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Award Number:

DAMD17-97-1-7282

TITLE:

Developmental Approach to Characterizing the Invasion Gene
Program in Breast Cancer

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REPORT DATE:

September 2000

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                    Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2000	3. REPORT TYPE AND DATES COVERED Annual (11 Aug 99 – 10 Aug 00)

4. TITLE AND SUBTITLE
Developmental Approach to Characterizing the Invasion Gene Program in Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7282

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The changes in the gene program of neoplastic cells that regulate the expression of an invasive phenotype are largely undefined. Direct comparisons of the gene expression profile displayed in normal and carcinomatous breast tissues have provided insights into the mechanisms underlying tumor progression. However, attempts to identify the gene products differentially expressed during invasion *in vivo* have been hampered by the fact that only a small percentage of the cells recovered from a tumor mass are actively engaged in invasive behavior at the time of isolation. Because tissue remodeling induced during mammary gland involution bears homology to early stages of carcinogenesis, the involuting mammary gland may be used to identify genes that control matrix turnover in cancerous states. To this end, we propose to *i) generate cDNA libraries from control versus involuting mouse mammary glands, ii) isolate differentially expressed genes during matrix remodeling, iii) identify differentially expressed genes that encode secretory proteins associated with the involution program and iv) identify human homologues of the mouse-derived matrix remodeling genes.* The approach should allow for the identification of gene products relevant to breast cancer invasion.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Page 2

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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I.   INTRODUCTION

Current evidence suggests that breast carcinoma cells invade local tissues and metastasize by i) altering their cell-cell and cell-matrix interactions, ii) displaying an aberrant motile phenotype, and iii) either synthesizing, or inducing the synthesis of, proteolytic enzymes that degrade the structural barriers established by the extracellular matrix[1-3]. The complex changes in the gene program of neoplastic cells that regulate the expression of this phenotype are largely undefined, but increased interest has focused on identifying those genes that are specifically overexpressed in human breast cancer[(e.g., 3-10)]. Such information not only provides new insights into the cellular factors that control tissue-invasive behavior, but may also lead to improvements in patient diagnosis and to the more rational design of therapeutic interventions[3-10]. Consistent with this rationale, direct comparisons of the gene expression profile displayed in normal versus neoplastic breast cancer cell lines, or normal and carcinomatous breast tissues, have provided a number of novel insights into the mechanisms and processes underlying tumor progression[6-11]. Interestingly, despite the power of the analytical techniques employed for these purposes, the number of differentially expressed genes identified thus far are - at first glance - perplexingly small, despite the striking changes known to occur in cellular behavior[(e.g., 7,8)]. However, analyses of breast cancer cell lines grown *in vitro* or static tumor masses recovered from *in vivo* sites of disease may be problematic. First, comparisons between normal and neoplastic breast cancer cell lines grown atop plastic substrata *in vitro* will not recapitulate the complex interactions known to occur across the carcinoma-mesenchymal cell axis *in vivo*[1,2]. Indeed, many of the most interesting gene products that have been associated with the expression of tissue-invasive phenotypes in breast cancer tissue are synthesized by surrounding stromal cells rather than the tumor itself[2,3,10]. Secondly, while the gene expression patterns identified in tissues recovered from *in vivo* sites clearly circumvent the limitations inherent in the *in vitro* studies, only a small percentage of the cells recovered from a tumor mass at a single, fixed time point would be expected to be actively engaged in invasive behavior. Given the many similarities between developmental/tissue repair processes and malignant growth (re; the ability of cancer cells inappropriately recapitulate developmental programs associated with epithelial-mesenchymal cell transitions or repair programs associated with wound healing[12,13]), we have considered the possibility that the *in situ* induction of a synchronous matrix remodeling program in normal tissues would allow for the more efficient isolation of those gene products critical to cancer cell invasion. Indeed, recent studies have demonstrated that gene expression patterns associated with the tissue remodeling program induced during the involution of the normal lactating mammary gland bear considerable overlap with those detected in the early stages of carcinogenesis (e.g., stromeylsin-1, stromeylsin-3, urokinase-type plasminogen activator, tissue inhibitor of metalloproteinases[14-16]). Hence, we propose to use the involuting mammary gland explant model as a means to rapidly enrich for, and identify, the subset of genes that control the disassembly of the extracellular matrix in cancerous states. Furthermore, by selectively identifying the subset of gene products that encode secreted proteins in breast cancer tissue, new diagnostics as well as novel targets for therapeutic intervention can be rapidly identified.

II.   BODY

Given the overall aim of identifying tissue-destructive genes up-regulated during tissue involution and problems encountered with the generation of subtraction libraries from our *in*

vitro model, we began using mammary glands isolated from lactating versus involuting tissues *in vivo*. Coincident with the use of this material, it became clear that differentially expressed genes could be more rapidly and accurately identified via the use of oligonucleotide arrays as opposed to subtraction libraries[17]. Consequently, in three experiments, RNA was isolated from glands with a Qiagen RNassy mini-kit and cRNA prepared for hybridization as described[17,18]. Oligonucleotide arrays (Gene Chip, Affymetrix) representing a total of 30,000 EST cluster sequences and/or full-length genes were used for hybridization according to the manufacturer's instructions. Arrays were then scanned using an Affymetrix confocal scanner and analyzed using Gene Chip 3.0 Software (Affymetrix). Expression data from the Affymetrix arrays were analyzed using a statistically based analysis methodology that estimates expression levels and provides confidence intervals for these estimates. It also allows for the normalization of array-based expression data to control for variations due to non-biological factors such as array-to-array variability, and variations in sample quality. For each gene, the presence or absence of a transcript was determined by testing the Null hypothesis. Briefly, the arrays included a set of probes derived from non-eukaryotic ("foreign") organisms (e.g., bacterial and bacteriophage sequences) which were defined as the "null set". This null set thus defines the intensity of nonspecific background/cross-hybridization. This null intensity distribution is modeled by a parametric statistical distribution. Since intensity is a positive random variable, this null distribution is modeled by either a Gamma or a Weibull class distribution. Once the parametric null distribution is determined, we computed the p-value for the hypothesis that the observed hybridization intensity values are also a random sample from the null distribution. Target genes with low p-values (i.e., not likely to have come from the same distribution as the null genes) are classified as present. The p-value provides a continuous measure of the confidence in the presence of a gene in the target sample. We also include a mathematical method to standardize the gene-expression levels between different samples, based on exogenous gene spikes, added at known concentrations, that constitute a calibration set[18]. Genes scored as "positive" (i.e., induced) in involuting tissues were i) more highly expressed in each of 3 independently performed experiments and ii) expressed at levels ≥2.5 than those detected in lactating glands in at least 2 of the 3 experiments.

Following data analysis of the more than 250 genes whose regulation were affected by the involution program (see representative examples in Appendix), message levels for three matrix-destructive cathepsins known to encode secretory proteins were found to be up-regulated during involution, the aspartyl proteinase cathepsin D, and the cysteinyl proteinases, cathepsin L and S (Table I). (Human homologues for each of these have already been identified.)

Table I
Fold-Increase in Gene Expression

	EXP #1	EXP #2	EXP #3
cathepsin D	4.7	3.9	2.3
cathepsin L	4.1	2.9	1.8
cathepsin S	7.4	6.2	2.3

While cathepsin D has been previously associated with human breast cancer and shown to express matrix-destructive activity *in vitro*[19,20], roles for cathepsin L and S in regulating extracellular matrix turnover *in vivo* are less clear. However, as we have recently identified cathepsin L and S as powerful matrix-destructive enzymes *in vitro*[18,21], we have received approval for a no-cost extension of the project to analyze the role of these enzymes in the resorption of breast tissues during involution in the corresponding knockout animals (animal use approval pending). Both cathepsin L and S have been deleted, and the single- as well as double-null animals are viable and fertile. Hence, mammary gland involution will be compared in wild-type and knockout littermates as described by Northern blot/*in situ* hybridization, ApoTag and transmission electron microscopy[15]. (Comparable experiments cannot be performed with cathepsin B knockout animals as the deleted-animals display a lethal phenotype.) Should matrix remodeling events be altered in the gene-deleted animals, lactating and involuting will be harvested and gene expression patterns detailed as described above.

In additional studies, we have also recently detected the matrix metalloproteinases (MMPs), stromelysin-1 and membrane type-1 MMP (MT1-MMP), in involuting gland tissues. While stromelysin-1 has already been linked to matrix-destructive events in the normal and neoplastic mammary gland[22], the role of MT1-MMP *in vivo* is less clear (though MT1-MMP expression, like of stromelysin-1, is up-regulated in breast cancer[23]). Though MT1-MMP knockout mice die soon after birth (thus precluding attempts to discern the role of the proteinase in mammary gland involution its link to matrix-destructive events in breast cancer), we have recently demonstrated that the function of the enzyme can be probed in cells engineered to stably overexpress the proteinase[24]. During the tissue-destructive event associated with both involution and cancer, the key extracellular matrix barrier that must be dissolved or perforated is the basement membrane[1-3]. Given the fact that MT1-MMP is expressed in involuting glands and that basement membrane disruption initiates apoptosis[15], we sought to determine whether MT1-MMP can directly degrade this structure. To this end, control or MT1-MMP-transfected epithelial cells were grown atop preformed basement membranes generated *in vitro* (see Figure 1A-d; Appendix). Interestingly, while control-transfected cells were unable to degrade the basement membrane, the MT1-MMP transfectants focally degraded the basement membrane and initiated invasive activity (Figs. 2A, B). These studies demonstrate that MT1-MMP, a protease expressed during mammary gland involution and in breast cancer, can degrade a key matrix barrier. These studies will be completed during the no-cost extension to highlight i) the association of involution-associated gene products with similar matrix-degradative programs in cancer cells and ii) the ability of these gene products to directly participate in invasive/matrix-remodeling states.

III. KEY RESEARCH ACCOMPLISHMENTS

- Murine proteinase gene products differentially expressed during tissue involution identified.

- Role for membrane type-1 matrix metalloproteinase in basement membrane degradation identified.

IV.   REPORTABLE OUTCOMES

Proposal to study role of membrane-type matrix metalloproteinases in breast cancer development submitted to The Susan B. Komen Breast Cancer Foundation.

V.   CONCLUSIONS

With the identification of suitable mammary gland tissues for isolating gene products differentially expressed during matrix-remodeling events, the model system has been used to identify the subset of genes that likely control the disassembly of the matrix during tumor invasion and metastasis. Furthermore, by selectively identifying those gene products that encode secreted proteins in breast cancer tissue, new diagnostics as well as novel targets for therapeutic intervention may be identified.

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VII. APPENDIX

Table II – Partial list of differentially expressed gene products in involuting (INV) versus lactating (LAC). Results are expressed as mean fluorescent intensity of the signals generated in 3 separate, paired experiments (experiments A, C, D for involuting glands and J, K, N for lactating glands).

Figure 1 – Basement membranes synthesized by MDCK epithelial cells are deposited atop a 3-dimensional type I collagen gel during a 3 wk culture period. In panel A, TEM analysis shows an epithelial cell depositing a ~50 nm thick basement membrane which is more readily observed after the overlying cell layer has been lysed (panel B). Panels C and D are scanning electron micrographs showing the type I collagen gel upon which is deposited an intact basement membrane, respectively.
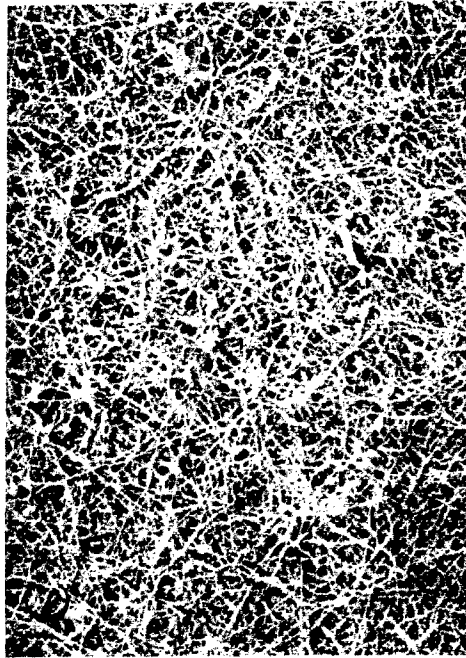
Figure 2 – A control transfected epithelial cell line is seeded atop a preformed basement membrane which remains intact during a 10 d culture period as visualized by transmission electron microscopy (panel A). In contrast, MT1-MMP-transfected cells perforate the basement membrane (panel B) and begin to invade into the underlying type I collagen gel (panel B).

TABLE II (page 1)

CHIP	PROBESET	Inv A	Inv C	Inv D	Lac J	Lac K	Lac N	DESC
A	108235_s	3894	4535	3521	915	643	652	Mus musculus clusterin mRNA, complete cds.
A	j03941_f	3894	4535	4207	3140	1098	1035	Mu ferritin heavy chain (MFH) mRNA, complete cds.
A	M24509_f	3205	3728	3433	3048	640	644	Mu ferritin heavy chain, complete cds.
A	112447_s	1861	2287	2039	943	297	297	Mus musculus insulin-like growth factor binding protein 5 (IGFBP5) mRNA, complete cds.
A	aa560093_f	1756	1242	1143	663	463	483	AA560093 vl29d03.r1 Stratagene Mu Tcell 937311 Mus musculus cDNA clone 973637 5' TIGR cluster TC30468
A	j04716_f	1705	1359	1135	838	528	512	Mu ferritin light chain, complete cds.
A	AA709861_f	1694	1926	1090	705	390	442	vt35a01.r1 Barstead Mu proximal colon MPLRB6 Mus musculus cDNA clone 1165032 5', mRNA sequence.
A	aa666971_f	1663	1084	994	618	404	423	vq87e06.r1 Knowles Solter Mu blastocyst B3 Mus musculus cDNA clone 1109314 5', mRNA sequence.
B	ET62056_f	1513	1939	1665	553	714	704	Mus musculus immunoglobulin rearranged kappa chain mRNA, partial cds.
B	V00802_f	1487	1827	1634	588	708	758	Mu gene fragment for kappa-immunoglobulin (constant region) (from cell line MOPC21).
B	ET62762_f	1468	1861	1584	473	609	656	Mus musculus anti-von Willebrand factor antibody NMC-4 kappa chain mRNA, partial cds.
B	ET61206_f	1313	1620	1429	495	680	678	Mus musculus Ig light chain (Fab 17/9) mRNA, partial cds.
B	x81627_s	1187	1309	1242	1000	441	384	M.musculus 24p3 gene.
B	Msa.1376.0_f	1174	1448	1334	1509	111	109	Mu mRNA for early T-lymphocyte activation 1 protein (ETa-1)
A	AA590859_f	1169	1368	1242	802	426	497	vn60f03.r1 Barstead Mu proximal colon MPLRB6 Mus musculus cDNA clone 1025597 5', mRNA sequence.
B	Msa.1271.0_s	1168	1258	1105	479	260	280	House Mu; Musculus domesticus mRNA for lactoferrin, complete cds
B	x13333_s	1074	1069	1059	611	278	249	Mu CD14 mRNA for myeloid cell-specific leucine-rich glycoprotein.
B	Msa.376.0_f	1072	1204	1085	1023	258	274	Mu adipocyte lipid binding protein gene, complete cds
A	d11468_s	1047	1148	1114	181	358	417	Mu gene for immunoglobulin alpha heavy chain, switch region and constant region complete sequence.
B	x52046_s	1039	1101	1159	802	400	335	M.musculus COL3A1 gene for collagen alpha-1.
B	ET62984_f	994	1253	1008	241	521	566	M.musculus mRNA (3C10) for IgA V-D-J-heavy chain.
A	D87896_f	947	1134	916	585	352	380	Mus musculus phgpx mRNA for phospholipid hydroperoxide glutathione peroxidase, complete cds.
B	Msa.2056.0_f	916	1148	1005	213	430	464	M.musculus mRNA (2F7) for IgA V-D-J-heavy chain
B	Msa.8919.0_f	906	1436	1295	1102	295	238	Homologous to sp P02568: ACTIN, ALPHA SKELETAL MUSCLE (ALPHA-ACTIN 1).
B	Msa.2129.0_s	882	780	867	725	235	180	Mu SV-40 induced 24p3 mRNA
B	Msa.29369.0_s	861	1543	1304	1065	377	369	Homologous to sp P00566: CREATINE KINASE, M CHAIN (EC 2.7.3.2) (NU-2 PROTEIN).
B	Msa.3025.0_s	856	1039	897	678	298	296	Mus musculus ceruloplasmin mRNA, complete cds
B	Msa.2213.0_s	832	1296	1125	852	357	354	Mu muscle creatine kinase mRNA (EC 2.7.3.2)
B	Msa.3420.0_f	809	819	793	541	345	321	Homologous to sp P36970: PHOSPHOLIPID HYDROPEROXIDE GLUTHATIONE PEROXIDASE (EC 1.11.9) (PHGPX).
A	u73004_s	799	778	695	186	89	105	Mus musculus secretory leukocyte protease inhibitor mRNA, complete cds.
A	AFFX-b-ActinMur/M12481_3	772	917	838	589	312	359	AFFX-b-ActinMur/M12481_3
★ B	x52886_s	768	628	682	337	161	145	M.musculus mRNA for cathepsin D.
A	j00544_s	739	941	786	236	359	403	Mu Ig active J chain, partial mRNA.
A	m16355_f	735	751	676	546	207	214	Mu major urinary protein I (MUP1) mRNA, complete cds.
B	ET62985_f	730	995	787	169	293	364	M.musculus mRNA (1B5) for IgA V-D-J-heavy chain.
B	X00496_s	725	600	593	339	69	33	Mu Ia-associated invariant chain (Ii) mRNA fragment.
B	x06046_f	725	575	644	336	228	149	Mu mRNA for major excreted protein (MEP).
A	k02109_f	627	554	530	482	140	163	Mu 3T3-L1 lipid binding protein mRNA, complete cds.
A	m21495_f	616	653	607	457	213	214	Mu cytoskeletal gamma-actin mRNA, complete cds.

TABLE II (page 2)

	ID							Description
A	M21050_s	593	625	550	347	163	137	Mu lysozyme M gene, exon 4.
B	Msa.18117.0_f	583	616	586	404	205	156	Homologous to sp P08226: APOLIPOPROTEIN E PRECURSOR (APO-E).
B	Msa.17332.0_f	566	466	504	288	176	123	Homologous to sp P36968: PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE (EC 1.11.1.9) (PHGPX).
B	Msa.547.0_f	548	545	534	313	187	129	Mu cathepsin L gene, complete cds, clones a-H-ras-1 and RIT-1
B	Msa.401.0_f	537	554	537	422	163	154	Mu cytoskeletal gamma-actin mRNA, complete cds
B	Msa.22134.0_s	534	480	465	312	69	66	Homologous to sp P13983: EXTENSIN PRECURSOR (CELL WALL HYD
B	Msa.3233.0_f	527	1029	853	587	141	124	Mus musculus myosin light chain 2 mRNA, complete cds.
B	Msa.739.0_s	526	643	632	307	127	142	C57BL/6J ob/ob haptoglobin mRNA, complete cds
B	Msa.1022.0_s	508	424	473	294	131	150	Mus musculus glucose phosphate isomerase mRNA, 3' end
B	X62940_s	499	421	439	196	98	67	M.musculus TSC-22 mRNA.
B	ET61420_f	487	405	407	118	253	232	Mus musculus anti-glycoprotein-B of human Cytomegalovirus immunoglobulin Vh chain gene, partial cds.
A	U62386_s	483	393	352	39	38	54	Mus musculus immunoglobulin heavy and light chain variable region mRNA, complete cds.
A	M73329_s	481	520	477	342	44	98	Mu phospholipase C-alpha (PLC-alpha) mRNA, complete cds.
B	v01527_s	467	335	382	238	143	185	Mu gene coding for major histocompatibility antigen. This is a class II antigen, I-A-beta.
B	Msa.23293.0_f	460	584	559	356	162	106	Homologous to sp P08226: APOLIPOPROTEIN E PRECURSOR (APO-E).
B	Msa.450.0_f	439	493	509	332	104	103	Mu 28 Kda mRNA encoding a possible serine protease, complete cds
A	aa255186_s	436	349	396	208	117	93	AA255186 mz77c03.r1 Soares Mu lymph node NbMLN Mus musculus cDNA clone 719452 5' similar to gb:M90696 CATHEPSIN S PRECURSOR (HUMAN); TIGR cluster
B	ET61815_f	432	367	342	112	221	208	Mus musculus anti-DNA immunoglobulin heavy chain IgG mRNA, antibody 373s.51, partial cds.
A	120276_s	432	365	337	228	155	151	Mu biglycan (Bgn) mRNA, complete cds.
A	u37222_s	431	375	402	369	72	111	Mus musculus 30kDa adipocyte complement-related protein Acrp30 mRNA, complete cds.
B	ET61876_f	425	341	330	104	203	201	Mus musculus anti-DNA immunoglobulin heavy chain IgM mRNA, antibody 452p.70, partial cds.
B	Msa.43207.0_f	421	425	390	284	163	156	Mu mRNA for matrix Gla protein (MGP)
B	X93038_s	418	317	308	247	109	92	M.musculus mRNA for MAT8 protein.
B	w08453_s	417	293	436	191	55	67	W08453 mb50a09.r1 Soares Mu p3NMF19.5 Mus musculus cDNA clone 332824 5' similar to gb:S65738 DESTRIN (HUMAN); TIGR cluster TC34375
B	ET61785_f	416	340	341	83	194	173	Mus musculus anti-DNA immunoglobulin heavy chain IgM mRNA, antibody 363p.168, partial cds.
B	x67141	415	778	805	383	59	35	M.musculus Pva mRNA for parvalbumin.
B	Msa.354.0_f	409	415	411	340	98	125	Mu mRNA for OSF-3, complete cds
B	ET61989_f	407	552	494	140	157	263	Mus musculus anti-DNA immunoglobulin light chain IgG, antibody 423s.17, partial cds.
B	X13605_s	406	274	364	219	50	40	Murine mRNA for replacement variant histone H3.3.
B	ET63039_f	402	323	345	62	172	150	M.musculus mRNA for variable heavy chain.
A	aa537404_f	396	339	324	275	108	103	AA537404 vj98c02.r1 Knowles Solter Mu blastocyst B1 Mus musculus cDNA clone 945122 5' similar to gb:S54005 THYMOSIN BETA-10 (HUMAN); TIGR cluster TC3885
B	ET61916_f	396	447	459	183	201	265	Mus musculus anti-DNA immunoglobulin light chain IgM mRNA, antibody 363p.193, partial cds.
B	ET63281_f	390	475	417	121	143	220	M.domesticus IgK variable region.)PIR:PH1085 (Ig light chain V region (clone 163.42) - Mu (fragment)
A	aa117835_s	390	377	397	264	130	139	AA117835 mn06c01.r1 Beddington Mu embryonic region Mus musculus cDNA clone 537120 5' similar to TR:G536926 G536926 MYELIN GENE EXPRESSION FACTOR ;
B	ET63295_f	385	318	326	31	126	126	M.domesticus IgM variable region.)PIR:S26747 (Ig heavy chain J region JH4 - Mu
A	M57590_s	384	603	605	320	194	190	Mu fast skeletal troponin C (sTnC) gene, complete cds.
B	Msa.4454.0_f	383	302	332	302	76	83	Homologous to sp P04117: FATTY ACID-BINDING PROTEIN, ADIPOCYTE (AFABP) (ADIPOCYTE LIPID-BINDING PROTEIN) (ALBP) (P2 ADIPOCYTE PROTEIN) (I
B	ET61839_f	374	298	281	105	164	182	Mus musculus anti-DNA immunoglobulin heavy chain IgG mRNA, antibody 384s.95, partial cds.
B	Msa.956.0_f	367	291	331	209	124	132	Mu fragment of mRNA encoding for the Ia antigen (heavy chain) from major histocompatibility complex (A-k-alpha). This is coded by the I-A region of the MHC and correspo
A	m94351	365	179	178	116	136	90	Mus musculus immunoglobulin lambda chain (IgL) mRNA, complete cds.