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Expression of **GENE X** in Embryonic & Adult Mice

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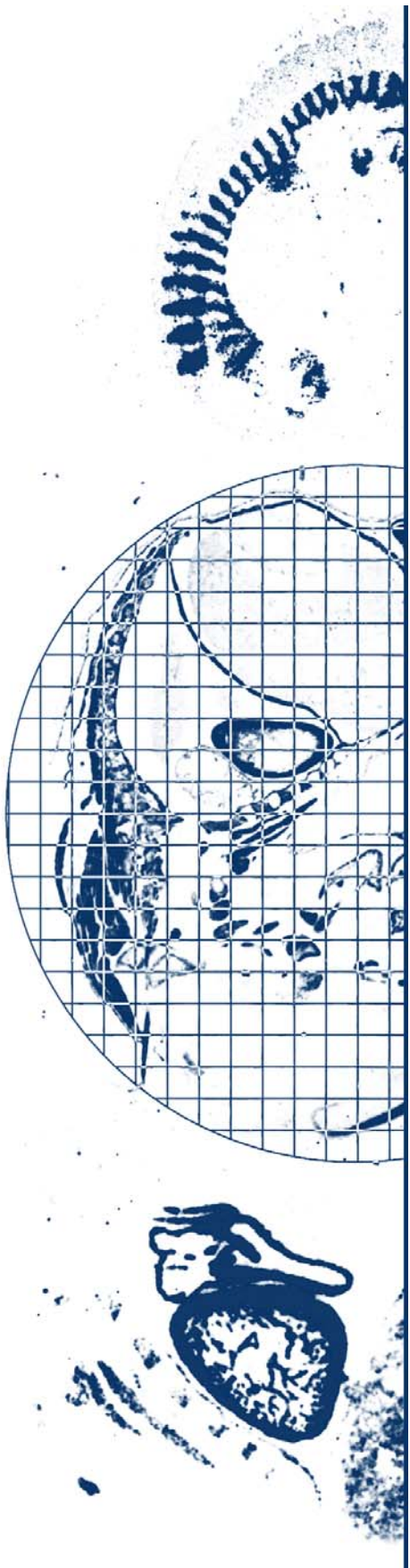


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CD-ROM (Images & Report)		
Additional Comments		

Please reference Phylogeny as having performed this study in any publications or presentations.

Summary

Enclosed are slides of select embryonic and adult mouse tissues that were assayed by *in situ* hybridization (ISH) with riboprobes for the transcript of the gene, GENE X. GENE X is a novel transcription factor expressed in mouse and human tissues¹. Previously published Northern blot data provided evidence for the presence of GENE X expression primarily in adult mouse kidney and uterus and to a lesser extent in 11 additional organs including brain, heart, lung, liver, spleen, stomach, intestine, thymus, testis, and placenta¹. Whole-mount ISH documented the presence of GENE X mRNA in a number of embryonic organs including kidney, lungs, testes, and limbs¹. In this work, the pattern of GENE X expression has been re-examined using cryostat sections from several embryonic and adult mouse stages with a special focus on kidney, testis, eye, lung, ovary, and limb (Table 1).

Table 1: Summary of Expression Over Course of Development

	Stage E11.5	Stage E12.5	Stage E13.5	Stage E14.5	Stage E16.5	Stage Adult
Kidney	x	x	x	x	x	
Testis				x		x
Eye/Optic Nerve		x		x (frontal)		x
Lung				x	x	x
Ovary						x
Limb		x		x	x	

The results confirmed GENE X expression in a number of embryonic tissues that were previously examined. Thus, rudimentary kidney contained GENE X-labeled pronephric and metanephric tubules (Figures 1 & 2). Rudimentary testes contained labeled testis cords (Figures 1 & 3). Embryonic eye contained some labeling within the sensory layer of the retina, while significantly higher GENE X labeling was noted in the primitive skin, in proximity of cornea (Figures 1 & 4). Lung primordium displayed GENE X labeling uniformly distributed throughout pulmonary bronchiole and alveoli (Figures 1 & 5). Developing limbs showed GENE X labeling within cell densities, most likely blastemas containing undifferentiated cells in the neighborhood of chondroification centers (Figures 1 & 6). In early-differentiated upper lips, there were intensely labeled mesenchymal cells present in between the rows of unlabeled primitive whisker roots (Figure 1). The findings showing GENE X expression in rudimentary pituitary gland (see Rathke's pocket) seen on day 11.5 (Figures 1 & 7) were unexpected. Circumventricular organs, choroid plexus, and ependyma in the brain ventricles were labeled (Figure 1). ISH labeling was also found in a number of adult tissues such as kidney, lung, eye, testis, ovary, and uterus (Figure 8). Note, however, that whereas the first 5 listed organs showed a low-density widespread GENE X mRNA distribution, the sixth (uterus) displayed comparatively high-level mRNA labeling of GENE X expressed specifically within epithelial cells around uterine cavity (Figure 8).

Conclusions and Significance

This work provides ISH evidence of a discrete pattern of developmentally regulated GENE X mRNA distribution in mouse embryo. Results were obtained using a relatively short cRNA probe (358 nts), giving weak ISH signal. To compensate, photographic exposure times were extended for x-ray films (12 days) and photographic emulsion (34 days).

The developmental portion of this study focused on five mid-gestational stages in mouse development, situated between day 11.5 and 16.5, when many primitive organs are formed. This revealed a developmentally regulated pattern of GENE X expression in the retina and posterior limb that peaked on day E13.5 and was followed by a decline on day E16.5. In comparison with adulthood, GENE X expression in the kidney, testis, and lung reached its maximum levels *in utero*, suggesting that it plays a role in development of these tissues. Finally, GENE X expression in the uterine epithelial cells may have significance in organ development and reproductive activity.

It is recommended that follow-up ISH studies include evaluation of GENE X expression patterns in prenatal and postnatal pituitary gland. This organ is a source of six hormones secreted throughout six endocrine cell types either before or after birth. GENE X could potentially play a role in pituitary cell differentiation and/or hormone secretion regulation.

1. Y-S Kim, G Nakanishi, M Lewandowski and AM Jetten. GENE X, a novel member of the X subfamily of Krüppel-like zinc finger proteins with repressor and activation functions. *Nucleic Acids Research*, 2003, 31:5513-5525.

Detailed Findings whole embryo

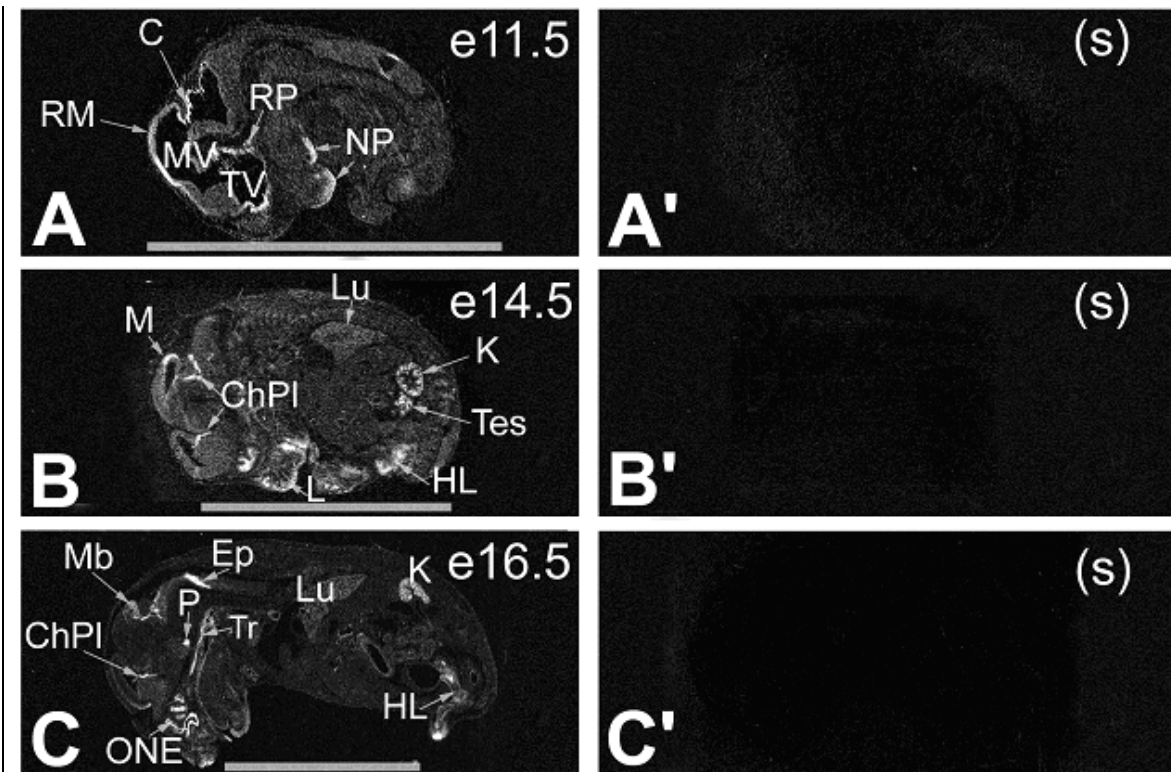


Figure 1: GENE X mRNA in mouse embryo at anatomical level

A	E11.5 embryo after hybridization with GENE X antisense probe. X-ray film autoradiography following 12-days exposure showed a discrete pattern of GENE X expression in 3 stages of mouse.
A'	Sense control result for (A).
B	E14.5 embryo after hybridization with GENE X antisense probe.
B'	Sense control result for (B).
C	E16.5 embryo after hybridization with GENE X antisense probe.
C'	Sense control result for (C).

Abbreviations: C- cerebellar primordium; ChPl – choroids plexus; Ep – ependyma around central canal; HL – hind limb; K – kidney, primordium; L- lip; Lu – lung, primordium; M – mesencephalon; Mb – midbrain, wall; MV – mesencephalic vesicle; NP – nasal processes; ONE – olfactory neuroepithelium in olfactory turbinates; P – pituitary gland, primordium; RP – Rathke pouch (future pituitary gland); Tes – testis; Tr – trachea; TV – telencephalic vesicle.

Magnifications: Bar in (A, B & C) = 1 cm.

Detailed Findings embryonic kidney

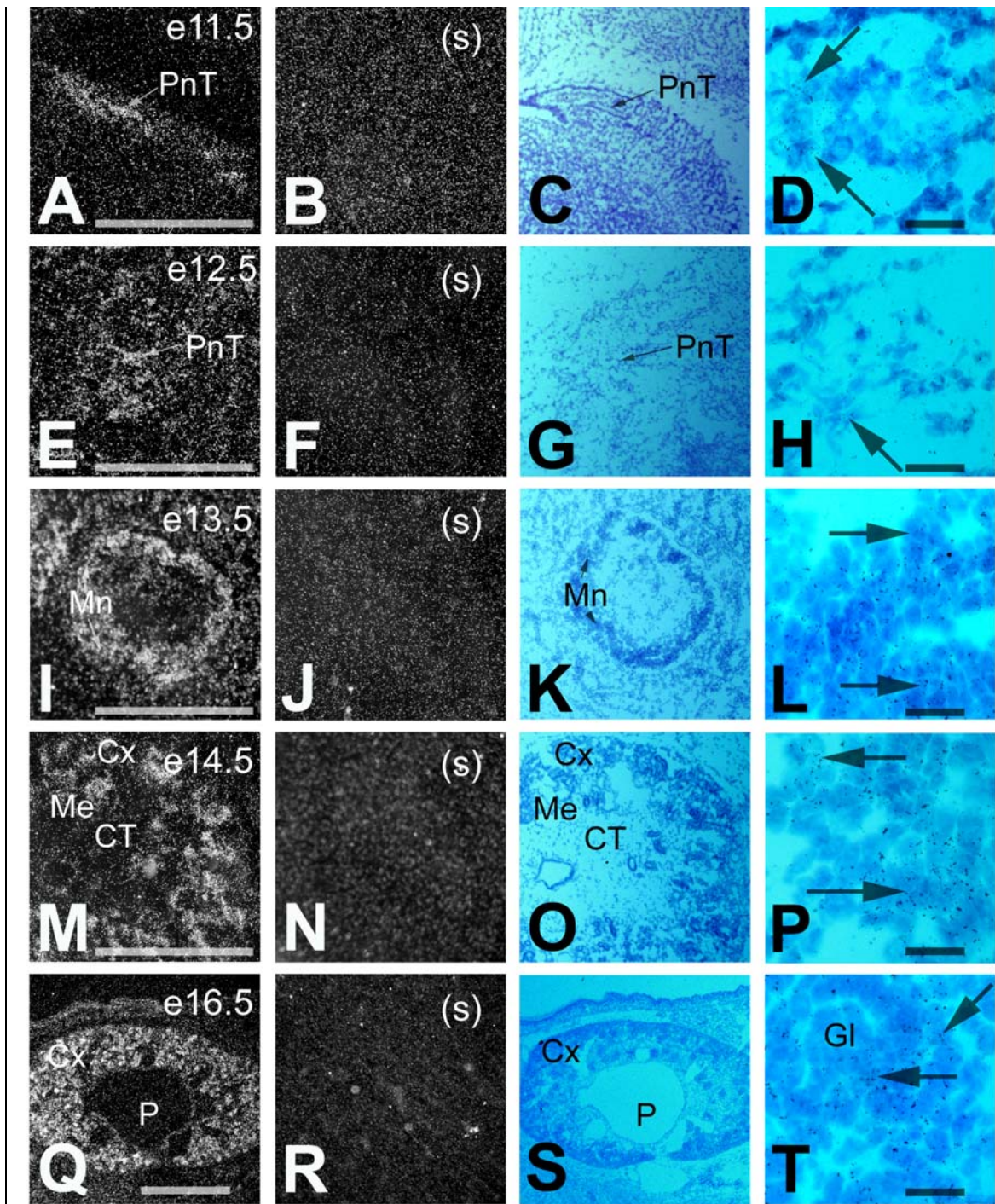


Figure 2: GENE X mRNA in kidney mid-gestational development

Detailed Findings embryonic kidney

Figure 2: GENE X mRNA in kidney mid-gestational development

A	Darkfield image of E11.5 embryo following hybridization with the GENE X antisense probe and emulsion autoradiography. GENE X mRNA appears in the kidney primordium.
B	Sense control result for (A).
C	E11.5 embryo following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
D	Close-up image of (C), showing labeling at cellular level (arrows).
E	Darkfield image of E12.5 mouse following ISH with GENE X antisense probe and emulsion autoradiography.
F	Sense control result for (E).
G	E12.5 embryo following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
H	Close-up image of (G), showing labeling at cellular level (arrows).
I	Darkfield image of E13.5 mouse following ISH with GENE X antisense probe and emulsion autoradiography.
J	Sense control result for (I).
K	E13.5 embryo following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
L	Close-up image of (K), showing labeling at cellular level (arrows).
M	Darkfield image of E14.5 mouse following ISH with GENE X antisense probe and emulsion autoradiography.
N	Sense control result for (M).
O	E14.5 embryo following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
P	Close-up image of (O), showing labeling at cellular level (arrows).
Q	Darkfield image of E16.5 mouse following ISH with GENE X antisense probe and emulsion autoradiography.
R	Sense control result for (Q).
S	E16.5 embryo following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
T	Close-up image of (S), showing labeling at cellular level (arrows).
	Abbreviations: CT – collecting tubules; Cx – renal cortex; Gl – glomerular apparatus, presumptive; Me – renal medulla; Mn – metanephrons; P – renal pelvis; PnT – pronephric tubules.
	Magnifications: Bar in (A, B, C, E, F, G, I, J, K, M, N & O) = 0.5 mm; Bar in (Q, R & S) = 0.5mm; Bar in (D, H, L, P & T) = 20 mm.

Detailed Findings embryonic testis

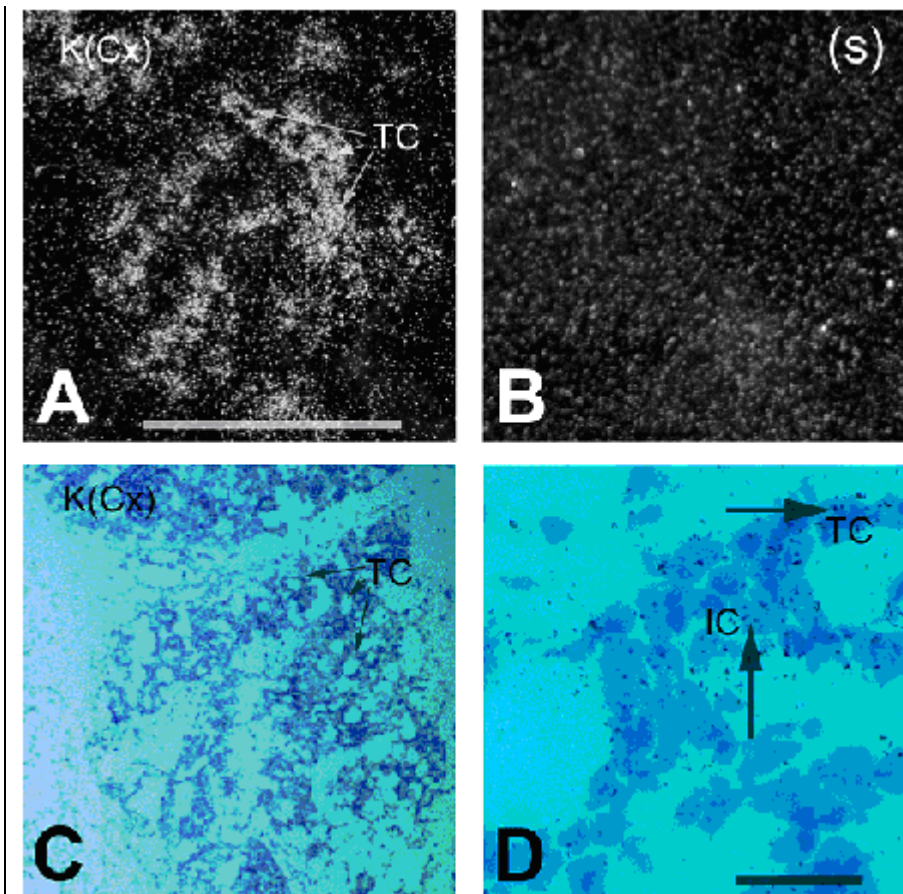


Figure 3: GENE X mRNA in testis of E14.5 mouse embryo

A	Darkfield imaging reveals GENE X expression in E14.5 testes cords following hybridization with the antisense probe. Note GENE X hybridization in kidney cortex rudiments.
B	Sense control result for (A).
C	Anatomical view of testis cords after hybridization with the antisense probe, emulsion autoradiography, and staining with hematoxylin.
D	Antisense hybridization at cellular level (arrows): mRNA occurs both in interstitial cells and cells of the testis cord that represent future seminiferous tubules.
Abbreviations: IC – interstitial cells; K(Cx) – kidney, cortical region; TC – testis cords.	
Magnifications: Bar in (A, B & C) = 0.5 mm; Bar in (D) = 25 mm.	

Detailed Findings embryonic eye

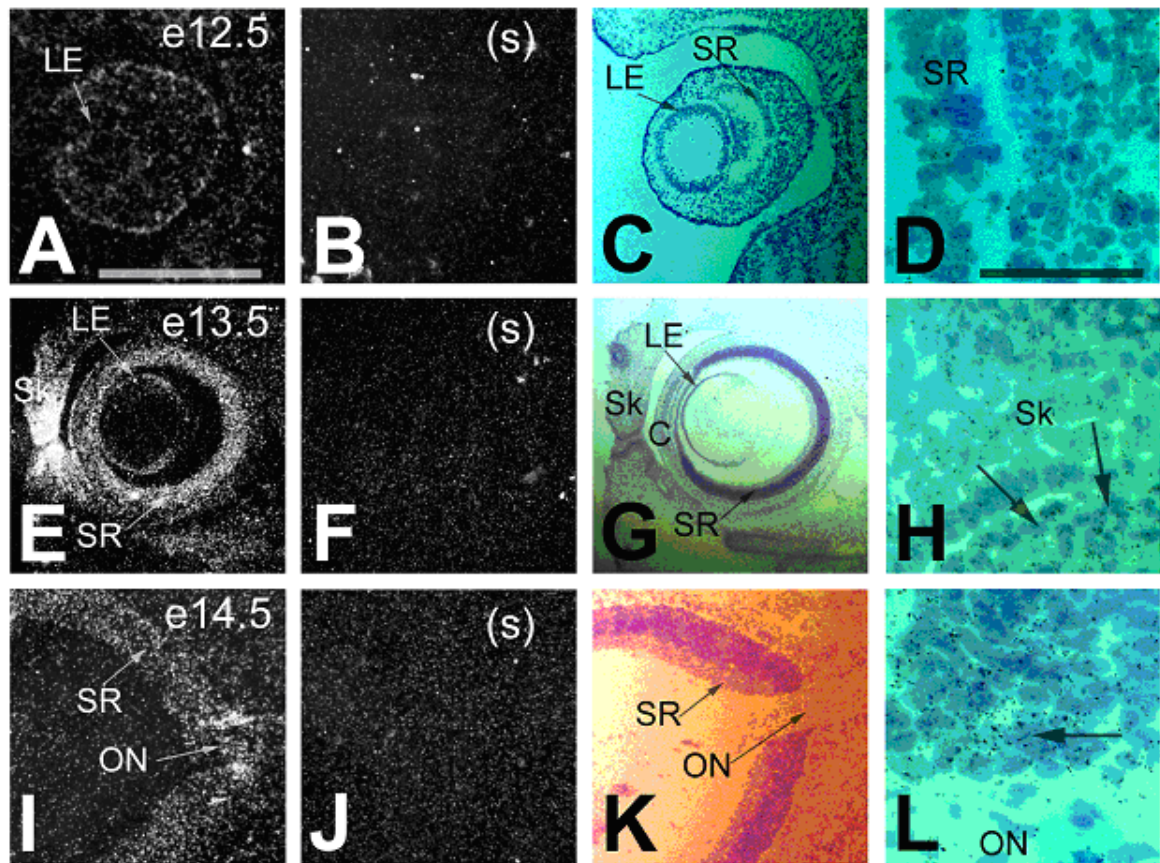


Figure 4: GENE X in eye development

Detailed Findings embryonic eye

Figure 4:	GENE X in eye development
A	GENE X mRNA was detected in developing eye. Varying levels of GENE X hybridization are found in sensory retina at stages E12.5 (A), E13.5 (E) and E14.5 (I) suggesting that developmental regulation of GENE X expression occurs in this tissue.
B	Sense control result for (A).
C	Anatomical view of E12.5 embryo following ISH with GENE X antisense probe, emulsion autoradiography, and hematoxylin staining.
D	Close-up image of (C), showing labeling at cellular level (arrows).
E	GENE X mRNA was detected in the eye of E13.5 mouse after hybridization with the antisense probe. Note the presence of high GENE X mRNA concentrations in primary skin facing the cornea.
F	Sense control result for (E).
G	Anatomical view of E13.5 embryo following ISH with GENE X antisense probe, emulsion autoradiography, and hematoxylin staining.
H	Close-up image of (G), showing labeling at cellular level. Here, mesenchymal cells delineated by a germinal cell layer along the cornea display high level labeling (arrows).
I	GENE X mRNA detected in E14.5 developing eye shown as bright labeling on dark background. Locally increased GENE X concentration was found to be at the junction between the optic nerve and sensory retina.
J	Sense control result for (I).
K	Anatomical view of E14.5 embryo following ISH with GENE X antisense probe, emulsion autoradiography, and hematoxylin staining.
L	Close-up image of (K), highlighting locally increased GENE X concentration (arrows) at the junction between the optic nerve and sensory retina. Abbreviations: C – cornea; LE – lens epithelium; ON – optic nerve; SR – sensory retina; Sk – skin. Magnifications: Bar in (A, B, C, E, F, G, I, J & K) = 0.5 mm; Bar in (D, H & L) = 50 mm.

Detailed Findings embryonic hind limb

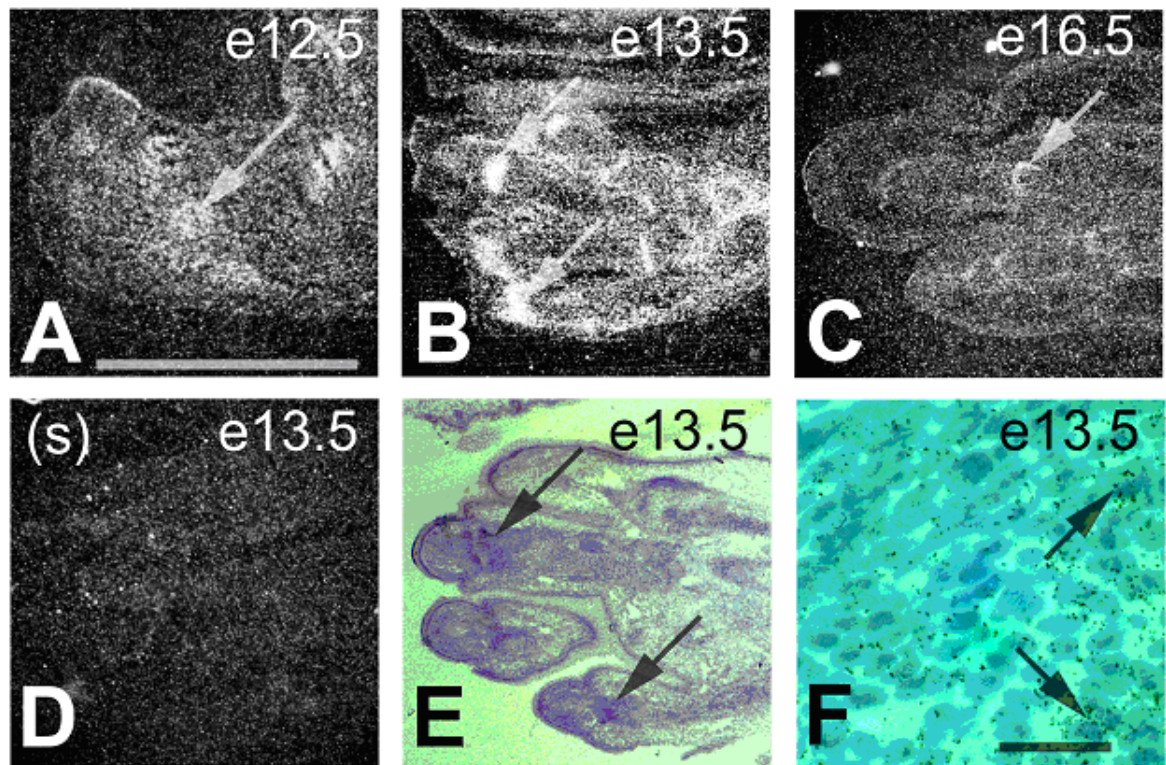


Figure 5: GENE X mRNA in embryonic hind limb

A	Hind limb at developmental stage E12.5 shown following hybridization with GENE X antisense probe. Bright mRNA labeling appears (arrows) with maximum hybridization levels noted on day E13.5.
B	Hind limb at E13.5 following hybridization with GENE X antisense probe.
C	Hind limb at E16.5 following hybridization with GENE X antisense probe.
D	Sense control result for (B).
E	Dense groups of cells at E13.5, likely composed of undifferentiated cells (blastemas), show GENE X expression (arrows) following antisense hybridization, emulsion autoradiography, and hematoxylin staining.
F	Close-up image of (E), showing GENE X labeling at cellular level in hind limb blastema (arrows) near chondroification centers.
Magnifications: Bar in (A, B, C, D & E) = 1 mm; Bar in (F) = 25 mm.	

Detailed Findings embryonic lung

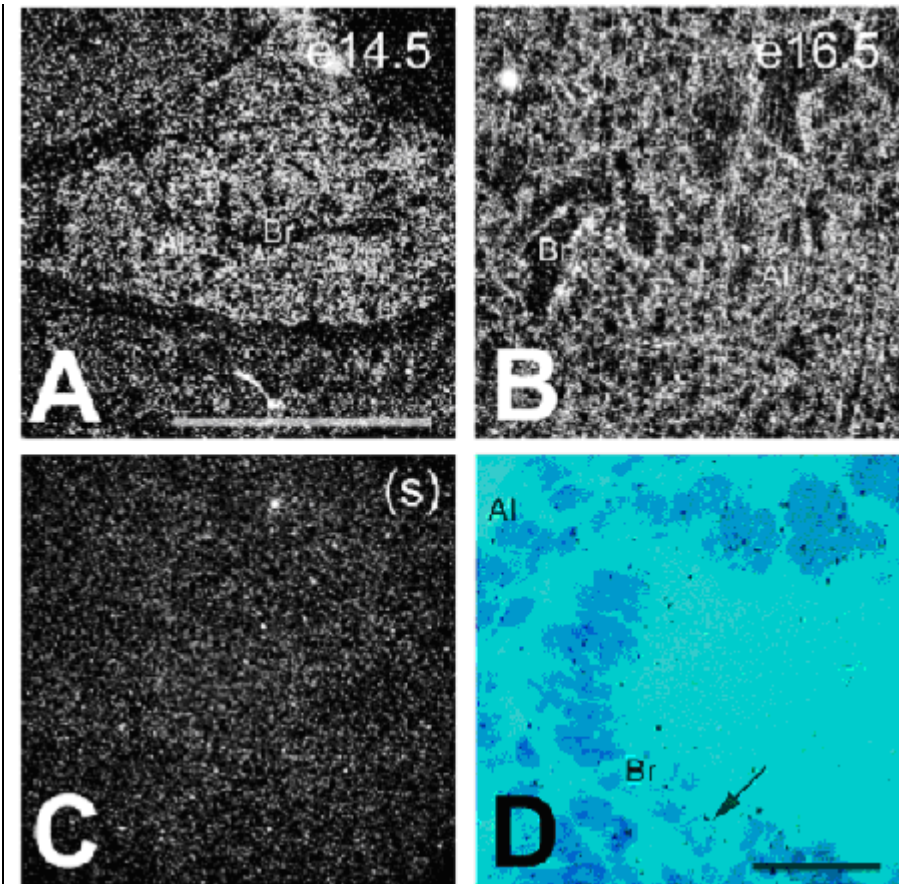


Figure 6: GENE X mRNA in rudimentary lungs

A	GENE X mRNA was detected in developing lungs on day E14.5. Hybridization of the GENE X antisense probe was found mainly in primitive pulmonary bronchi and alveoli.
B	GENE X mRNA was also detected in developing lungs on day E16.5.
C	Sense control result for (B).
D	High magnification image of E16.5 lung following hybridization with GENE X antisense probe. Note labeling at arrows.
Abbreviations: Br - bronchi and Al - alveoli.	
Magnifications: Bar in (A, B & C) = 0.5 mm; Bar in (D) = 25 mm.	

Detailed Findings embryonic pituitary gland

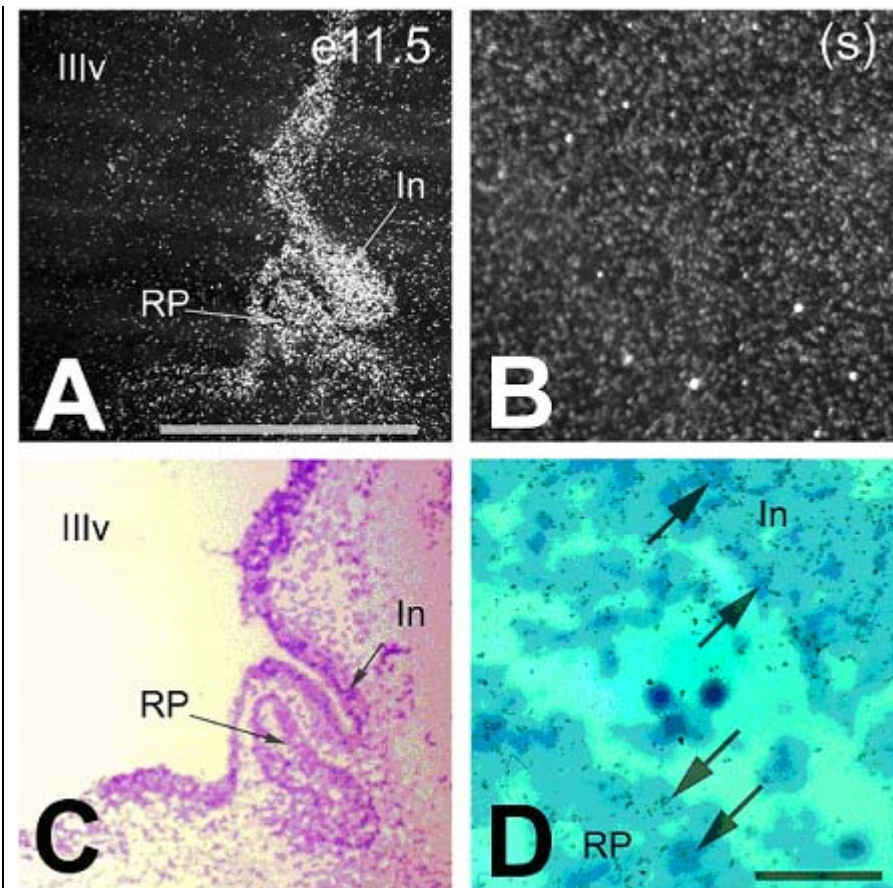


Figure 7: GENE X mRNA in primitive pituitary gland

- A** GENE X mRNA was detected in developing pituitary gland on day E11.5. Hybridization of the GENE X antisense probe was found mainly in the primitive infundibulum and Rathke's pocket. Note that the infundibulum shows the evagination of the diencephalon, giving rise to a pars intermedia (intermediate lobe) of the pituitary gland, whereas Rathke's pocket through cellular proliferation gives rise to a pars distalis (anterior lobe) of the pituitary gland.
- B** Sense control result for (A).
- C** Topographic hematoxylin staining of E11.5 pituitary following hybridization with the GENE X antisense probe and emulsion autoradiography.
- D** Close-up of (C), showing GENE X labeling at cellular level (arrows).

Abbreviations: IIIv – third ventricle; In - infundibulum; RP - Rathke's pocket.

Magnifications: Bar in (A, B & C) = 0.5 mm; Bar in (D) = 25 mm.

Detailed Findings selected adult tissues

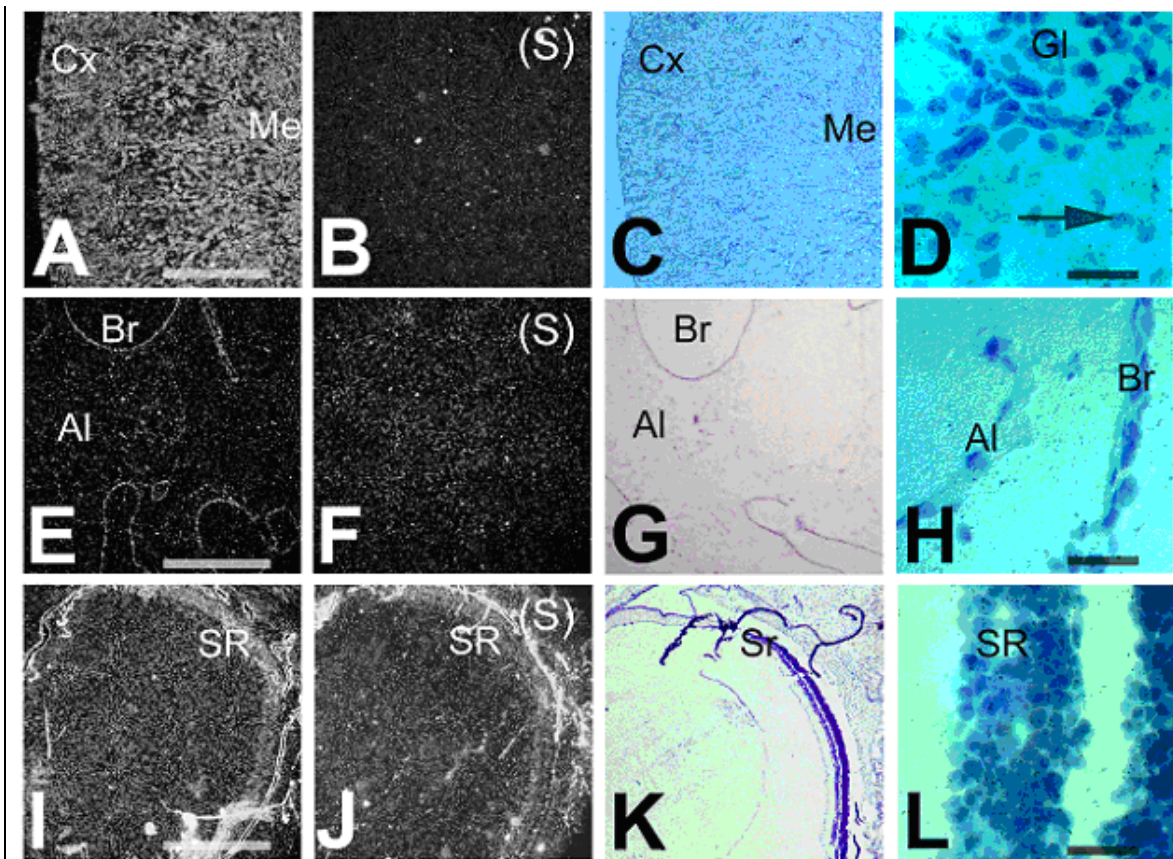


Figure 8: GENE X mRNA in adult kidney, lung and eye

Detailed Findings selected adult tissues

Figure 8:	GENE X mRNA in adult kidney, lung and eye
A	After ISH with GENE X antisense probe, adult kidney tissue displays relatively uniform low-level labeling found throughout kidney cortex (Cx) and medulla (Me).
B	Sense control result for (A).
C	Kidney following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
D	Close-up of (C), showing labeling at cellular level (arrows). Glomeruli containing little, if any labeling, are surrounded by tubular structures showing slightly-labeled GENE X cells (arrow).
E	Pulmonary tissue displays little GENE X mRNA concentration in bronchi (Br) and alveoli (Al).
F	Sense control result for (E).
G	Pulmonary tissue following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
H	Close-up of (G), showing labeling at cellular level.
I	Eye tissue seems to exhibit negligible GENE X mRNA after ISH with the antisense probe. Antisense and sense hybridization levels in sensory retina (SR) are very similar.
J	Sense control result for (I).
K	Eye tissue following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
L	Close-up of (K), showing labeling at cellular level.
Magnifications: Bar in (A, B, C, E, F, G, I, J and K) bar = 0.5 mm; Bar in (D, H & L) = 20 mm.	

Detailed Findings selected adult tissues

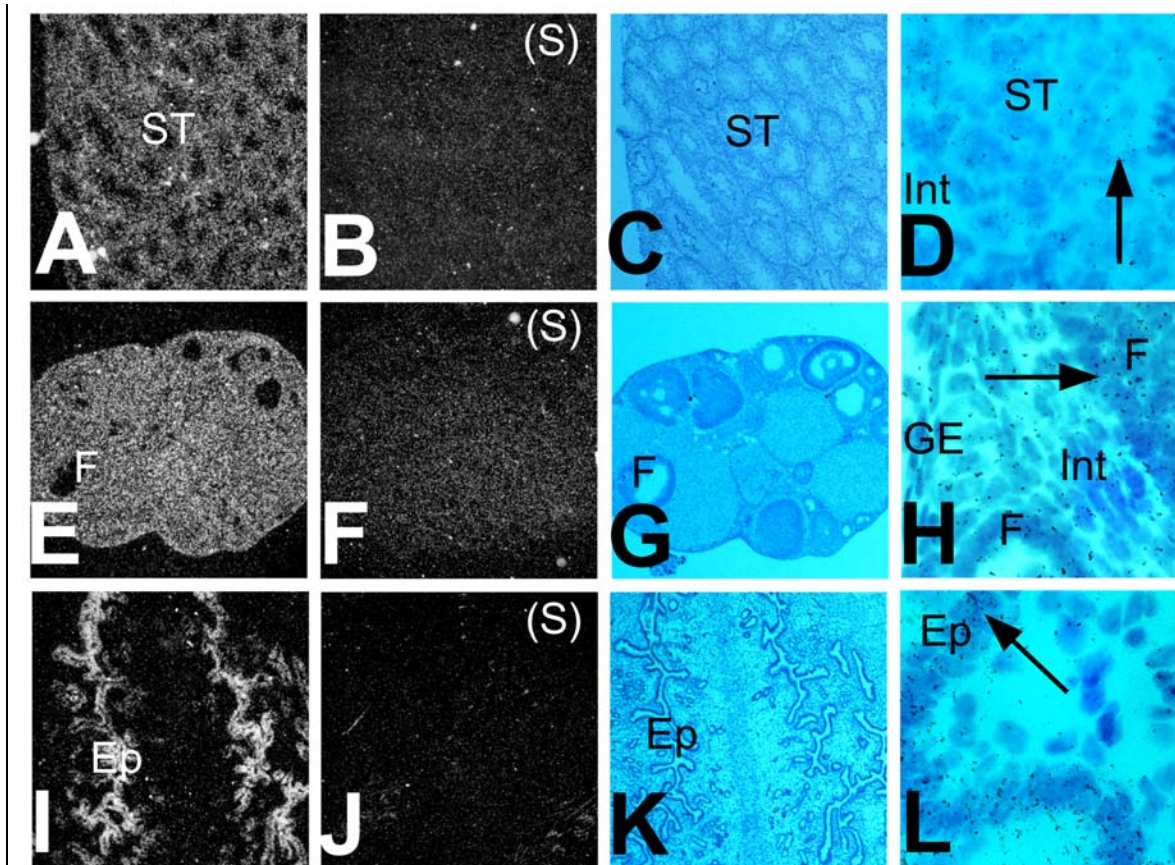


Figure 9: GENE X mRNA in adult testis, ovary and uterus

Detailed Findings selected adult tissues

Figure 9:	GENE X mRNA in adult testis, ovary and uterus
A	Testis displays low-level GENE X labeling distributed uniformly along seminiferous tubules (ST, arrow in P) and interstitial space cells (Int in P).
B	Sense control result for (M).
C	Testis following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
D	Close-up of (O), showing labeling at cellular level (arrows).
E	Ovary displays a low-level GENE X labeling distributed uniformly throughout the follicles (F) and interstitial cells (Int).
F	Sense control result for (Q).
G	Ovary following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
H	Close-up of (S), showing labeling at cellular level in follicles (arrows).
I	Uterus shows a discrete pattern of GENE X mRNA distribution with relatively high-level accumulation of the labeling in epithelial cell layers (Ep) that delineate the lumen of the organ.
J	Sense control result for (U).
K	Uterus following ISH with GENE X antisense, emulsion autoradiography, and hematoxylin staining.
L	Close-up of (W), showing labeling at cellular level. Note labeling in epithelial cell layers (Ep) that delineate the lumen of the organ (arrows).
Magnifications: Bar in (A, B, C, E, F, G, I, J and K) = 0.5 mm; Bar in (D, H & L) = 20 mm.	

Methods

Tissue Fixation, Embedding, and Pretreatment

Frozen tissues were cryosectioned into 8 μm to 10 μm sections, mounted on gelatin-coated slides and stored at -80°C . Between 1 and 10 sections were placed on each slide. Prior to ISH, sections were fixed in 4% paraformaldehyde in phosphate buffered saline, treated with triethanolamine/acetic anhydride, washed, and dehydrated with a series of ethanol solutions. Prior to use in this study, tissues were validated by ISH using probes for the mouse LDL receptor, which experiment showed an mRNA distribution/abundance pattern that was as expected (data not shown).

cRNA Probe Preparation

The cRNA transcripts were synthesized in vitro according to manufacturer's conditions (Ambion) and labeled with ^{35}S -UTP (>1000 Ci/mmol; Amersham). Both sense and antisense DNA templates (358 nts) were used as provided.

Hybridization and Washing Procedures

Tissues were hybridized with ^{35}S -labeled cRNA probes, antisense and sense, generating positive and negative (control) signals, respectively. Two sets of sections were hybridized with antisense riboprobes and one set of sections was hybridized with control sense riboprobes. Sections were hybridized overnight at 55°C in 50% deionized formamide, 0.3 M NaCl, 20mM Tris-HCl pH 7.4, 5 mM EDTA, 10 mM NaH_2PO_4 , 10% dextran sulphate, 1 x Denhardt's, 50 $\mu\text{g}/\text{ml}$ total yeast RNA, and 50-80,000 cpm/ μl ^{35}S -labeled cRNA probe. The tissue was subjected to stringent washing at 65°C in 50% formamide, 2 x SSC, 10 mM DTT and washed in PBS before treatment with 20 $\mu\text{g}/\text{ml}$ RNase A at 37°C for 30 minutes. Following washes in 2 x SSC and 0.1 x SSC for 10 minutes at 37°C , the slides were dehydrated, exposed to X-Rays Film for twelve 12 days, and dipped in Kodak NTB-2 nuclear track emulsion and exposed for 34 days in light-tight boxes with desiccant at 4°C .

Imaging

Following ISH, the gene expression patterns were analyzed by both x-ray film autoradiography (12 days exposure time) and emulsion autoradiography (34 days exposure time). Emulsion autoradiography was carried out in Kodak D-19. Slides were counterstained lightly with hematoxylin and eosin and analyzed using both light- and darkfield optics. Sense control cRNA probes (identical to the mRNAs) always gave background levels of hybridization signal. Selected slides were then scanned into Photoshop 6.0 files.

Storage and Rehydration

Any "crystallized" section may be repaired by allowing the coverslip to fall off after soaking the slide in xylene for 24-48 hours. Rehydrate the slide to 70% EtOH and then re-dehydrate them again in a series of ethanol (80%, 96% and 2 x 100% for 2 minutes each). After three changes of xylene, mount the coverslips with Cytoseal (VWR Scientific) or other comparable mounting medium. Using the same method, coverslips can be removed for histological staining to take brightfield micrographs. Histological stains that require acidic conditions may dissolve silver grains. Overstaining may obscure the silver grains. Any excess mounting medium or residual emulsion on the back of the slides can be removed with a single-edged razor. Dry the re-coverslipped slides flat for 24 hours. These slides can be stored indefinitely at room temperature.

Viewing Original Slides

The results are best viewed with darkfield illumination, or with a 20x or 40x phase-contrast objective, the silver grains can be localized over particular cells. The antisense probe (AS) detects the mRNA and the sense control probe (S) shows the background level of silver grains for the experiments.

Table of Figures

Figure	Panel	Slide	Tissue	GENE X Probe	Total Magnification	Photo Filename
1	A	X-ray	E11.5 Embryo	AS	5.5	FX-1
	A'	X-ray	E14.5 Embryo	S	5.5	FX-2
	B	X-ray	E16.5 Embryo	AS	3.9	FX-3
	B'	X-ray	E11.5 Embryo	S	3.9	FX-4
	C	X-ray	E14.5 Embryo	AS	3.0	FX-5
	C'	X-ray	E16.5 Embryo	S	3.0	FX-6
	2	A	21	E11.5 Kidney	AS	48
B		41	E11.5 Kidney	S	48	F1-2
C		1	E11.5 Kidney	AS	48	F1-3
D		1	E11.5 Kidney	AS	500	F1-4
E		22	E12.5 Kidney	AS	48	F1-5
F		42	E12.5 Kidney	S	48	F1-6
G		2	E12.5 Kidney	AS	48	F1-7
H		2	E12.5 Kidney	AS	500	F1-8
I		23	E13.5 Kidney	AS	48	F1-9
J		43	E13.5 Kidney	S	48	F1-10
K		3	E13.5 Kidney	AS	48	F1-11
L		3	E13.5 Kidney	AS	500	F1-12
M		26	E14.5 Kidney	AS	48	F1-13
N		46	E14.5 Kidney	S	48	F1-14
O		6	E14.5 Kidney	AS	48	F1-17
P		6	E14.5 Kidney	AS	500	F1-19
Q		29	E16.5 Kidney	AS	30	F1-21
R		49	E16.5 Kidney	S	30	F1-22
S		9	E16.5 Kidney	AS	30	F1-23
T		9	E16.5 Kidney	AS	500	F1-24
3	A	26	E14.5 Testis	AS	80	F1-15
	B	46	E14.5 Testis	S	80	F1-16
	C	6	E14.5 Testis	AS	80	F1-18
	D	6	E14.5 Testis	AS	800	F1-20
4	A	22	E12.5 Eye	AS	48	F2-6
	B	42	E12.5 Eye	S	48	F2-7
	C	2	E12.5 Eye	AS	48	F2-8
	D	2	E12.5 Eye	AS	500	F2-9
	E	24	E13.5 Eye	AS	48	F2-10
	F	44	E13.5 Eye	S	48	F2-11
	G	4	E13.5 Eye	AS	48	F2-12
	H	4	E13.5 Eye	AS	500	F2-15
	I	27	E14.5 Eye	AS	48	F2-19
	J	47	E14.5 Eye	S	48	F2-20
	K	7	E14.5 Eye	AS	48	F2-16
	L	7	E14.5 Eye	AS	500+	F2-17

Table of Figures continued

Figure	Panel	Slide	Tissue	GENE X Probe	Total Magnification	Photo Filename
5	A	22	E12.5 Hind Limb	AS	35	F2-23
	B	24	E13.5 Hind Limb	AS	35	F2-24
	C	29	E16.5 Hind Limb	AS	35	F2-25
	D	44	E13.5 Hind Limb	S	35	F2-29
	E	24	E13.5 Hind Limb	AS	35	F2-26
	F	24	E13.5 Hind Limb	AS	565	F2-27
6	A	26	E14.5 Lung	AS	80	F2-34
	B	29	E16.5 Lung	AS	80	F2-35
	C	49	E14.5 Lung	S	80	F4-11
	D	8	E16.5 Lung	AS	800	F3-1
7	A	21	E11.5 Pituitary Gland	AS	80	F3-5
	B	41	E11.5 Pituitary Gland	S	80	F3-6
	C	1	E11.5 Pituitary Gland	AS	80	F3-4
	D	1	E11.5 Pituitary Gland	AS	800	F3-3
8	A	32	Adult Kidney	AS	5	F1-25
	B	52	Adult Kidney	S	5	F1-26
	C	12	Adult Kidney	AS	5	F1-27
	D	12	Adult Kidney	AS	80	F1-28
	E	32	Adult Lung	AS	5	F2-36
	F	52	Adult Lung	S	5	F2-37
	G	2	Adult Lung	AS	5	F4-1
	H	2	Adult Lung	AS	80	F3-2
	I	30	Adult Eye	AS	5	F2-30
	J	50	Adult Eye	S	5	F2-31
	K	10	Adult Eye	AS	5	F2-32
	L	10	Adult Eye	AS	80	F2-33
9	A	31	Adult Testis	AS	5	F1-30
	B	51	Adult Testis	S	5	F1-32
	C	11	Adult Testis	AS	5	F1-33
	D	11	Adult Testis	AS	80	F1-29
	E	31	Adult Ovary	AS	5	F1-35
	F	51	Adult Ovary	S	5	F1-36
	G	11	Adult Ovary	AS	5	F1-34
	H	11	Adult Ovary	AS	80	F1-37
	I	31	Adult Uterus	AS	30	F2-4
	J	51	Adult Uterus	S	30	F2-5
	K	11	Adult Uterus	AS	30	F2-3
	L	11	Adult Uterus	AS	500	F2-1