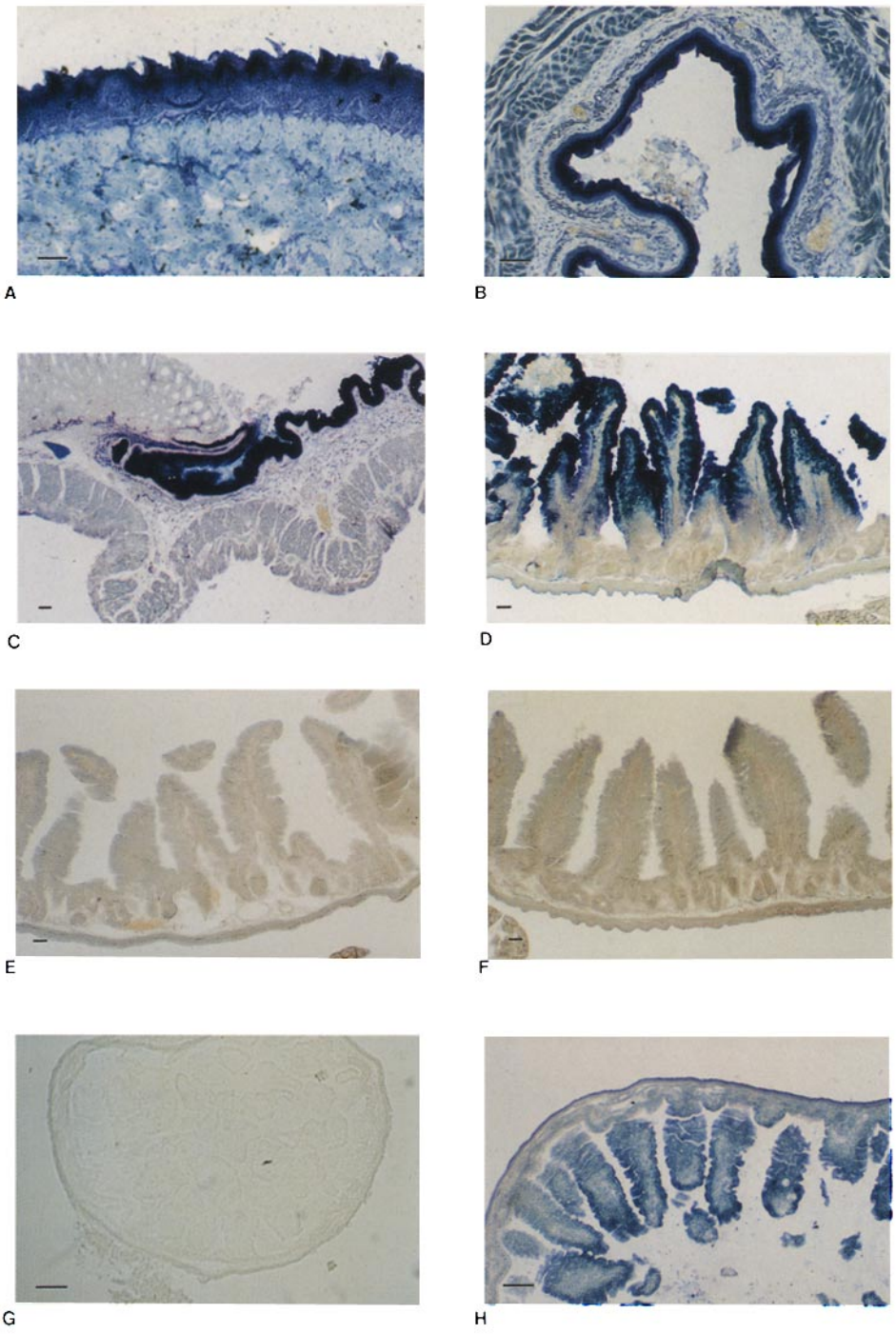


Figure 4. Histochemical analyses of catabolic enzymes in situ. Tissue sections were stained for either individual purine catabolic enzyme activities, or the entire purine catabolic pathway as described in Materials and Methods (see Fig. 1). Each of the cell types that exhibited strong signal by in situ hybridization versus ADA mRNA also exhibits high-level expression in each purine catabolic enzyme and these enzymes function in situ in a linked fashion. The tissues and the individual enzyme activities, or enzyme pathway activities, that are illustrated in this figure are as follows: (*A*) mouse tongue PNP, (*B*) mouse esophagus PNP, (*C*) mouse stomach XO, (*D*) mouse duodenum 5′NT → XO, (*E*) mouse duodenum 5′NT → XO preincubated with dCF, (*F*) mouse duodenum 5′NT → XO preincubated with oxypurinol, (*G*) term-fetal mouse duodenum 5′NT → XO, (*H*) post-natal day 5 mouse duodenum 5′NT → XO, (*I*) human duodenum ADA, (*J*) human duodenum 5′NT → XO, (*K*) mouse duodenum XO, (*L*) day 6.5 mouse implantation site 5′NT → XO, (*M*) day 6.5 mouse implantation site stained with hematoxylin and eosin, and (*N*) day 16 mouse placenta XO. In the mouse tongue, esophagus, and forestomach, the blue formazan reaction product localizes to the mature surface squamous epithelial cells. The apical and basalar limits of the villous enterocytes are demarcated by arrowheads in *I–K*. Note the strongly sublocalized reaction in the brush border region of the cells and in addition in a granular pattern at the basalar portion of the enterocytes. At the mouse implantation site (*L* and *M*) note that only the decidual cells immediately adjacent to the embryo express the purine catabolic enzymes strongly. In the mature placenta (*N*) XO and each of the catabolic enzymes are localized to the decidua basalis (*DB*), and spongiotrophoblast (*ST*) with little or no activity within the chorioallantoic plate (*CA*) or in the uterine myometrial wall (*arrowhead*). The results of these and other histochemical analyses are summarized in Table 1. Bars, 50 μm.

trations of adenosine, salvage might be expected. Alternatively, if high-level ADA expression were part of a metabolic pathway leading to the net breakdown to nonreusable compounds, localized coexpression of XO and GUAase might be expected.

Histochemical Demonstration of Purine Catabolic Enzymes and their Functional Linkage In Situ

One approach to distinguishing among the above possibilities for tissue-specific ADA function is to evaluate the relative expression of other enzymes in the purine catabolic pathway within specific types of cells. To do this, we modified standard histochemical techniques (Spencer et al., 1968; Knudsen et al., 1988) to allow the detection of each of the enzymes of the purine catabolic pathway. Cocktails were devised to demonstrate the activity of each enzyme (Fig. 1 *B*). We further sought to modify this approach to demonstrate that the product of one enzymatic step could efficiently provide substrate for the next, in situ. The potential of this approach is that an entire metabolic pathway, with a series of

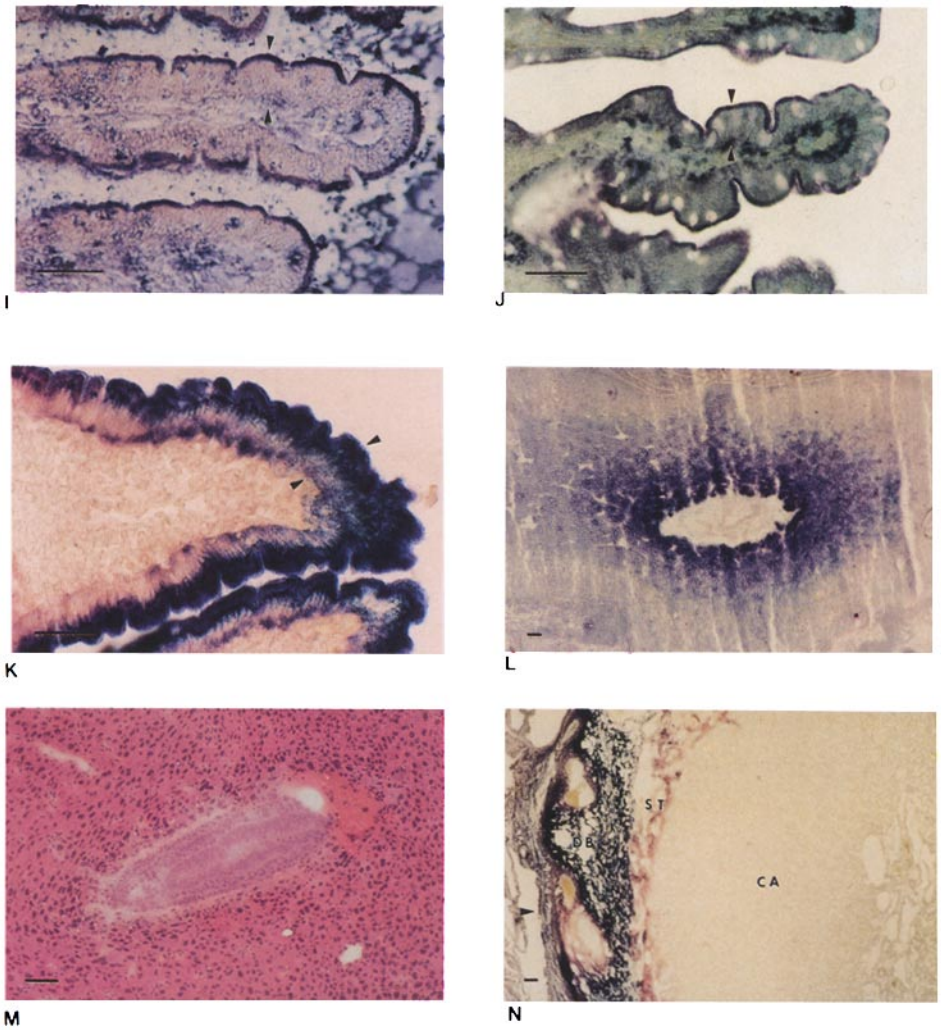


Figure 4 (continued).

individual enzymes, could be demonstrated in situ. To do this for the complete purine catabolic pathway, AMP and NBT were used as the substrates without the addition of exogenous enzymes to the cocktail.

The results of these studies are shown in part in Fig. 4, and summarized in Table I, and indicate that each of the catabolic enzymes (5′NT, ADA, PNP, XO, and GUAase) is present in the same cell types within the proximal GI tract (Fig. 4, *A–K*, Table I). Table I indicates only the relative intensities of the staining reactions, and should not be construed as strict quantitative measures of the amount of enzyme present. For each tissue examined there was complete concordance with results that were obtained by in situ hybridization for ADA mRNA. Specifically, the great majority of the differentiated mucosal epithelial cells of the tongue, esophagus, forestomach, and duodenum exhibited intense expression of each of the five enzyme activities that were tested. A strong signal was also detected when the complete purine catabolic pathway was tested indicating that the enzymes were also able to function in a linked fashion in situ.

In all tissue sections that we have analyzed, for each site of high-level ADA expression, the omission of purine substrate from the cocktail fully prevented the appearance of formazan precipitate. This indicates that the reduction of NBT was dependent upon the purine compound, and that under the conditions of our histochemical reactions, there was not a detectable level of reductase activity capable of acting directly upon NBT. That the reaction is specific to the purine catabolic enzymes is implied by the use of pharmacologic inhibitors. Thus when the complete pathway was assayed using AMP, the preincubation of the tissue with dCF, a potent inhibitor of ADA, or with oxypurinol, the active metabolite of the clinically potent XO inhibitor allopurinol (for review, Rundles, 1985), there was complete inhibition of formazan appearance (Fig. 4, *E* and *F*, respectively). These results strongly support the specificity of the histochemical reactions, and taken with the results described above, indicate that the entire set of purine catabolic enzymes function together in situ.

Coordinate Regulation of Purine Catabolic Enzyme Expression during Postnatal GI Tract Development

Because ADA activity has been noted to increase progressively in the proximal mouse GI tract over the first 1–3 wk after birth (Lee, 1973; Chinsky, 1990) and immediately after implantation in the reproductive tract (Knudsen et al., 1988;

Table I. Summary of Histochemical Analyses

Tissue	Location of enzyme activity	Histochemically tested enzyme activities					
		5′ NT/AP	ADA	PNP	XO	GUAase	AMP→XO
Mouse GI tract							
adult							
Tongue	Mature squamous mucosal epithelial cells	++	++	++	++	++	++
Esophagus		++	++	++	++	++	++
Forestomach		++	++	++	++	++	++
Duodenum	Mature villous enterocytes: brush border and basalar	++	++	++	++	++	++
7-d-old pup							
Tongue	Surface epithelial	+	+	+	+	+	+
Duodenum	Villous epithelial	+	+	+	+	+	+
3-d-old pup							
Tongue	Weak signals, surface epithelial	+	+	+	+	+	+
Duodenum	Weak signals, villous epithelial	+	+	+	+	+	+
1-d-old pup							
Tongue	Trace signals, surface epithelial	tr.	tr.	tr.	–	tr.	–
Duodenum	Trace signals, villous epithelial	tr.	tr.	tr.	–	tr.	–
Term-fetal pup							
Tongue	No signals	–	–	–	–	–	–
Duodenum	No signals	–	–	–	–	–	–
Human GI tissues							
Tongue	No signals	–	–	–	–	–	–
Esophagus	No signals	–	–	–	–	–	–
Duodenum	Villous enterocytes; brush border and basalar	++	++	++	++	++	++
Mouse							
Reproductive tract							
Day 7	Circumferential periembryonic decidual cells	++	++	++	++	–	++
Day 9	Antimesometrial decidual cells	++	++	++	++	–	++
Day 16	Decidua basalis cells and spongiocyto-trophoblasts	++	++	++	++	–	++

Summary of histochemical staining reactions performed for each of the purine catabolic enzyme activities as described in Figure 3. Visual assessment of each enzyme reaction was made and recorded as trace (*tr*) when just visible; +, light staining; and ++, dark staining.

ourselves, unpublished), we evaluated histologically whether the expression of each of the other purine catabolic enzymes increased in parallel. In the GI tract, as summarized in Table I, term fetal duodenum (~day 19.5 p.c.) failed to express any of the purine catabolic enzymes (see also Fig. 4 *G*). However, even 1 day following birth, the histochemical staining technique demonstrated faintly detectable 5′NT, ADA, and PNP in the duodenum. XO, however, was not detected until day 3. By postnatal day 7, however, each of the purine catabolic enzymes was present at low yet easily detectable levels within the same cell types of tongue and duodenum (see also Fig. 4 *H*). These results are fully consistent with the results reported by Lee (1973) in which there were parallel increases in the specific activity of several of these enzymes in crude homogenates over the first several weeks after birth. Since our results indicate that there are also increases in the activities of 5′NT, PNP, and GUAase, we conclude that each of the purine catabolic pathway enzymes is subject to postnatal developmental activation in a coordinated manner.

Mouse versus Human GI Tract

We have previously shown a distinction between humans and mice in that unlike mice, extracts of tongue and esophagus from humans express ADA at only a low level (Aronow et al., 1989). To evaluate whether the expression of the other catabolic enzymes was also limited to the duodenum, we applied the histochemical stains to cross-sections of human tongue and esophagus. No signal was detected for any of the enzymes (Table I). In contrast, terminal villous epithelial cells of the human duodenum did coexpress all of the enzymes of the purine catabolic pathway (Table I, and Fig. 4 *J*). Thus, while the tissues that express purine catabolic enzymes within the GI tract are not conserved between mice and humans, coregulation of the entire pathway is conserved in the duodenum.

Subcellular Localization of Each Purine Catabolic Enzyme in the Duodenal Brush Border

In the duodenum, for each enzyme and for the entire purine catabolic pathway, formazan reaction product was not deposited uniformly over the entire cell body of the terminally differentiated epithelial cells. Rather, deposition occurred most intensely over the brush border/apical portion of the enterocyte, with a less intense accumulation of product occurring in the basalar aspect of the enterocyte next to the lamina propria. A similar staining pattern was observed for each of the enzymes in both mouse and human enterocytes although perhaps the ratio of apical to basalar staining varied depending on the specific enzyme (Fig. 4, *I–K*, others not shown). Compared to the apically stained portion of mouse duodenal enterocytes, apical staining in human duodenal enterocytes was more obviously restricted to a tight brush border band. The basis for this difference is not clear at this time. The diffuseness of the staining reaction is attributable at least in part to high activity of the enzymes with rapid deposition of formazan. Shorter incubations and inspection of enterocytes nearer to the base of the villi show more apparent restriction to the brush border (Fig. 4 *K*).

Coordinate Expression of Purine Catabolic Enzyme Expression during the Postimplantation Maternal Decidual Reaction

Each of the purine catabolic enzymes also was expressed intensely by a series of distinct cell types of the uterus and placenta during the postimplantation maternal decidual reaction (days 6.5–9) and as well in the maturing placenta (days 11–18) (Fig. 4, *L–N*). The only exception to this statement is that GUAase activity was not detectable at any of these stages. At an early postimplantation stage (~day 6.5 p.c.), each of the enzymes of the purine catabolic pathway exhibited strong expression and colocalization in a subset of maternal decidual cells immediately surrounding the embryo (Fig. 4 *L*; Table I). In contrast, decidual cells further from the embryo failed to express the purine catabolic enzymes at a detectable level. By examination of an adjacent section stained with hematoxylin and eosin (Fig. 4 *M*), it is apparent that among the positively stained decidual cells are many with fragmented and pyknotic nuclei and occasional cytoplasmic vacuolation. At the next stage that we have studied in detail, day 9 p.c., there is a very high level of expression of the purine catabolic enzymes within the maternal antimesometrial decidual cells (decidua capsularis shown in Fig. 3 *H*), a population of cells that has been characterized to be in a stage of degeneration and regression (Welsh and Enders, 1985; Katz and Abrahamsohn, 1987). Except for their anatomic location, these cells appear morphologically identical to the periembryonic decidual cells at day 6.5 p.c.. Nearer to term (day 16 p.c.), a major zone of the placenta that is immediately adjacent to the myometrium was strikingly positive for ADA mRNA as assessed by in situ hybridization (Fig. 3, *H–J*, and the purine catabolic enzymes (Table I; Fig. 4 *M*, others not shown). Three subzones can be recognized: a layer containing fetal spongiotrophoblasts, maternal decidual basalis cells, and maternal derived metrial gland cells (Stewart and Peel, 1978). There may be some differences among these cell types with respect to their quantitative expression of each purine catabolic enzyme, but the histochemical method is not sufficiently quantitative or of sufficient resolution to be sure. What is clear however, is that each enzyme is expressed by the predominant cell type of each zone at much higher levels than surrounding placental cell types (Fig. 4 *N*).

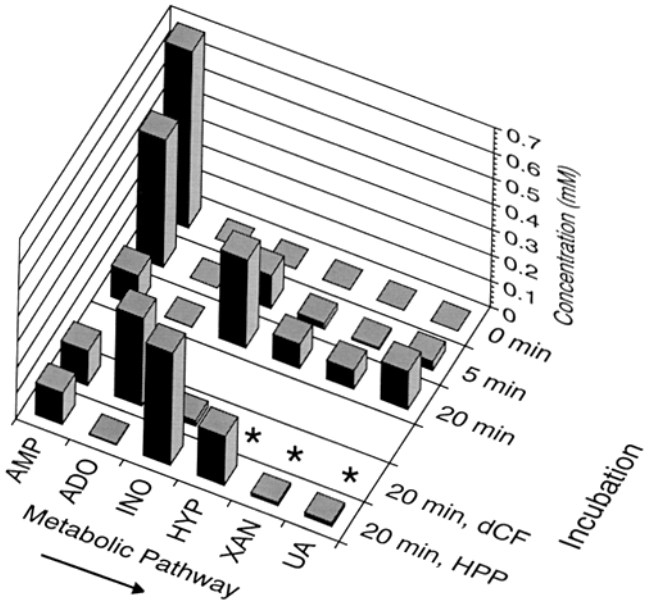


Figure 5. HPLC analysis of purine metabolites generated from incubation of AMP with mouse duodenum. Mouse duodenum was opened lengthwise and cut into squares (~50 mg each) and placed into 1 ml of 1 mM AMP at 37°C as described in experimental procedures. At the indicated times, aliquots were removed, acid extracted, neutralized, and analyzed by HPLC to identify each purine metabolite. The amount of accumulated metabolite is indicated in the *z*-axis for each time point. Samples incubated in the presence of dCF and Hpp show accumulations of adenosine and hypoxanthine, respectively. Note that adenosine is never accumulated in the absence of dCF. Asterisk (*) indicates that hypoxanthine, xanthine, and UA could not be quantitated from incubations that included dCF because of the presence of low-abundance unidentified compounds with overlapping retention times. Most likely, these compounds represent acid hydrolysis products of dCF.

Analysis of Purine Metabolites by HPLC

As further proof that the histochemical observations were

representative of authentic purine catabolism, analyses of purine metabolites were carried out by HPLC. To do this, small fragments of intact duodenum were incubated with AMP in solution. As indicated in Fig. 5, all of the expected catabolites of AMP accumulated in the incubation medium, except for adenosine. However, adenosine did accumulate when a tissue fragment was first preincubated with 2′ deoxycoformycin (dCF). Similarly, dCF treatment strongly interfered with the accumulation of Ino. Preincubation of duodenum with allopurinol caused the accumulation of hypoxanthine and inosine, and no accumulation of xanthine or UA. Since adenosine did not appear in the medium in the absence of dCF, it is likely that AMP dephosphorylation and ADA-mediated conversion of adenosine to inosine are normally quite tightly coupled at the duodenal brush border. The accumulation of inosine and each of the nucleobases in the incubation medium that occurred in the absence of the inhibitors suggests that the metabolic conversions that follow ADA may not be as stringently coupled in situ as assayed under our conditions. Incubation of human duodenum with AMP gave similar results with no adenosine accumulation. These results confirm that the GI tract has the capability of catabolizing purines at high level (see introduction) and demonstrate that the histochemical staining reactions are specific for these metabolic reactions.

Discussion

Multilevel Coordination of Purine Catabolic Enzyme Expression

These studies have indicated that there is a high level of coordination in the expression of purine catabolic enzymes along the proximal mouse GI tract, and in the postimplantation reproductive tract. This occurs at several distinct levels. First, each of the enzymes comprising the complete metabolic pathway is expressed together at high level in distinct and structurally diverse tissues of the proximal GI tract: tongue, esophagus, forestomach, and duodenum. This concordance extends in a cell type–specific manner: in each cell type with high level expression of one of the enzymes, there is also high-level expression of each of the other enzymes. Moreover, in each tissue location, there is lumenal coordination such that expression of the metabolic pathway is directed at or very near to the lumenal surface of the proximal GI tract. Second, within each tissue type, the expression of each enzyme occurs only in cells that are in the process of undergoing a postmitotic differentiation program. This pattern is matched by the in situ hybridization results obtained for the presence of ADA mRNA. Third, during postnatal GI tract maturation, there is an indistinguishable time course over which each enzyme becomes highly expressed. Finally, in the reproductive tract, except for GUAase, each of the enzymes is coexpressed by a subset of mouse maternal decidua cells that are undergoing a rapid program of postimplantation development and are potentially at an early stage of regression and/or programmed cell death (see below).

Subcellular Localization of Purine Catabolic Enzymes in the Duodenum

The histochemical assays, despite their somewhat limited histological resolution, suggest that there is a strong degree of localization of the purine catabolic enzymes to the apical and/or brush border membrane of the duodenal enterocyte in adult mice and humans. It is important to note that while NBT reduction exhibits an effective signal, its ability to sublocalize the signal to membrane versus cytoplasmic segments of the expressing cell types is limited. In results not shown, antibody localization of ADA in the mouse duodenum provides an identical signal, highly predominant in the brush border. Immunohistochemical localization of the other enzymes remains to be done. Brush border localization has been reported for a wide variety of digestive enzymes such as disaccharidases and aminopeptidases. However, the mechanism(s) for localizing (what are normally) cytoplasmic and/or soluble enzymes (ADA, PNP, GUAase, XO) are not clear. Chicken gizzard 5′NT, extracellularly localized, has been shown to have the capability of direct carboxyl terminal interaction with laminin and fibronectin (Stochaj et al., 1989). That ADA is easily releasable from its duodenal localization is suggested by its specific activity in soluble extracts as shown in Fig. 2. Several mechanisms by which extracellular proteins are localized to the brush border, such as covalent linkage to phophatidyl inositol, have been discussed by Semenza (1989). Other means of localization could involve interactions with specific binding proteins such as the ADA complexing protein (Schrader and West, 1990). In fact, Dinjens et al. (1989) have shown that immuno–cross-reactive ADA complexing protein is precisely localized to the apical enterocyte brush border membrane in mice and humans. Interestingly, the complexing protein is also expressed in other cell types such as the proximal tubular epithelium of kidney, but the form isolated from kidney does not bind ADA, nor is ADA expressed at high level in these cells.

Physiological Significance of High-Level Purine Degradation?

The deamination of adenosine or deoxyadenosine by ADA could be essential for regulating local concentrations of Ado. This could be important if Ado were to act as a neuro-transmitter in these tissues (Nagy et al., 1984; Norstrand, 1985; Yamamoto et al., 1987; Williams, 1987), or if resident cell types such as intra-epithelial lymphocytes were unable to function in regions with high concentrations of these purines. Also, high-level ADA expression could be part of a metabolic pathway leading either to the net salvage of preformed purines or the net breakdown to nonreusable compounds. Since our results have shown that high-level ADA expression is accompanied by high-level expression of each of the other purine catabolic enzymes, including xanthine oxidase, the general implication of these results is that there appears to be a commitment by the above described cell types to the digestion and degradation of purine nucleotides. It is likely that there are common regulatory mechanisms that control the expression of these enzymes, but this remains to be determined. Perhaps a clue for the future is that since GUAase expression is separable from the expression of the other enzymes in the reproductive tract, the regulatory factors directing gene expression in the female reproductive tract cannot be the same as those in the proximal GI tract. It also certainly remains to be determined what the biological significance is of this elaborately organized expression pattern, but a variety of evidence from several sources suggests

several considerations. Since epithelial cells of colon, cervix, and other types of mucosae differentiate and mature in a manner similar to the epithelial cells of the proximal GI tract but do not exhibit focal high-level expression of ADA, high level ADA expression in the proximal GI tract must have some local significance and not be related to the mechanism of epithelial cell differentiation per se. Similarly, only a select population of decidual cells of the gravid uterus exhibits high level ADA expression. Therefore, high-level ADA expression in the proximal GI tract and restricted cell types of the placenta is most likely related to specific local inductive influences, and perhaps, to the function of the enzyme in these locations. Several of these potential functions will be discussed briefly in turn.

GI Tract Expression

Despite the evolutionary conservation of GI tract purine degradation in mice and humans, there is little evidence for abnormal GI tract function in humans with three different gene defects affecting purine catabolism. ADA-deficient children fail to develop the immune system, but have remarkably little other pathology (Giblett et al., 1972; Hirschhorn et al., 1981; and for reviews Carrera and Carson, 1987; Kredich and Hershfield, 1989). For example, ADA-deficient children do not exhibit striking abnormality of GI tract function after a bone marrow transplantation that has restored immune function (Kredich and Hershfield, 1989). Thus, it is unlikely that low concentrations of free adenosine must be maintained locally for the basic functioning (e.g., peristalsis or circulatory control) of the GI tract. Further, PNP-deficient children do not exhibit any striking abnormalities of GI tract function, and finally, clinically normal adult humans have been found with apparently complete deficiency of XO (for review see Holmes and Wyngaarden, 1989). Thus, whatever physiological role is played by GI tract expression of the complete purine catabolic pathway, a failure to do so is not a cause of marked GI tract dysfunction per se. An additional consideration is the suggestion that nucleotides may be semi-essential nutrients (for review, see Uauy, 1989). Specifically, young mice fed a nucleoside-free diet show a significant decrease in their ability to survive sepsis (Kulkarni et al., 1986*a*,*b*), exhibit delayed allograft rejection (Van Buren et al., 1985), and have striking alterations in their fecal bacterial flora (Gil et al., 1986). The addition of nucleic acids to nucleoside-free chow at least partly reverses these effects. However, in light of the high level of purine catabolism in the proximal GI tract, it would seem paradoxical to suppose that exogenous purine nucleotides were nutritionally significant.

An alternate consideration might be that the in situ degradation of nucleotides could itself be of benefit to the gastrointestinal microenvironment. Such could be the case if the degradation of reutilizable purine was inhibitory to the growth of some microorganisms. For example, parasitic protozoa, causative agents of a plethora of human diseases, are entirely auxotrophic for purines and contain powerful mechanisms for purine salvage (Aronow et al., 1986). Since the GI tract is a frequent target site in the life cycles of a variety of parasites, such as *Toxoplasma*, *Entamoeba*, *Giardia*, and *Helminthic* organisms, it is possible that the in situ degradation of purines has served as an evolutionarily significant line of defense against parasitism.

Reproductive Tract Expression

As described in Results, there is high-level expression of purine degradative enzymes in an intricate series of locations within the expanding implantation chamber and the developing placenta during mouse pregnancy. Despite the diverse locations in which the catabolic enzymes are expressed, the actual number of different cell types may be rather limited. During the first half of pregnancy, the major expressing cell types are a subset of maternal decidual cells previously known to express high levels of alkaline phosphatase and 5′NT (Hall, 1971; Finn, 1971), and more recently ADA (Knudsen et al., 1988, 1991). The maternal decidualization reaction is the result of the proliferation and differentiation of uterine stromal cells (Loeb, 1908; Krehbiel, 1937; Enders et al., 1985), and represents a major portion of the overall growth that occurs during the first half of postimplantation pregnancy in the mouse (Rugh, 1968; Theiler, 1972). The decidual reaction can be divided into two phases. In the first (days 6–7), there is a concentric proliferative development of decidual cells extending from the embryo to the myometrium. However, only the small fraction of the resultant decidual cells that are immediately adjacent to the embryo express the purine catabolic enzymes. In the second phase (days 8–10), there is intense regression of the antimesometrial decidua capsularis cells (Welsh and Enders, 1985; Katz and Abrahamson, 1987), and these are quite uniformly positive for high level expression of the purine catabolic enzymes. By colcemid arrest and radiolabeled thymidine incorporation, Finn and Martin (1967) have shown that unlike the surrounding decidual cells, those from these two regions in which we see purine catabolic enzyme expression consist of non- or postmitotic cells. Thus the nonmitotic cells, which are clearly in the process of regression, appear to express the purine catabolic enzymes as a gene regulatory event that precedes their undergoing programmed cell death. The exception to this is that GUAase was not expressed by any of the cell types in the reproductive tract. Consistent with the lack of guanase expression by the decidual cell types, and our proposal that the role of the catabolic enzymes is to metabolize large quantities of nucleosides generated in situ, Van Kveel (1982) has observed that both uric acid and guanine accumulate in amniotic fluid. Expression of the catabolic enzymes may be especially critical in the early postimplantation period as Knudsen et al. (1989) have shown that the inhibition of ADA activity at day 7 or 8 p.c., but not at day 6 or 9 causes pronounced embryolethality with neural plate cell degeneration. Also, the administration of allopurinol to day 9 p.c. mice has also been reported to result in a significant degree of fetal loss and teratogenicity (Fujii and Nishimura, 1972).

The final stages of implantation chamber development are somewhat more difficult to understand in the context of the purine catabolic enzyme expression. From day 11 through term, the labyrinth zone of the chorioallantoic placenta, composed chiefly of fetal trophoblastic cells, is organizing and expanding into the maternal decidua basalis along the mesometrial side of the uterus. During this phase, the maternal decidua basalis that underlies this region undergoes a prominent and continuous regression (Stewart and Peel, 1978). Consistent with earlier stages, the regressing decidual cells exhibit intense expression of the purine catabolic en-

zymes. Further, only a subset of the decidual cells in the basalis are positive. One or two days prior to term, the decidua basalis layer has become quite thin, but it is virtually completely positive for the purine catabolic enzymes. At this stage, there is also purine catabolic enzyme expression by a subpopulation of fetal-derived cells of the placenta (spongiotrophoblasts) that are interposed between the embryo-derived chorioallantoic plate and the maternal-derived decidua basalis (Fig. 3, *I* and *J*). Consistent with this, Knudsen et al. (1991) have shown by adoptive transfer of embryos that unlike days 7–11 p.c., ADA expression at day 13 occurs in both materna and fetal derived cell types. These cells may play an additional role in the protection of the embryo from the potentially cytotoxic effects of high concentrations of purine nucleotides such as adenosine and deoxyadenosine released by regressing cells undergoing programmed cell death.

Decidual cells have been long considered to play a role in the nutrition of the early postimplantation embryo, but given the high level of XO expression by the periembryonic decidual cells, it is unlikely that decidual cell degeneration leads to the production of preformed and nontoxic purines for the rapidly proliferating cells of the embryo. More likely, the purine catabolic enzymes are expressed to cope with a considerable amount of purine nucleotides that are generated by a high level of cell death that accompanies remodeling of the maternal decidua (Welsh and Enders, 1985; Katz and Abrahamson, 1987). Since induction of the purine catabolic enzymes precedes and accompanies programmed cell death in these tissues, we hypothesize that before actual cell death, there is induced expression of enzymes to detoxify cellular breakdown products. Thus, a segment of the cell death program may be directed towards the expression of catabolic enzymes. In this manner, tissues undergoing programmed cell death may share some of the characteristics of tissues dedicated to digestive metabolism such as the proximal GI tract. This "concerted catabolic clean-up hypothesis" suggests that in the process of marking a cell for death, some of the genes that must be activated are those that are necessary for the metabolism of potentially toxic or bioactive compounds released by cellular autolysis. A partly analogous situation could be imagined in the thymus. Regulatory elements that direct high level ADA expression in human thymus (Aronow et al., 1989) could also be a response or adaptation to the occurrence of programmed cell death among thymocytes marked for cell death. However, unlike the proximal GI and the maternal fetal interface, in the thymus there is discoordinate expression of ADA and PNP in separate cell-type subpopulations (Barton et al., 1980; Ma et al., 1982; ourselves unpublished), and there is no detectable xanthine oxidase activity in the thymus (Aronow, B., and D. Witte, unpublished). Thus nontoxic purines may be salvaged by rapidly proliferating thymocytes. In contrast, preformed purines are less likely to be salvaged by the embryo because uric acid is not reusable. But similar to intrathymic T cell development, the rapid development of the maternal–fetal interface may also require a high level of programmed cell death and therefore the need for purine catabolic enzyme expression. It is possible that a failure to express this or any of several catabolic pathways during implantation, placental, and embryonic development could lead to infertility, intrauterine growth retardation, birth defects, or fetal death.

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