7.2 Evolutionary conservation of core processes

Because of the various "model organism" genome-sequencing projects that have already been completed, reasonable comparative information is available for beginning the analysis of the evolution of the human genome. The genomes of *S. cerevisiae* ("bakers' yeast") (*118*) and two diverse invertebrates, *C. elegans* (a nematode worm) (*119*) and *D. melanogaster* (fly) (*26*), as well as the first plant genome, *A. thaliana*, recently completed (*92*), provide a diverse background for genome comparisons.

We enumerated the "strict orthologs" conserved between human and fly, and between human and worm (Fig. 16) to address the question, What are the core functions that appear to be common across the animals? The concept of orthology is important because if two genes are orthologs, they can be traced by descent to the common ancestor of the two organisms (an "evolutionarily conserved protein set"), and therefore are likely to perform similar conserved functions in the different organisms. It is critical in this analysis to separate orthologs (a gene that appears in two organisms by descent from a common ancestor) from paralogs (a gene that appears in more than one copy in a given organism by a duplication event) because paralogs may subsequently diverge in function. Following the yeast-worm ortholog comparison in (*120*), we identified two different cases for each pairwise comparison (human-fly and human-worm). The first case was a pair of genes, one from each organism, for which there was no other close homolog in either organism. These are straightforwardly identified as orthologous, because there are no additional members of the families that complicate separating orthologs from paralogs. The second case is a family of genes with more than one member in either or both of the organisms being compared. Chervitz *et al.* (*120*) deal with this case by analyzing a phylogenetic tree that described the relationships between all of the sequences in both organisms, and then looked for pairs of genes that were nearest neighbors in the tree. If the nearest-neighbor pairs were from different organisms, those genes were presumed to be orthologs. We note that these nearest neighbors can often be confidently identified from pairwise sequence comparison without having to examine a phylogenetic tree (see legend to Fig. 16). If the nearest neighbors are not from different organisms, there has been a paralogous expansion in one or both organisms after the speciation event (and/or a gene loss by one organism). When this one-to-one correspondence is lost, defining an ortholog becomes ambiguous. For our initial computational overview of the predicted human protein set, we could not answer this question for every predicted protein. Therefore, we consider only "strict orthologs," i.e., the proteins with unambiguous one-to-one relationships (Fig. 16). By these criteria, there are 2758 strict human-fly orthologs, 2031 human-worm (1523 in common between these sets). We define the evolutionarily conserved set as those 1523 human proteins that have strict orthologs in both *D. melanogaster* and *C. elegans*.

The distribution of the functions of the conserved protein set is shown in Fig. 16. Comparison with Fig. 15 shows that, not surprisingly, the set of conserved proteins is not distributed among molecular functions in the same way as the whole human protein set. Compared with the whole human set (Fig. 15), there are several categories that are overrepresented in the conserved set by a factor of ~2 or more. The first category is nucleic acid enzymes, primarily the transcriptional machinery (notably DNA/RNA methyltransferases, DNA/RNA polymerases, helicases, DNA ligases, DNA- and RNA-processing factors, nucleases, and ribosomal proteins). The basic transcriptional and translational machinery is well known to have been conserved over evolution, from bacteria through to the most complex eukaryotes. Many ribonucleoproteins involved in RNA splicing also appear to be conserved among the animals. Other enzyme types are also overrepresented (transferases, oxidoreductases, ligases, lyases, and isomerases). Many of these en-

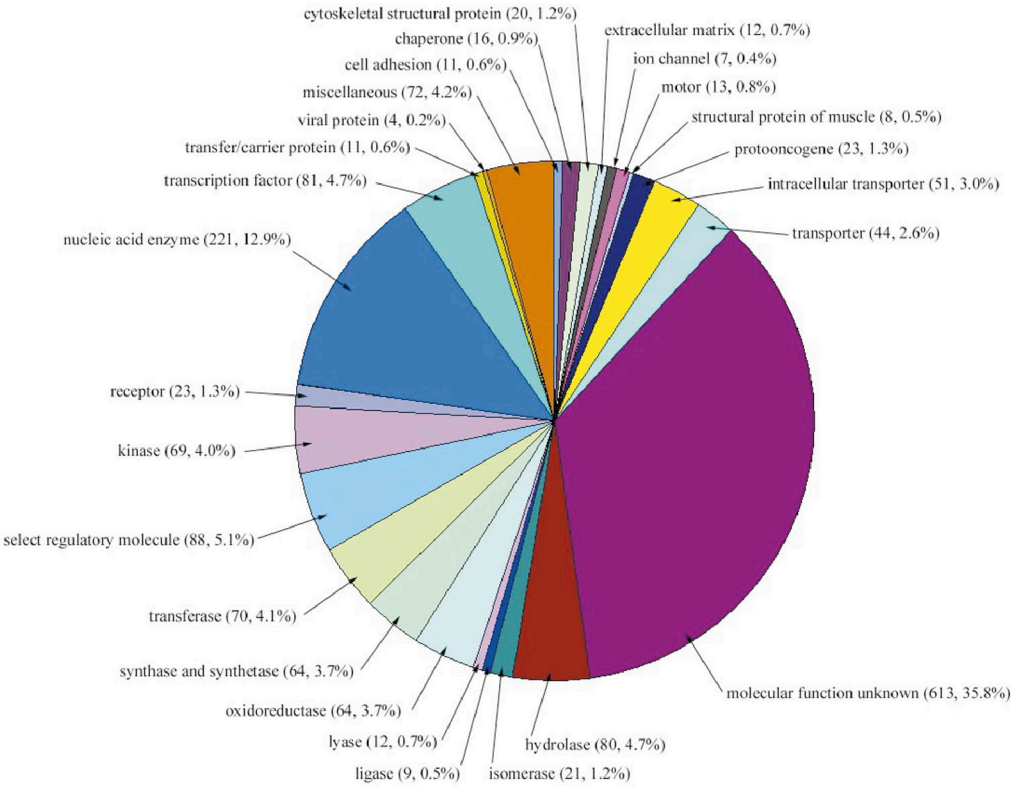

Fig. 16. Functions of putative orthologs across vertebrate and invertebrate genomes. Each slice lists the number and percentages (in parentheses) of "strict orthologs" between the human, fly, and worm genomes involved in a given category of molecular function. "Strict orthologs" are defined here as bi-directional BLAST best hits (*180*) such that each orthologous pair (i) has a BLASTP *P*-value of $\leq 10^{-10}$ (*120*), and (ii) has a more significant BLASTP score than any paralogs in either organism, i.e., there has likely been no duplication subsequent to speciation that might make the orthology ambiguous. This measure is quite strict and is a lower bound on the number of orthologs. By these criteria, there are 2758 strict human-fly orthologs, and 2031 human-worm orthologs (1523 in common between these sets).

zymes are involved in intermediary metabolism. The only exception is the hydrolase category, which is not significantly overrepresented in the shared protein set. Proteases form the largest part of this category, and several large protease families have expanded in each of these three organisms after their divergence. The category of select regulatory molecules is also overrepresented in the conserved set. The major conserved families are small guanosine triphosphatases (GTPases) (especially the Ras-related superfamily, including ADP ribosylation factor) and cell cycle regulators (particularly the cullin family, cyclin C family, and several cell division protein kinases). The last two significantly overrepresented categories are protein transport and trafficking, and chaperones. The most conserved groups in these categories are proteins involved in coated vesicle-mediated transport, and chaperones involved in protein folding and heat-shock response [particularly the DNAJ family, and heat-shock protein 60 (HSP60), HSP70, and HSP90 families]. These observations provide only a conservative estimate of the protein families in the context of specific cellular processes that were likely derived from the last common ancestor of the human, fly, and worm. As stated before, this analysis does not provide a complete estimate of conservation across the three animal genomes, as paralogous duplication makes the determination of true orthologs difficult within the members of conserved protein families.

7.3 Differences between the human genome and other sequenced eukaryotic genomes

To explore the molecular building blocks of the vertebrate taxon, we have compared the human genome with the other sequenced eukaryotic genomes at three levels: molecular functions, protein families, and protein domains.

Molecular differences can be correlated with phenotypic differences to begin to reveal the developmental and cellular processes that are unique to the vertebrates. Tables 18 and 19 display a comparison among all sequenced eukaryotic genomes, over selected protein/domain families (defined by sequence similarity, e.g., the serine-threonine protein kinases) and superfamilies (defined by shared molecular function, which may include several sequence-related families, e.g., the cytokines). In these tables we have focused on (super) families that are either very large or that differ significantly in humans compared with the other sequenced eukaryote genomes. We have found that the most prominent human expansions are in proteins involved in (i) acquired immune functions; (ii) neural development, structure, and functions; (iii) intercellular and intracellular signaling pathways in development and homeostasis; (iv) hemostasis; and (v) apoptosis.

Acquired immunity. One of the most striking differences between the human genome and the *Drosophila* or *C. elegans* genome is the appearance of genes involved in acquired immunity (Tables 18 and 19). This is expected, because the acquired immune response is a defense system that only occurs in vertebrates. We observe 22 class I and 22 class II major histocompatibility complex (MHC) antigen genes and 114 other immunoglobulin genes in the human genome. In addition, there are 59 genes in the cognate immunoglobulin receptor family. At the domain level, this is exemplified by an expansion and recruitment of the ancient immunoglobulin fold to constitute molecules such as MHC, and of the integrin fold to form several of the cell adhesion molecules that mediate interactions between immune effector cells and the extracellular matrix. Vertebrate-specific proteins include the paracrine immune regulators family of secreted 4-alpha helical bundle proteins, namely the cytokines and chemokines. Some of the cytoplasmic signal transduction components associated with cytokine receptor signal transduction are also features that are poorly represented in the fly and worm. These include protein domains found in the signal transducer and activator of transcription (STATs), the suppressors of cytokine signaling (SOCS), and protein inhibitors of activated STATs (PIAS). In contrast, many of the animal-specific protein domains that play a role in innate immune response, such as the Toll receptors, do not appear to be significantly expanded in the human genome.

Neural development, structure, and function. In the human genome, as compared with the worm and fly genomes, there is a marked increase in the number of members of protein families that are involved in neural development. Examples include neurotrophic factors such as ependymin, nerve growth factor, and signaling molecules such as semaphorins, as well as the number of proteins involved directly in neural structure and function such as myelin proteins, voltage-gated ion channels, and synaptic proteins such as synaptotagmin. These observations correlate well with the known phenotypic differences between the nervous systems of these taxa, notably (i) the increase in the number and connectivity of neurons; (ii) the increase in number of distinct neural cell types (as many as a thousand or more in human compared with a few hundred in fly and worm) (*121*); (iii) the increased length of individual axons; and (iv) the significant increase in glial cell number, especially the appearance of myelinating glial cells, which are electrically inert supporting cells differentiated from the same stem cells as neurons. A number of prominent protein expansions are involved in the processes of neural development. Of the extracellular domains that mediate cell adhesion, the connexin domain–containing proteins (*122*) exist only in humans. These proteins, which are not present in the *Drosophila* or *C. elegans* genomes, appear to provide the constitutive subunits of intercellular channels and the structural basis for electrical coupling. Pathway finding by axons and neuronal network formation is mediated through a subset of ephrins and their cognate receptor tyrosine kinases that act as positional labels to establish topographical projections (*123*). The probable biological role for the semaphorins (22 in human compared with 6 in the fly and 2 in the worm) and their receptors (neuropilins and plexins) is that of axonal guidance molecules (*124*). Signaling molecules such as neurotrophic factors and some cytokines have been shown to regulate neuronal cell survival, proliferation, and axon guidance (*125*). Notch receptors and ligands play important roles in glial cell fate determination and gliogenesis (*126*).

Other human expanded gene families play key roles directly in neural structure and function. One example is synaptotagmin (expanded more than twofold in humans relative to the invertebrates), originally found to regulate synaptic transmission by serving as a Ca^{2+} sensor (or receptor) during synaptic vesicle fusion and release (*127*). Of interest is the increased co-occurrence in humans of PDZ and the SH3 domains in neuronal-specific adaptor molecules; examples include proteins that likely modulate channel activity at synaptic junctions (*128*). We also noted expansions in several ion-channel families (Table 19), including the EAG subfamily (related to cyclic nucleotide gated channels), the voltage-gated calcium/sodium channel family, the inward-rectifier potassium channel family, and the voltage-gated potassium channel, alpha subunit family. Voltage-gated sodium and potassium channels are involved in the generation of action potentials in neurons. Together with voltage-gated calcium channels, they also play a key role in coupling action potentials to neurotransmitter release, in the development of neurites, and in short-term memory. The recent observation of a calcium-regulated association between sodium channels and synaptotagmin may have consequences for the establishment and regulation of neuronal excitability (*129*).

Myelin basic protein and myelin-associated glycoprotein are major classes of protein components in both the central and peripheral nervous system of vertebrates. Myelin P0 is a major component of peripheral myelin, and myelin proteolipid and myelin oligodendrocyte glycopotein are found in the central nervous system. Mutations in any of these

Table 18. Domain-based comparative analysis of proteins in *H. sapiens* (H), *D. melanogaster* (F), *C. elegans* (W), *S. cerevisiae* (Y), and *A. thaliana* (A). The predicted protein set of each of the above eukaryotic organisms was analyzed with Pfam version 5.5 using E value cutoffs of 0.001. The number of proteins containing the specified Pfam domains as well as the total number of domains (in parentheses) are shown in each column. Domains were categorized into cellular processes for presentation. Some domains (i.e., SH2) are listed in more than one cellular process. Results of the Pfam analysis may differ from results obtained based on human curation of protein families, owing to the limitations of large-scale automatic classifications. Representative examples of domains with reduced counts owing to the stringent E value cutoff used for this analysis are marked with a double asterisk (**). Examples include short divergent and predominantly alpha-helical domains, and certain classes of cysteine-rich zinc finger proteins.

Accession number	Domain name	Domain description	H	F	W	Y	A
		Developmental and homeostatic regulators					
PF02039	Adrenomedullin	Adrenomedullin	1	0	0	0	0
PF00212	ANP	Atrial natriuretic peptide	2	0	0	0	0
PF00028	Cadherin	Cadherin domain	100 (550)	14 (157)	16 (66)	0	0
PF00214	Calc_CGRP_IAPP	Calcitonin/CGRP/IAPP family	3	0	0	0	0
PF01110	CNTF	Ciliary neurotrophic factor	1	0	0	0	0
PF01093	Clusterin	Clusterin	3	0	0	0	0
PF00029	Connexin	Connexin	14 (16)	0	0	0	0
PF00976	ACTH_domain	Corticotropin ACTH domain	1	0	0	0	0
PF00473	CRF	Corticotropin-releasing factor family	2	1	0	0	0
PF00007	Cys_knot	Cystine-knot domain	10 (11)	2	0	0	0
PF00778	DIX	Dix domain	5	2	4	0	0
PF00322	Endothelin	Endothelin family	3	0	0	0	0
PF00812	Ephrin	Ephrin	7 (8)	2	4	0	0
PF01404	EPh_lbd	Ephrin receptor ligand binding domain	12	2	1	0	0
PF00167	FGF	Fibroblast growth factor	23	1	1	0	0
PF01534	Frizzled	Frizzled/Smoothened family membrane region	9	7	3	0	0
PF00236	Hormone6	Glycoprotein hormones	1	0	0	0	0
PF01153	Glypican	Glypican	14	2	1	0	0
PF01271	Granin	Grainin (chromogranin or secretogranin)	3	0	0	0	0
PF02058	Guanylin	Guanylin precursor	1	0	0	0	0
PF00049	Insulin	Insulin/IGF/Relaxin family	7	4	0	0	0
PF00219	IGFBP	Insulin-like growth factor binding proteins	10	0	0	0	0
PF02024	Leptin	Leptin	1	0	0	0	0
PF00193	Xlink	LINK (hyaluron binding)	13 (23)	0	1	0	0
PF00243	NGF	Nerve growth factor family	3	0	0	0	0
PF02158	Neuregulin	Neuregulin family	4	0	0	0	0
PF00184	Hormone5	Neurohypophysial hormones	1	0	0	0	0
PF02070	NMU	Neuromedin U	1	0	0	0	0
PF00066	Notch	Notch (DSL) domain	3 (5)	2 (4)	2 (6)	0	0
PF00865	Osteopontin	Osteopontin	1	0	0	0	0
PF00159	Hormone3	Pancreatic hormone peptides	3	0	0	0	0
PF01279	Parathyroid	Parathyroid hormone family	2	0	0	0	0
PF00123	Hormone2	Peptide hormone	5 (9)	0	0	0	0
PF00341	PDGF	Platelet-derived growth factor (PDGF)	5	1	0	0	0
PF01403	Sema	Sema domain	27 (29)	8 (10)	3 (4)	0	0
PF01033	Somatomedin_B	Somatomedin B domain	5 (8)	3	0	0	0
PF00103	Hormone	Somatotropin	1	0	0	0	0
PF02208	Sorb	Sorbin homologous domain	2	0	0	0	0
PF02404	SCF	Stem cell factor	2	0	0	0	0
PF01034	Syndecan	Syndecan domain	3	1	1	0	0
PF00020	TNFR_c6	TNFR/NGFR cysteine-rich region	17 (31)	1	0	0	0
PF00019	TGF-β	Transforming growth factor β-like domain	27 (28)	6	4	0	0
PF01099	Uteroglobin	Uteroglobin family	3	0	0	0	0
PF01160	Opiods_neuropep	Vertebrate endogenous opioids neuropeptide	3	0	0	0	0
PF00110	Wnt	Wnt family of developmental signaling proteins	18	7 (10)	5	0	0
		Hemostasis					
PF01821	ANATO	Anaphylotoxin-like domain	6 (14)	0	0	0	0
PF00386	C1q	C1q domain	24	0	0	0	0
PF00200	Disintegrin	Disintegrin	18	2	3	0	0
PF00754	F5_F8_type_C	F5/8 type C domain	15 (20)	5 (6)	2	0	0
PF01410	COLFI	Fibrillar collagen C-terminal domain	10	0	0	0	0
PF00039	Fn1	Fibronectin type I domain	5 (18)	0	0	0	0
PF00040	Fn2	Fibronectin type II domain	11 (16)	0	0	0	0
PF00051	Kringle	Kringle domain	15 (24)	2	2	0	0
PF01823	MACPF	MAC/Perforin domain	6	0	0	0	0
PF00354	Pentaxin	Pentaxin family	9	0	0	0	0
PF00277	SAA_proteins	Serum amyloid A protein	4	0	0	0	0
PF00084	Sushi	Sushi domain (SCR repeat)	53 (191)	11 (42)	8 (45)	0	0
PF02210	TSPN	Thrombospondin N-terminal–like domains	14	1	0	0	0
PF01108	Tissue_fac	Tissue factor	1	0	0	0	0
PF00868	Transglutamin_N	Transglutaminase family	6	1	0	0	0
PF00927	Transglutamin_C	Transglutaminase family	8	1	0	0	0

Table 18 (*Continued*)

Accession number	Domain name	Domain description	H	F	W	Y	A
PF00594	Gla	Vitamin K-dependent carboxylation/gamma-carboxyglutamic (GLA) domain	11	0	0	0	0
		Immune response					
PF00711	Defensin_beta	Beta defensin	1	0	0	0	0
PF00748	Calpain_inhib	Calpain inhibitor repeat	3 (9)	0	0	0	0
PF00666	Cathelicidins	Cathelicidins	2	0	0	0	0
PF00129	MHC_I	Class I histocompatibility antigen, domains alpha 1 and 2	18 (20)	0	0	0	0
PF00993	MHC_II_alpha**	Class II histocompatibility antigen, alpha domain	5 (6)	0	0	0	0
PF00969	MHC_II_beta**	Class II histocompatibility antigen, beta domain	7	0	0	0	0
PF00879	Defensin_propep	Defensin propeptide	3	0	0	0	0
PF01109	GM_CSF	Granulocyte-macrophage colony-stimulating factor	1	0	0	0	0
PF00047	Ig	Immunoglobulin domain	381 (930)	125 (291)	67 (323)	0	0
PF00143	Interferon	Interferon alpha/beta domain	7 (9)	0	0	0	0
PF00714	IFN-gamma	Interferon gamma	1	0	0	0	0
PF00726	IL10	Interleukin-10	1	0	0	0	0
PF02372	IL15	Interleukin-15	1	0	0	0	0
PF00715	IL2	Interleukin-2	1	0	0	0	0
PF00727	IL4	Interleukin-4	1	0	0	0	0
PF02025	IL5	Interleukin-5	1	0	0	0	0
PF01415	IL7	Interleukin-7/9 family	1	0	0	0	0
PF00340	IL1	Interleukin-1	7	0	0	0	0
PF02394	IL1_propep	Interleukin-1 propeptide	1	0	0	0	0
PF02059	IL3	Interleukin-3	1	0	0	0	0
PF00489	IL6	Interleukin-6/G-CSF/MGF family	2	0	0	0	0
PF01291	LIF_OSM	Leukemia inhibitory factor (LIF)/oncostatin (OSM) family	2	0	0	0	0
PF00323	Defensins	Mammalian defensin	2	0	0	0	0
PF01091	PTN_MK	PTN/MK heparin-binding protein	2	0	0	0	0
PF00277	SAA_proteins	Serum amyloid A protein	4	0	0	0	0
PF00048	IL8	Small cytokines (intecrine/chemokine), interleukin-8 like	32	0	0	0	0
PF01582	TIR	TIR domain	18	8	2	0	131 (143)
PF00229	TNF	TNF (tumor necrosis factor) family	12	0	0	0	0
PF00088	Trefoil	Trefoil (P-type) domain	5 (6)	0	2	0	0
		PI-PY-rho GTPase signaling					
PF00779	BTK	BTK motif	5	1	0	0	0
PF00168	C2	C2 domain	73 (101)	32 (44)	24 (35)	6 (9)	66 (90)
PF00609	DAGKa	Diacylglycerol kinase accessory domain (presumed)	9	4	7	0	6
PF00781	DAGKc	Diacylglycerol kinase catalytic domain (presumed)	10	8	8	2	11 (12)
PF00610	DEP	Domain found in Dishevelled, Egl-10, and Pleckstrin (DEP)	12 (13)	4	10	5	2
PF01363	FYVE	FYVE zinc finger	28 (30)	14	15	5	15
PF00996	GDI	GDP dissociation inhibitor	6	2	1	1	3
PF00503	G-alpha	G-protein alpha subunit	27 (30)	10	20 (23)	2	5
PF00631	G-gamma	G-protein gamma like domains	16	5	5	1	0
PF00616	RasGAP	GTPase-activator protein for Ras-like GTPase	11	5	8	3	0
PF00618	RasGEFN	Guanine nucleotide exchange factor for Ras-like GTPases; N-terminal motif	9	2	3	5	0
PF00625	Guanylate_kin	Guanylate kinase	12	8	7	1	4
PF02189	ITAM	Immunoreceptor tyrosine-based activation motif	3	0	0	0	0
PF00169	PH	PH domain	193 (212)	72 (78)	65 (68)	24	23
PF00130	DAG_PE-bind	Phorbol esters/diacylglycerol binding domain (C1 domain)	45 (56)	25 (31)	26 (40)	1 (2)	4
PF00388	PI-PLC-X	Phosphatidylinositol-specific phospholipase C, X domain	12	3	7	1	8
PF00387	PI-PLC-Y	Phosphatidylinositol-specific phospholipase C, Y domain	11	2	7	1	8
PF00640	PID	Phosphotyrosine interaction domain (PTB/PID)	24 (27)	13	11 (12)	0	0
PF02192	PI3K_p85B	PI3-kinase family, p85-binding domain	2	1	1	0	0
PF00794	PI3K_rbd	PI3-kinase family, ras-binding domain	6	3	1	0	0
PF01412	ArfGAP	Putative GTP-ase activating protein for Arf	16	9	8	6	15
PF02196	RBD	Raf-like Ras-binding domain	6 (7)	4	1	0	0
PF02145	Rap_GAP	Rap/ran-GAP	5	4	2	0	0
PF00788	RA	Ras association (RalGDS/AF-6) domain	18 (19)	7 (9)	6	1	0
PF00071	Ras	Ras family	126	56 (57)	51	23	78
PF00617	RasGEF	RasGEF domain	21	8	7	5	0
PF00615	RGS	Regulator of G protein signaling domain	27	6 (7)	12 (13)	1	0
PF02197	RIIa	Regulatory subunit of type II PKA R-subunit	4	1	2	1	0

Table 18 (*Continued*)

Accession number	Domain name	Domain description	H	F	W	Y	A
PF00620	RhoGAP	RhoGAP domain	59	19	20	9	8
PF00621	RhoGEF	RhoGEF domain	46	23 (24)	18 (19)	3	0
PF00536	SAM	SAM domain (Sterile alpha motif)	29 (31)	15	8	3	6
PF01369	Sec7	Sec7 domain	13	5	5	5	9
PF00017	SH2	Src homology 2 (SH2) domain	87 (95)	33 (39)	44 (48)	1	3
PF00018	SH3	Src homology 3 (SH3) domain	143 (182)	55 (75)	46 (61)	23 (27)	4
PF01017	STAT	STAT protein	7	1	1 (2)	0	0
PF00790	VHS	VHS domain	4	2	4	4	8
PF00568	WH1	WH1 domain	7	2	2 (3)	1	0
		Domains involved in apoptosis					
PF00452	Bcl-2	Bcl-2	9	2	1	0	0
PF02180	BH4	Bcl-2 homology region 4	3	0	1	0	0
PF00619	CARD	Caspase recruitment domain	16	0	2	0	0
PF00531	Death	Death domain	16	5	7	0	0
PF01335	DED	Death effector domain	4 (5)	0	0	0	0
PF02179	BAG	Domain present in Hsp70 regulators	5 (8)	3	2	1	5
PF00656	ICE_p20	ICE-like protease (caspase) p20 domain	11	7	3	0	0
PF00653	BIR	Inhibitor of Apoptosis domain	8 (14)	5 (9)	2 (3)	1 (2)	0
		Cytoskeletal					
PF00022	Actin	Actin	61 (64)	15 (16)	12	9 (11)	24
PF00191	Annexin	Annexin	16 (55)	4 (16)	4 (11)	0	6 (16)
PF00402	Calponin	Calponin family	13 (22)	3	7 (19)	0	0
PF00373	Band_41	FERM domain (Band 4.1 family)	29 (30)	17 (19)	11 (14)	0	0
PF00880	Nebulin_repeat	Nebulin repeat	4 (148)	1 (2)	1	0	0
PF00681	Plectin_repeat	Plectin repeat	2 (11)	0	0	0	0
PF00435	Spectrin	Spectrin repeat	31 (195)	13 (171)	10 (93)	0	0
PF00418	Tubulin-binding	Tau and MAP proteins, tubulin-binding	4 (12)	1 (4)	2 (8)	0	0
PF00992	Troponin	Troponin	4	6	8	0	0
PF02209	VHP	Villin headpiece domain	5	2	2	0	5
PF01044	Vinculin	Vinculin family	4	2	1	0	0
		ECM adhesion					
PF01391	Collagen	Collagen triple helix repeat (20 copies)	65 (279)	10 (46)	174 (384)	0	0
PF01413	C4	C-terminal tandem repeated domain in type 4 procollagen	6 (11)	2 (4)	3 (6)	0	0
PF00431	CUB	CUB domain	47 (69)	9 (47)	43 (67)	0	0
PF00008	EGF	EGF-like domain	108 (420)	45 (186)	54 (157)	0	1
PF00147	Fibrinogen_C	Fibrinogen beta and gamma chains, C-terminal globular domain	26	10 (11)	6	0	0
PF00041	Fn3	Fibronectin type III domain	106 (545)	42 (168)	34 (156)	0	1
PF00757	Furin-like	Furin-like cysteine rich region	5	2	1	0	0
PF00357	Integrin_A	Integrin alpha cytoplasmic region	3	1	2	0	0
PF00362	Integrin_B	Integrins, beta chain	8	2	2	0	0
PF00052	Laminin_B	Laminin B (Domain IV)	8 (12)	4 (7)	6 (10)	0	0
PF00053	Laminin_EGF	Laminin EGF-like (Domains III and V)	24 (126)	9 (62)	11 (65)	0	0
PF00054	Laminin_G	Laminin G domain	30 (57)	18 (42)	14 (26)	0	0
PF00055	Laminin_Nterm	Laminin N-terminal (Domain VI)	10	6	4	0	0
PF00059	Lectin_c	Lectin C-type domain	47 (76)	23 (24)	91 (132)	0	0
PF01463	LRRCT	Leucine rich repeat C-terminal domain	69 (81)	23 (30)	7 (9)	0	0
PF01462	LRRNT	Leucine rich repeat N-terminal domain	40 (44)	7 (13)	3 (6)	0	0
PF00057	Ldl_recept_a	Low-density lipoprotein receptor domain class A	35 (127)	33 (152)	27 (113)	0	0
PF00058	Ldl_recept_b	Low-density lipoprotein receptor repeat class B	15 (96)	9 (56)	7 (22)	0	0
PF00530	SRCR	Scavenger receptor cysteine-rich domain	11 (46)	4 (8)	1 (2)	0	0
PF00084	Sushi	Sushi domain (SCR repeat)	53 (191)	11 (42)	8 (45)	0	0
PF00090	Tsp_1	Thrombospondin type 1 domain	41 (66)	11 (23)	18 (47)	0	0
PF00092	Vwa	von Willebrand factor type A domain	34 (58)	0	17 (19)	0	1
PF00093	Vwc	von Willebrand factor type C domain	19 (28)	6 (11)	2 (5)	0	0
PF00094	Vwd	von Willebrand factor type D domain	15 (35)	3 (7)	9	0	0
		Protein interaction domains					
PF00244	14-3-3	14-3-3 proteins	20	3	3	2	15
PF00023	Ank	Ank repeat	145 (404)	72 (269)	75 (223)	12 (20)	66 (111)
PF00514	Armadillo_seg	Armadillo/beta-catenin-like repeats	22 (56)	11 (38)	3 (11)	2 (10)	25 (67)
PF00168	C2	C2 domain	73 (101)	32 (44)	24 (35)	6 (9)	66 (90)
PF00027	cNMP_binding	Cyclic nucleotide-binding domain	26 (31)	21 (33)	15 (20)	2 (3)	22
PF01556	DnaJ_C	DnaJ C terminal region	12	9	5	3	19
PF00226	DnaJ	DnaJ domain	44	34	33	20	93
PF00036	Efhand**	EF hand	83 (151)	64 (117)	41 (86)	4 (11)	120 (328)
PF00611	FCH	Fes/CIP4 homology domain	9	3	2	4	0
PF01846	FF	FF domain	4 (11)	4 (10)	3 (16)	2 (5)	4 (8)
PF00498	FHA	FHA domain	13	15	7	13 (14)	17

myelin proteins result in severe demyelination, which is a pathological condition in which the myelin is lost and the nerve conduction is severely impaired (*130*). Humans have at least 10 genes belonging to four different families involved in myelin production (five myelin P0, three myelin proteolipid, myelin basic protein, and myelin-oligodendrocyte glycoprotein, or MOG), and possibly more-remotely related members of the MOG family. Flies have only a single myelin proteolipid, and worms have none at all.

Intercellular and intracellular signaling pathways in development and homeostasis. Many protein families that have expanded in humans relative to the invertebrates are involved in signaling processes, particularly in response to development and differentiation

Table 18 (*Continued*)

Accession number	Domain name	Domain description	H	F	W	Y	A
PF00254	FKBP	FKBP-type peptidyl-prolyl cis-trans isomerases	15 (20)	7 (8)	7 (13)	4	24 (29)
PF01590	GAF	GAF domain	7 (8)	2 (4)	1	0	10
PF01344	Kelch	Kelch motif	54 (157)	12 (48)	13 (41)	3	102 (178)
PF00560	LRR**	Leucine Rich Repeat	25 (30)	24 (30)	7 (11)	1	15 (16)
PF00917	MATH	MATH domain	11	5	88 (161)	1	61 (74)
PF00989	PAS	PAS domain	18 (19)	9 (10)	6	1	13 (18)
PF00595	PDZ	PDZ domain (Also known as DHR or GLGF)	96 (154)	60 (87)	46 (66)	2	5
PF00169	PH	PH domain	193 (212)	72 (78)	65 (68)	24	23
PF01535	PPR**	PPR repeat	5	3 (4)	0	1	474 (2485)
PF00536	SAM	SAM domain (Sterile alpha motif)	29 (31)	15	8	3	6
PF01369	Sec7	Sec7 domain	13	5	5	5	9
PF00017	SH2	Src homology 2 (SH2) domain	87 (95)	33 (39)	44 (48)	1	3
PF00018	SH3	Src homology 3 (SH3) domain	143 (182)	55 (75)	46 (61)	23 (27)	4
PF01740	STAS	STAS domain	5	1	6	2	13
PF00515	TPR**	TPR domain	72 (131)	39 (101)	28 (54)	16 (31)	65 (124)
PF00400	WD40**	WD40 domain	136 (305)	98 (226)	72 (153)	56 (121)	167 (344)
PF00397	WW	WW domain	32 (53)	24 (39)	16 (24)	5 (8)	11 (15)
PF00569	ZZ	ZZ-Zinc finger present in dystrophin, CBP/p300	10 (11)	13	10	2	10
		Nuclear interaction domains					
PF01754	Zf-A20	A20-like zinc finger	2 (8)	2	2	0	8
PF01388	ARID	ARID DNA binding domain	11	6	4	2	7
PF01426	BAH	BAH domain	8 (10)	7 (8)	4 (5)	5	21 (25)
PF00643	Zf-B_box**	B-box zinc finger	32 (35)	1	2	0	0
PF00533	BRCT	BRCA1 C Terminus (BRCT) domain	17 (28)	10 (18)	23 (35)	10 (16)	12 (16)
PF00439	Bromodomain	Bromodomain	37 (48)	16 (22)	18 (26)	10 (15)	28
PF00651	BTB	BTB/POZ domain	97 (98)	62 (64)	86 (91)	1 (2)	30 (31)
PF00145	DNA_methylase	C-5 cytosine-specific DNA methylase	3 (4)	1	0	0	13 (15)
PF00385	Chromo	chromo' (CHRromatin Organization MOdifier) domain	24 (27)	14 (15)	17 (18)	1 (2)	12
PF00125	Histone	Core histone H2A/H2B/H3/H4	75 (81)	5	71 (73)	8	48
PF00134	Cyclin	Cyclin	19	10	10	11	35
PF00270	DEAD	DEAD/DEAH box helicase	63 (66)	48 (50)	55 (57)	50 (52)	84 (87)
PF01529	Zf-DHHC	DHHC zinc finger domain	15	20	16	7	22
PF00646	F-box**	F-box domain	16	15	309 (324)	9	165 (167)
PF00250	Fork_head	Fork head domain	35 (36)	20 (21)	15	4	0
PF00320	GATA	GATA zinc finger	11 (17)	5(6)	8 (10)	9	26
PF01585	G-patch	G-patch domain	18	16	13	4	14 (15)
PF00010	HLH**	Helix-loop-helix DNA-binding domain	60 (61)	44	24	4	39
PF00850	Hist_deacetyl	Histone deacetylase family	12	5 (6)	8 (10)	5	10
PF00046	Homeobox	Homeobox domain	160 (178)	100 (103)	82 (84)	6	66
PF01833	TIG	IPT/TIG domain	29 (53)	11 (13)	5 (7)	2	1
PF02373	JmjC	JmjC domain	10	4	6	4	7
PF02375	JmjN	JmjN domain	7	4	2	3	7
PF00013	KH-domain	KH domain	28 (67)	14 (32)	17 (46)	4 (14)	27 (61)
PF01352	KRAB	KRAB box	204 (243)	0	0	0	0
PF00104	Hormone_rec	Ligand-binding domain of nuclear hormone receptor	47	17	142 (147)	0	0
PF00412	LIM	LIM domain containing proteins	62 (129)	33 (83)	33 (79)	4 (7)	10 (16)
PF00917	MATH	MATH domain	11	5	88 (161)	1	61 (74)
PF00249	Myb_DNA-binding	Myb-like DNA-binding domain	32 (43)	18 (24)	17 (24)	15 (20)	243 (401)
PF02344	Myc-LZ	Myc leucine zipper domain	1	0	0	0	0
PF01753	Zf-MYND	MYND finger	14	14	9	1	7
PF00628	PHD	PHD-finger	68 (86)	40 (53)	32 (44)	14 (15)	96 (105)
PF00157	Pou	Pou domain—N-terminal to homeobox domain	15	5	4	0	0
PF02257	RFX_DNA_binding	RFX DNA-binding domain	7	2	1	1	0
PF00076	Rrm	RNA recognition motif (a.k.a. RRM, RBD, or RNP domain)	224 (324)	127 (199)	94 (145)	43 (73)	232 (369)
PF02037	SAP	SAP domain	15	8	5	5	6 (7)
PF00622	SPRY	SPRY domain	44 (51)	10 (12)	5 (7)	3	6
PF01852	START	START domain	10	2	6	0	23
PF00907	T-box	T-box	17 (19)	8	22	0	0

Table 18 (*Continued*)

Accession number	Domain name	Domain description	H	F	W	Y	A
PF02135	Zf-TAZ	TAZ finger	2 (3)	1 (2)	6 (7)	0	10 (15)
PF01285	TEA	TEA domain	4	1	1	1	0
PF02176	Zf-TRAF	TRAF-type zinc finger	6 (9)	1 (3)	1	0	2
PF00352	TBP	Transcription factor TFIID (or TATA-binding protein, TBP)	2 (4)	4 (8)	2 (4)	1 (2)	2 (4)
PF00567	TUDOR	TUDOR domain	9 (24)	9 (19)	4 (5)	0	2
PF00642	Zf-CCCH	Zinc finger C-x8-C-x5-C-x3-H type (and similar)	17 (22)	6 (8)	22 (42)	3 (5)	31 (46)
PF00096	Zf-C2H2**	Zinc finger, C2H2 type	564 (4500)	234 (771)	68 (155)	34 (56)	21 (24)
PF00097	Zf-C3HC4	Zinc finger, C3HC4 type (RING finger)	135 (137)	57	88 (89)	18	298 (304)
PF00098	Zf-CCHC	Zinc knuckle	9 (17)	6 (10)	17 (33)	7 (13)	68 (91)

(Tables 18 and 19). They include secreted hormones and growth factors, receptors, intracellular signaling molecules, and transcription factors.

Developmental signaling molecules that are enriched in the human genome include growth factors such as wnt, transforming growth factor–β (TGF-β), fibroblast growth factor (FGF), nerve growth factor, platelet derived growth factor (PDGF), and ephrins. These growth factors affect tissue differentiation and a wide range of cellular processes involving actin-cytoskeletal and nuclear regulation. The corresponding receptors of these developmental ligands are also expanded in humans. For example, our analysis suggests at least 8 human ephrin genes (2 in the fly, 4 in the worm) and 12 ephrin receptors (2 in the fly, 1 in the worm). In the wnt signaling pathway, we find 18 wnt family genes (6 in the fly, 5 in the worm) and 12 frizzled receptors (6 in the fly, 5 in the worm). The Groucho family of transcriptional corepressors downstream in the wnt pathway are even more markedly expanded, with 13 predicted members in humans (2 in the fly, 1 in the worm).

Extracellular adhesion molecules involved in signaling are expanded in the human genome (Tables 18 and 19). The interactions of several of these adhesion domains with extracellular matrix proteoglycans play a critical role in host defense, morphogenesis, and tissue repair (*131*). Consistent with the well-defined role of heparan sulfate proteoglycans in modulating these interactions (*132*), we observe an expansion of the heparin sulfate sulfotransferases in the human genome relative to worm and fly. These sulfotransferases modulate tissue differentiation (*133*). A similar expansion in humans is noted in structural proteins that constitute the actin-cytoskeletal architecture. Compared with the fly and worm, we observe an explosive expansion of the nebulin (35 domains per protein on average), aggrecan (12 domains per protein on average), and plectin (5 domains per protein on average) repeats in humans. These repeats are present in proteins involved in modulating the actin-cytoskeleton with predominant expression in neuronal, muscle, and vascular tissues.

Comparison across the five sequenced eukaryotic organisms revealed several expanded protein families and domains involved in cytoplasmic signal transduction (Table 18). In particular, signal transduction pathways playing roles in developmental regulation and acquired immunity were substantially enriched. There is a factor of 2 or greater expansion in humans in the Ras superfamily GTPases and the GTPase activator and GTP exchange factors associated with them. Although there are about the same number of tyrosine kinases in the human and *C. elegans* genomes, in humans there is an increase in the SH2, PTB, and ITAM domains involved in phosphotyrosine signal transduction. Further, there is a twofold expansion of phosphodiesterases in the human genome compared with either the worm or fly genomes.

The downstream effectors of the intracellular signaling molecules include the transcription factors that transduce developmental fates. Significant expansions are noted in the ligand-binding nuclear hormone receptor class of transcription factors compared with the fly genome, although not to the extent observed in the worm (Tables 18 and 19). Perhaps the most striking expansion in humans is in the C2H2 zinc finger transcription factors. Pfam detects a total of 4500 C2H2 zinc finger domains in 564 human proteins, compared with 771 in 234 fly proteins. This means that there has been a dramatic expansion not only in the number of C2H2 transcription factors, but also in the number of these DNA-binding motifs per transcription factor (8 on average in humans, 3.3 on average in the fly, and 2.3 on average in the worm). Furthermore, many of these transcription factors contain either the KRAB or SCAN domains, which are not found in the fly or worm genomes. These domains are involved in the oligomerization of transcription factors and increase the combinatorial partnering of these factors. In general, most of the transcription factor domains are shared between the three animal genomes, but the reassortment of these domains results in organism-specific transcription factor families. The domain combinations found in the human, fly, and worm include the BTB with C2H2 in the fly and humans, and homeodomains alone or in combination with Pou and LIM domains in all of the animal genomes. In plants, however, a different set of transcription factors are expanded, namely, the myb family, and a unique set that includes VP1 and AP2 domain–containing proteins (*134*). The yeast genome has a paucity of transcription factors compared with the multicellular eukaryotes, and its repertoire is limited to the expansion of the yeast-specific C6 transcription factor family involved in metabolic regulation.

While we have illustrated expansions in a subset of signal transduction molecules in the human genome compared with the other eukaryotic genomes, it should be noted that most of the protein domains are highly conserved. An interesting observation is that worms and humans have approximately the same number of both tyrosine kinases and serine/threonine kinases (Table 19). It is important to note, however, that these are merely counts of the catalytic domain; the proteins that contain these domains also display a wide repertoire of interaction domains with significant combinatorial diversity.

Hemostasis. Hemostasis is regulated primarily by plasma proteases of the coagulation pathway and by the interactions that occur between the vascular endothelium and platelets. Consistent with known anatomical and physiological differences between vertebrates and invertebrates, extracellular adhesion domains that constitute proteins integral to hemostasis are expanded in the human relative to the fly and worm (Tables 18 and 19). We note the evolution of domains such as FIMAC, FN1, FN2, and C1q that mediate surface interactions between hematopoeitic cells and the vascular matrix. In addition, there has been extensive recruitment of more-ancient animal-specific domains such as VWA, VWC, VWD, kringle, and FN3 into multidomain proteins that are involved in hemostatic regulation. Although we do not find a large expansion in the total number of serine proteases, this enzymatic domain has been specifically recruited into several of these multidomain proteins for proteolytic regulation in the vascular compartment. These are represented in plasma proteins that belong to the kinin and complement pathways. There is a

significant expansion in two families of matrix metalloproteases: ADAM (a disintegrin and metalloprotease) and MMPs (matrix metalloproteases) (Table 19). Proteolysis of extracellular matrix (ECM) proteins is critical for tissue development and for tissue degradation in diseases such as cancer, arthritis, Alzheimer's disease, and a variety of inflammatory conditions (*135*, *136*). ADAMs are a family of integral membrane proteins with a pivotal role in fibrinogenolysis and modulating interactions between hematopoietic components and the vascular matrix components. These proteins have been shown to cleave matrix proteins, and even signaling molecules: ADAM-17 converts tumor necrosis factor–α, and ADAM-10 has been implicated in the Notch signaling pathway (*135*). We have identified 19 members of the matrix metalloprotease family, and a total of 51 members of the ADAM and ADAM-TS families.

Apoptosis. Evolutionary conservation of some of the apoptotic pathway components across eukarya is consistent with its central role in developmental regulation and as a response to pathogens and stress signals. The signal transduction pathways involved in programmed cell death, or apoptosis, are mediated by interactions between well-characterized domains that include extracellular domains, adaptor (protein-protein interaction) domains, and those found in effector and regulatory enzymes (*137*). We enumerated the protein counts of central adaptor and effector enzyme domains that are found only in the apoptotic pathways to provide an estimate of divergence across eukarya and relative expansion in the human genome when compared with the fly and worm (Table 18). Adaptor domains found in proteins restricted only to apoptotic regulation such as the DED domains are vertebrate-specific, whereas others like BIR, CARD, and Bcl2 are represented in the fly and worm (although the number of Bcl2 family members in humans is significantly expanded). Although plants and yeast lack the caspases, caspase-like molecules, namely the para- and meta-caspases, have been reported in these organisms (*138*). Compared with other animal genomes, the human genome shows an expansion in the adaptor and effector domain–containing proteins involved in apoptosis, as well as in the proteases involved in the cascade such as the caspase and calpain families.

Expansions of other protein families. *Metabolic enzymes.* There are fewer cytochrome P450 genes in humans than in either the fly or worm. Lipoxygenases (six in humans), on the other hand, appear to be specific to the vertebrates and plants, whereas the lipoxygenase-activating proteins (four in humans) may be vertebrate-specific. Lipoxygenases are involved in arachidonic acid metabolism, and they and their activators have been implicated in diverse human pathology ranging from allergic responses to cancers. One of the most surprising human expansions, however, is in the number of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (46 in humans, 3 in the fly, and 4 in the worm). There is, however, evidence for many retrotransposed GAPDH pseudogenes (*139*), which may account for this apparent expansion. However, it is interesting that GAPDH, long known as a conserved enzyme involved in basic metabolism found across all phyla from bacteria to humans, has recently been shown to have other functions. It has a second cat-

Table 19. Number of proteins assigned to selected Panther families or subfamilies in *H. sapiens* (H), *D. melanogaster* (F), *C. elegans* (W), *S. cerevisiae* (Y), and *A. thaliana* (A).

Panther family/subfamily*	H	F	W	Y	A
Neural structure, function, development					
Ependymin	1	0	0	0	0
Ion channels					
Acetylcholine receptor	17	12	56	0	0
Amiloride-sensitive/degenerin	11	24	27	0	0
CNG/EAG	22	9	9	0	30
IRK	16	3	3	0	0
ITP/ryanodine	10	2	4	0	0
Neurotransmitter-gated	61	51	59	0	19
P2X purinoceptor	10	0	0	0	0
TASK	12	12	48	1	5
Transient receptor	15	3	3	1	0
Voltage-gated Ca^{2+} alpha	22	4	8	2	2
Voltage-gated Ca^{2+} alpha-2	10	3	2	0	0
Voltage-gated Ca^{2+} beta	5	2	2	0	0
Voltage-gated Ca^{2+} gamma	1	0	0	0	0
Voltage-gated K^{+} alpha	33	5	11	0	0
Voltage-gated KQT	6	2	3	0	0
Voltage-gated Na^{+}	11	4	4	9	1
Myelin basic protein	1	0	0	0	0
Myelin PO	5	0	0	0	0
Myelin proteolipid	3	1	0	0	0
Myelin-oligodendrocyte glycoprotein	1	0	0	0	0
Neuropilin	2	0	0	0	0
Plexin	9	2	0	0	0
Semaphorin	22	6	2	0	0
Synaptotagmin	10	3	3	0	0
Immune response					
Defensin	3	0	0	0	0
Cytokine†	86	14	1	0	0
GCSF	1	0	0	0	0
GMCSF	1	0	0	0	0
Intercrine alpha	15	0	0	0	0
Intercrine beta	5	0	0	0	0
Inteferon	8	0	0	0	0
Interleukin	26	1	1	0	0
Leukemia inhibitory factor	1	0	0	0	0
MCSF	1	0	0	0	0
Peptidoglycan recognition protein	2	13	0	0	0
Pre-B cell enhancing factor	1	0	0	0	0
Small inducible cytokine A	14	0	0	0	0
Sl cytokine	2	0	0	0	0
TNF	9	0	0	0	0
Cytokine receptor†	62	1	0	0	0
Bradykinin/C-C chemokine receptor	7	0	0	0	0
Fl cytokine receptor	2	0	0	0	0
Interferon receptor	3	0	0	0	0
Interleukin receptor	32	0	0	0	0
Leukocyte tyrosine kinase receptor	3	0	0	0	0
MCSF receptor	1	0	0	0	0
TNF receptor	3	0	0	0	0
Immunoglobulin receptor†	59	0	0	0	0
T-cell receptor alpha chain	16	0	0	0	0
T-cell receptor beta chain	15	0	0	0	0
T-cell receptor gamma chain	1	0	0	0	0
T-cell receptor delta chain	1	0	0	0	0
Immunoglobulin FC receptor	8	0	0	0	0
Killer cell receptor	16	0	0	0	0
Polymeric-immunoglobulin receptor	4	0	0	0	0

alytic activity, as a uracil DNA glycosylase (*140*) and functions as a cell cycle regulator (*141*) and has even been implicated in apoptosis (*142*).

Translation. Another striking set of human expansions has occurred in certain families involved in the translational machinery. We identified 28 different ribosomal subunits that each have at least 10 copies in the genome; on average, for all ribosomal proteins there is about an 8- to 10-fold expansion in the number of genes relative to either the worm or fly. Retrotransposed pseudogenes may account for many of these expansions [see the discussion above and (*143*)]. Recent evidence suggests that a number of ribosomal proteins have secondary functions independent of their involvement in protein biosynthesis; for example, L13a and the related L7 subunits (36 copies in humans) have been shown to induce apoptosis (*144*).

There is also a four- to fivefold expansion in the elongation factor 1-alpha family (eEF1A; 56 human genes). Many of these expansions likely represent intronless paralogs that have presumably arisen from retrotransposition, and again there is evidence that many of these may be pseudogenes (*145*). However, a second form (eEF1A2) of this factor has been identied with tissue-specific expression in skeletal muscle and a complementary expression pattern to the ubiquitously expressed eEF1A (*146*).

Ribonucleoproteins. Alternative splicing results in multiple transcripts from a single gene, and can therefore generate additional diversity in an organism's protein complement. We have identified 269 genes for ribonucleoproteins. This represents over 2.5 times the number of ribonucleoprotein genes in the worm, two times that of the fly, and about the same as the 265 identified in the *Arabidopsis* genome. Whether the diversity of ribonucleoprotein genes in humans contributes to gene regulation at either the splicing or translational level is unknown.

Posttranslational modifications. In this set of processes, the most prominent expansion is the transglutaminases, calcium-dependent enzymes that catalyze the cross-linking of proteins in cellular processes such as hemostasis and apoptosis (*147*). The vitamin K–dependent gamma carboxylase gene product acts on the GLA domain (missing in the fly and worm) found in coagulation factors, osteocalcin, and matrix GLA protein (*148*). Tyrosylprotein sulfotransferases participate in the posttranslational modification of proteins involved in inflammation and hemostasis, including coagulation factors and chemokine receptors (*149*). Although there is no significant numerical increase in the counts for domains involved in nuclear protein modification, there are a number of domain arrangements in the predicted human proteins that are not found in the other currently sequenced genomes. These include the tandem association of two histone deacetylase domains in HD6 with a ubiquitin finger domain, a feature lacking in the fly genome. An additional example is the co-occurrence of important nuclear regulatory enzyme PARP (poly-ADP ribosyl transferase) domain fused to protein-interaction domains—BRCT and VWA in humans.

Concluding remarks. There are several possible explanations for the differences in phenotypic complexity observed in humans when compared to the fly and worm. Some of these relate to the prominent differences in the immune system, hemostasis, neuronal, vascular, and cytoskeletal complexity. The finding that the human genome contains fewer genes than previously predicted might be compensated for by combinatorial diversity generated at the levels of protein architecture, transcriptional and translational control, posttranslational modification of proteins, or posttranscriptional regulation. Extensive domain shuffling to increase or alter combinatorial diversity can provide an exponential

Table 19 (*Continued*)

Panther family/subfamily*	H	F	W	Y	A
MHC class I	22	0	0	0	0
MHC class II	20	0	0	0	0
Other immunoglobulin†	114	0	0	0	0
Toll receptor–related	10	6	0	0	0
Developmental and homeostatic regulators					
Signaling molecules†					
Calcitonin	3	0	0	0	0
Ephrin	8	2	4	0	0
FGF	24	1	1	0	0
Glucagon	4	0	0	0	0
Glycoprotein hormone beta chain	2	0	0	0	0
Insulin	1	0	0	0	0
Insulin-like hormone	3	0	0	0	0
Nerve growth factor	3	0	0	0	0
Neuregulin/heregulin	6	0	0	0	0
neuropeptide Y	4	0	0	0	0
PDGF	1	1	0	0	0
Relaxin	3	0	0	0	0
Stannocalcin	2	0	0	0	0
Thymopoeitin	2	0	1	0	0
Thyomosin beta	4	2	0	0	0
TGF-β	29	6	4	0	0
VEGF	4	0	0	0	0
Wnt	18	6	5	0	0
Receptors†					
Ephrin receptor	12	2	1	0	0
FGF receptor	4	4	0	0	0
Frizzled receptor	12	6	5	0	0
Parathyroid hormone receptor	2	0	0	0	0
VEGF receptor	5	0	0	0	0
BDNF/NT-3 nerve growth factor receptor	4	0	0	0	0
Kinases and phosphatases					
Dual-specificity protein phosphatase	29	8	10	4	11
S/T and dual-specificity protein kinase†	395	198	315	114	1102
S/T protein phosphatase	15	19	51	13	29
Y protein kinase†	106	47	100	5	16
Y protein phosphatase	56	22	95	5	6
Signal transduction					
ARF family	55	29	27	12	45
Cyclic nucleotide phosphodiesterase	25	8	6	1	0
G protein-coupled receptors†‡	616	146	284	0	1
G-protein alpha	27	10	22	2	5
G-protein beta	5	3	2	1	1
G-protein gamma	13	2	2	0	0
Ras superfamily	141	64	62	26	86
G-protein modulators†					
ARF GTPase-activating	20	8	9	5	15
Neurofibromin	7	2	0	2	0
Ras GTPase-activating	9	3	8	1	0
Tuberin	7	3	2	0	0
Vav proto-oncogene family	35	15	13	3	0

increase in the ability to mediate protein-protein interactions without dramatically increasing the absolute size of the protein complement (*150*). Evolution of apparently new (from the perspective of sequence analysis) protein domains and increasing regulatory complexity by domain accretion both quantitatively and qualitatively (recruitment of novel domains with preexisting ones) are two features that we observe in humans. Perhaps the best illustration of this trend is the C2H2 zinc finger–containing transcription factors, where we see expansion in the number of domains per protein, together with vertebrate-specific domains such as KRAB and SCAN. Recent reports on the prominent use of internal ribosomal entry sites in the human genome to regulate translation of specific classes of proteins suggests that this is an area that needs further research to identify the full extent of this process in the human genome (*151*). At the posttranslational level, although we provide examples of expansions of some protein families involved in these modifications, further experimental evidence is required to evaluate whether this is correlated with increased complexity in protein processing. Posttranscriptional processing and the extent of isoform generation in the human remain to be cataloged in their entirety. Given the conserved nature of the spliceosomal machinery, further analysis will be required to dissect regulation at this level.

8 Conclusions

8.1 The whole-genome sequencing approach versus BAC by BAC

Experience in applying the whole-genome shotgun sequencing approach to a diverse group of organisms with a wide range of genome sizes and repeat content allows us to assess its strengths and weaknesses. With the success of the method for a large number of microbial genomes, *Drosophila*, and now the human, there can be no doubt concerning the utility of this method. The large number of microbial genomes that have been sequenced by this method (*15*, *80*, *152*) demonstrate that megabase-sized genomes can be sequenced efficiently without any input other that the de novo mate-paired sequences. With more complex genomes like those of *Drosophila* or human, map information, in the form of well-ordered markers, has been critical for long-range ordering of scaffolds. For joining scaffolds into chromosomes, the quality of the map (in terms of the order of the markers) is more important than the number of markers per se. Although this mapping could have been performed concurrently with sequencing, the prior existence of mapping data was beneficial. During the sequencing of the *A. thaliana* genome, sequencing of individual BAC clones permitted extension of the se-

Table 19 (*Continued*)

Panther family/subfamily*	H	F	W	Y	A
Transcription factors/chromatin organization					
C2H2 zinc finger–containing†	607	232	79	28	8
COE	7	1	1	0	0
CREB	7	1	2	0	0
ETS-related	25	8	10	0	0
Forkhead-related	34	19	15	4	0
FOS	8	2	1	0	0
Groucho	13	2	1	0	0
Histone H1	5	0	1	0	0
Histone H2A	24	1	17	3	13
Histone H2B	21	1	17	2	12
Histone H3	28	2	24	2	16
Histone H4	9	1	16	1	8
Homeotic†	168	104	74	4	78
ABD-B	5	0	0	0	0
Bithoraxoid	1	8	1	0	0
Iroquois class	7	3	1	0	0
Distal-less	5	2	1	0	0
Engrailed	2	2	1	0	0
LIM-containing	17	8	3	0	0
MEIS/KNOX class	9	4	4	2	26
NK-3/NK-2 class	9	4	5	0	0
Paired box	38	28	23	0	2
Six	5	3	4	0	0
Leucine zipper	6	0	0	0	0
Nuclear hormone receptor†	59	25	183	1	4
Pou-related	15	5	4	1	0
Runt-related	3	4	2	0	0
ECM adhesion					
Cadherin	113	17	16	0	0
Claudin	20	0	0	0	0
Complement receptor-related	22	8	6	0	0
Connexin	14	0	0	0	0
Galectin	12	5	22	0	0
Glypican	13	2	1	0	0
ICAM	6	0	0	0	0
Integrin alpha	24	7	4	0	1
Integrin beta	9	2	2	0	0
LDL receptor family	26	19	20	0	2
Proteoglycans	22	9	7	0	5
Apoptosis					
Bcl-2	12	1	0	0	0
Calpain	22	4	11	1	3
Calpain inhibitor	4	0	0	0	1
Caspase	13	7	3	0	0
Hemostasis					
ADAM/ADAMTS	51	9	12	0	0
Fibronectin	3	0	0	0	0
Globin	10	2	3	0	3
Matrix metalloprotease	19	2	7	0	3
Serum amyloid A	4	0	0	0	0
Serum amyloid P (subfamily of Pentaxin)	2	0	0	0	0
Serum paraoxonase/arylesterase	4	0	3	0	0
Serum albumin	4	0	0	0	0
Transglutaminase	10	1	0	0	0
Other enzymes					
Cytochrome p450	60	89	83	3	256
GAPDH	46	3	4	3	8
Heparan sulfotransferase	11	4	2	0	0
Splicing and translation					
EF-1alpha	56	13	10	6	13
Ribonucleoproteins†	269	135	104	60	265
Ribosomal proteins†	812	111	80	117	256

*The table lists Panther families or subfamilies relevant to the text that either (i) are not specifically represented by Pfam (Table 18) or (ii) differ in counts from the corresponding Pfam models. †This class represents a number of different families in the same Panther molecular function subcategory. ‡This count includes only rhodopsin-class, secretin-class, and metabotropic glutamate-class GPCRs.

quence well into centromeric regions and allowed high-quality resolution of complex repeat regions. Likewise, in *Drosophila*, the BAC physical map was most useful in regions near the highly repetitive centromeres and telomeres. WGA has been found to deliver excellent-quality reconstructions of the unique regions of the genome. As the genome size, and more importantly the repetitive content, increases, the WGA approach delivers less of the repetitive sequence.

The cost and overall efficiency of clone-by-clone approaches makes them difficult to justify as a stand-alone strategy for future large-scale genome-sequencing projects. Specific applications of BAC-based or other clone mapping and sequencing strategies to resolve ambiguities in sequence assembly that cannot be efficiently resolved with computational approaches alone are clearly worth exploring. Hybrid approaches to whole-genome sequencing will only work if there is sufficient coverage in both the whole-genome shotgun phase and the BAC clone sequencing phase. Our experience with human genome assembly suggests that this will require at least 3× coverage of both whole-genome and BAC shotgun sequence data.

8.2 The low gene number in humans

We have sequenced and assembled ~95% of the euchromatic sequence of *H. sapiens* and used a new automated gene prediction method to produce a preliminary catalog of the human genes. This has provided a major surprise: We have found far fewer genes (26,000 to 38,000) than the earlier molecular predictions (50,000 to over 140,000). Whatever the reasons for this current disparity, only detailed annotation, comparative genomics (particularly using the *Mus musculus* genome), and careful molecular dissection of complex phenotypes will clarify this critical issue of the basic "parts list" of our genome. Certainly, the analysis is still incomplete and considerable refinement will occur in the years to come as the precise structure of each transcription unit is evaluated. A good place to start is to determine why the gene estimates derived from EST data are so discordant with our predictions. It is likely that the following contribute to an inflated gene number derived from ESTs: the variable lengths of 3′- and 5′-untranslated leaders and trailers; the little-understood vagaries of RNA processing that often leave intronic regions in an unspliced condition; the finding that nearly 40% of human genes are alternatively spliced (*153*); and finally, the unsolved technical problems in EST library construction where contamination from heterogeneous nuclear RNA and genomic DNA are not uncommon. Of course, it is possible that there are genes that remain unpredicted owing to the absence of EST or protein data to support them, although our use of mouse genome data for predicting genes should limit this number. As was true at the beginning of genome sequencing, ultimately it will be necessary to measure mRNA in specific cell types to demonstrate the presence of a gene.

J. B. S. Haldane speculated in 1937 that a population of organisms might have to pay a price for the number of genes it can possibly carry. He theorized that when the number of genes becomes too large, each zygote carries so many new deleterious mutations that the population simply cannot maintain itself. On the basis of this premise, and on the basis of available mutation rates and x-ray–induced mutations at specific loci, Muller, in 1967 (*154*), calculated that the mammalian genome would contain a maximum of not much more than 30,000 genes (*155*). An estimate of 30,000 gene loci for humans was also arrived at by Crow and Kimura (*156*). Muller's estimate for *D. melanogaster* was 10,000 genes, compared to 13,000 derived by annotation of the fly genome (*26*, *27*). These arguments for the theoretical maximum gene number were based on simplified ideas of genetic load—that all genes have a certain low rate of mutation to a deleterious state. However, it is clear that many mouse, fly, worm, and yeast knockout mutations lead to almost no discernible phenotypic perturbations.

The modest number of human genes means that we must look elsewhere for the mechanisms that generate the complexities inherent in human development and the sophisticated signaling systems that maintain homeostasis. There are a large number of ways in which the functions of individual genes and gene products are regulated. The degree of "openness" of chromatin structure and hence transcriptional activity is regulated by protein complexes that involve histone and DNA enzymatic modifications. We enumerate many of the proteins that are likely involved in nuclear regulation in Table 19. The location, timing, and quantity of transcription are intimately linked to nuclear signal transduction events as well as by the tissue-specific expression of many of these proteins. Equally important are regulatory DNA elements that include insulators, repeats, and endogenous viruses (*157*); methylation of CpG islands in imprinting (*158*); and promoter-enhancer and intronic regions that modulate transcription. The spliceosomal machinery consists of multisubunit proteins (Table 19) as well as structural and catalytic RNA elements (*159*) that regulate transcript structure through alternative start and termination sites and splicing. Hence, there is a need to study different classes of RNA molecules (*160*) such as small nucleolar RNAs, antisense riboregulator RNA, RNA involved in X-dosage compensation, and other structural RNAs to appreciate their precise role in regulating gene expression. The phenomenon of RNA editing in which coding changes occur directly at the level of mRNA is of clinical and biological relevance (*161*). Finally, examples of translational control include internal ribosomal entry sites that are found in proteins involved in cell cycle regulation and apoptosis (*162*). At the protein level, minor alterations in the nature of protein-protein interactions, protein modifications, and localization can have dramatic effects on cellular physiology (*163*). This dynamic system therefore has many ways to modulate activity, which suggests that definition of complex systems by analysis of single genes is unlikely to be entirely successful.

In situ studies have shown that the human genome is asymmetrically populated with G+C content, CpG islands, and genes (*68*). However, the genes are not distributed quite as unequally as had been predicted (Table 9) (*69*). The most G+C-rich fraction of the genome, H3 isochores, constitute more of the genome than previously thought (about 9%), and are the most gene-dense fraction, but contain only 25% of the genes, rather than the predicted ~40%. The low G+C L isochores make up 65% of the genome, and 48% of the genes. This inhomogeneity, the net result of millions of years of mammalian gene duplication, has been described as the "desertification" of the vertebrate genome (*71*). Why are there clustered regions of high and low gene density, and are these accidents of history or driven by selection and evolution? If these deserts are dispensable, it ought to be possible to find mammalian genomes that are far smaller in size than the human genome. Indeed, many species of bats have genome sizes that are much smaller than that of humans; for example, *Miniopterus*, a species of Italian bat, has a genome size that is only 50% that of humans (*164*). Similarly, *Muntiacus*, a species of Asian barking deer, has a genome size that is ~70% that of humans.

8.3 Human DNA sequence variation and its distribution across the genome

This is the first eukaryotic genome in which a nearly uniform ascertainment of polymorphism has been completed. Although we have identified and mapped more than 3 million SNPs, this by no means implies that the task of finding and cataloging SNPs is complete. These represent only a fraction of the SNPs present in the human population as a whole. Nevertheless, this first glimpse at genome-wide variation has revealed strong inhomogeneities in the distribution of SNPs across the genome. Polymorphism in DNA carries with it a snapshot of the past operation of population genetic forces, including mutation, migration, selection, and genetic drift. The availability of a dense array of SNPs will allow questions related to each of these factors to be addressed on a genome-wide basis. SNP studies can establish the range of haplo-

types present in subjects of different ethnogeographic origins, providing insights into population history and migration patterns. Although such studies have suggested that modern human lineages derive from Africa, many important questions regarding human origins remain unanswered, and more analyses using detailed SNP maps will be needed to settle these controversies. In addition to providing evidence for population expansions, migration, and admixture, SNPs can serve as markers for the extent of evolutionary constraint acting on particular genes. The correlation between patterns of intraspecies and interspecies genetic variation may prove to be especially informative to identify sites of reduced genetic diversity that may mark loci where sequence variations are not tolerated.

The remarkable heterogeneity in SNP density implies that there are a variety of forces acting on polymorphism—sparse regions may have lower SNP density because the mutation rate is lower, because most of those regions have a lower fraction of mutations that are tolerated, or because recent strong selection in favor of a newly arisen allele "swept" the linked variation out of the population (*165*). The effect of random genetic drift also varies widely across the genome. The nonrecombining portion of the Y chromosome faces the strongest pressure from random drift because there are roughly one-quarter as many Y chromosomes in the population as there are autosomal chromosomes, and the level of polymorphism on the Y is correspondingly less. Similarly, the X chromosome has a smaller effective population size than the autosomes, and its nucleotide diversity is also reduced. But even across a single autosome, the effective population size can vary because the density of deleterious mutations may vary. Regions of high density of deleterious mutations will see a greater rate of elimination by selection, and the effective population size will be smaller (*166*). As a result, the density of even completely neutral SNPs will be lower in such regions. There is a large literature on the association between SNP density and local recombination rates in *Drosophila*, and it remains an important task to assess the strength of this association in the human genome, because of its impact on the design of local SNP densities for disease-association studies. It also remains an important task to validate SNPs on a genomic scale in order to assess the degree of heterogeneity among geographic and ethnic populations.

8.4 Genome complexity

We will soon be in a position to move away from the cataloging of individual components of the system, and beyond the simplistic notions of "this binds to that, which then docks on this, and then the complex moves there. . . ." (*167*) to the exciting area of network perturbations, nonlinear responses and thresholds, and their pivotal role in human diseases.

The enumeration of other "parts lists" reveals that in organisms with complex nervous systems, neither gene number, neuron number, nor number of cell types correlates in any meaningful manner with even simplistic measures of structural or behavioral complexity. Nor would they be expected to; this is the realm of nonlinearities and epigenesis (*168*). The 520 million neurons of the common octopus exceed the neuronal number in the brain of a mouse by an order of magnitude. It is apparent from a comparison of genomic data on the mouse and human, and from comparative mammalian neuroanatomy (*169*), that the morphological and behavioral diversity found in mammals is underpinned by a similar gene repertoire and similar neuroanatomies. For example, when one compares a pygmy marmoset (which is only 4 inches tall and weighs about 6 ounces) to a chimpanzee, the brain volume of this minute primate is found to be only about 1.5 cm^3, two orders of magnitude less than that of a chimp and three orders less than that of humans. Yet the neuroanatomies of all three brains are strikingly similar, and the behavioral characteristics of the pygmy marmoset are little different from those of chimpanzees. Between humans and chimpanzees, the gene number, gene structures and functions, chromosomal and genomic organizations, and cell types and neuroanatomies are almost indistinguishable, yet the developmental modifications that predisposed human lineages to cortical expansion and development of the larynx, giving rise to language, culminated in a massive singularity that by even the simplest of criteria made humans more complex in a behavioral sense.

Simple examination of the number of neurons, cell types, or genes or of the genome size does not alone account for the differences in complexity that we observe. Rather, it is the interactions within and among these sets that result in such great variation. In addition, it is possible that there are "special cases" of regulatory gene networks that have a disproportionate effect on the overall system. We have presented several examples of "regulatory genes" that are significantly increased in the human genome compared with the fly and worm. These include extracellular ligands and their cognate receptors (e.g., wnt, frizzled, TGF-β, ephrins, and connexins), as well as nuclear regulators (e.g., the KRAB and homeodomain transcription factor families), where a few proteins control broad developmental processes. The answers to these "complexities" perhaps lie in these expanded gene families and differences in the regulatory control of ancient genes, proteins, pathways, and cells.

8.5 Beyond single components

While few would disagree with the intuitive conclusion that Einstein's brain was more complex than that of *Drosophila*, closer comparisons such as whether the set of predicted human proteins is more complex than the protein set of *Drosophila*, and if so, to what degree, are not straightforward, since protein, protein domain, or protein-protein interaction measures do not capture context-dependent interactions that underpin the dynamics underlying phenotype.

Currently, there are more than 30 different mathematical descriptions of complexity (*170*). However, we have yet to understand the mathematical dependency relating the number of genes with organism complexity. One pragmatic approach to the analysis of biological systems, which are composed of nonidentical elements (proteins, protein complexes, interacting cell types, and interacting neuronal populations), is through graph theory (*171*). The elements of the system can be represented by the vertices of complex topographies, with the edges representing the interactions between them. Examination of large networks reveals that they can self-organize, but more important, they can be particularly robust. This robustness is not due to redundancy, but is a property of inhomogeneously wired networks. The error tolerance of such networks comes with a price; they are vulnerable to the selection or removal of a few nodes that contribute disproportionately to network stability. Gene knockouts provide an illustration. Some knockouts may have minor effects, whereas others have catastrophic effects on the system. In the case of vimentin, a supposedly critical component of the cytoplasmic intermediate filament network of mammals, the knockout of the gene in mice reveals them to be reproductively normal, with no obvious phenotypic effects (*172*), and yet the usually conspicuous vimentin network is completely absent. On the other hand, ~30% of knockouts in *Drosophila* and mice correspond to critical nodes whose reduction in gene product, or total elimination, causes the network to crash most of the time, although even in some of these cases, phenotypic normalcy ensues, given the appropriate genetic background. Thus, there are no "good" genes or "bad" genes, but only networks that exist at various levels and at different connectivities, and at different states of sensitivity to perturbation. Sophisticated mathematical analysis needs to be constantly evaluated against hard biological data sets that specifically address network dynamics. Nowhere is this more critical than in attempts to come to grips with "complexity," particularly because deconvoluting and correcting complex networks that have undergone perturbation, and have resulted in human diseases, is the greatest significant challenge now facing us.

It has been predicted for the last 15 years that complete sequencing of the human ge-

nome would open up new strategies for human biological research and would have a major impact on medicine, and through medicine and public health, on society. Effects on biomedical research are already being felt. This assembly of the human genome sequence is but a first, hesitant step on a long and exciting journey toward understanding the role of the genome in human biology. It has been possible only because of innovations in instrumentation and software that have allowed automation of almost every step of the process from DNA preparation to annotation. The next steps are clear: We must define the complexity that ensues when this relatively modest set of about 30,000 genes is expressed. The sequence provides the framework upon which all the genetics, biochemistry, physiology, and ultimately phenotype depend. It provides the boundaries for scientific inquiry. The sequence is only the first level of understanding of the genome. All genes and their control elements must be identified; their functions, in concert as well as in isolation, defined; their sequence variation worldwide described; and the relation between genome variation and specific phenotypic characteristics determined. Now we know what we have to explain.

Another paramount challenge awaits: public discussion of this information and its potential for improvement of personal health. Many diverse sources of data have shown that any two individuals are more than 99.9% identical in sequence, which means that all the glorious differences among individuals in our species that can be attributed to genes falls in a mere 0.1% of the sequence. There are two fallacies to be avoided: determinism, the idea that all characteristics of the person are "hard-wired" by the genome; and reductionism, the view that with complete knowledge of the human genome sequence, it is only a matter of time before our understanding of gene functions and interactions will provide a complete causal description of human variability. The real challenge of human biology, beyond the task of finding out how genes orchestrate the construction and maintenance of the miraculous mechanism of our bodies, will lie ahead as we seek to explain how our minds have come to organize thoughts sufficiently well to investigate our own existence.

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32. Eligibility criteria for participation in the study were as follows: prospective donors had to be 21 years of age or older, not pregnant, and capable of giving an informed consent. Donors were asked to self-define their ethnic backgrounds. Standard blood bank screens (screening for HIV, hepatitis viruses, and so forth) were performed on all samples at the clinical laboratory prior to DNA extraction in the Celera laboratory. All samples that tested positive for transmissible viruses were ineligible and were discarded. Karyotype analysis was performed on peripheral blood lymphocytes from all samples selected for sequencing; all were normal. A two-staged consent process for prospective donors was employed. The first stage of the consent process provided information about the genome project, procedures, and risks and benefits of participating. The second stage of the consent process involved answering follow-up questions and signing consent forms, and was conducted about 48 hours after the first.
33. DNA was isolated from blood (*173*) or sperm. For sperm, a washed pellet (100 μl) was lysed in a suspension (1 ml) containing 0.1 M NaCl, 10 mM tris-Cl–20 mM EDTA (pH 8), 1% SDS, 1 mg proteinase K, and 10 mM dithiothreitol for 1 hour at 37°C. The lysate was extracted with aqueous phenol and with phenol/chloroform. The DNA was ethanol precipitated and dissolved in 1 ml TE buffer. To make genomic libraries, DNA was randomly sheared, end-polished with consecutive BAL31 nuclease and T4 DNA polymerase treatments, and size-selected by electrophoresis on 1% low-melting-point agarose. After ligation to Bst XI adapters (Invitrogen, catalog no. N408-18), DNA was purified by three rounds of gel electrophoresis to remove excess adapters, and the fragments, now with 3′-CACA overhangs, were inserted into Bst XI-linearized plasmid vector with 3′-TGTG overhangs. Libraries with three different average sizes of inserts were constructed: 2, 10, and 50 kbp. The 2-kbp fragments were cloned in a high-copy pUC18 derivative. The 10- and 50-kbp fragments were cloned in a medium-copy pBR322 derivative. The 2- and 10-kbp libraries yielded uniform-sized large colonies on plating. However, the 50-kbp libraries produced many small colonies and inserts were unstable. To remedy this, the 50-kbp libraries were digested with Bgl II, which does not cleave the vector, but generally cleaved several times within the 50-kbp insert. A 1264-bp Bam HI kanamycin resistance cassette (purified from pUCK4; Amersham Pharmacia, catalog no. 27-4958-01) was added and ligation was carried out at 37°C in the continual presence of Bgl II. As Bgl II–Bgl II ligations occurred, they were continually cleaved, whereas Bam HI–Bgl II ligations were not cleaved. A high yield of internally deleted circular library molecules was obtained in which the residual insert ends were separated by the kanamycin cassette DNA. The internally deleted libraries, when plated on agar containing ampicillin (50 μg/ml), carbenicillin (50 μg/ml), and kanamycin (15 μg/ml), produced relatively uniform large colonies. The resulting clones could be prepared for sequencing using the same procedures as clones from the 10-kbp libraries.
34. Transformed cells were plated on agar diffusion plates prepared with a fresh top layer containing no antibiotic poured on top of a previously set bottom layer containing excess antibiotic, to achieve the correct final concentration. This method of plating permitted the cells to develop antibiotic resistance before being exposed to antibiotic without the potential clone bias that can be introduced through liquid outgrowth protocols. After colonies had grown, QBot (Genetix, UK) automated colony-picking robots were used to pick colonies meeting stringent size and shape criteria and to inoculate 384-well microtiter plates containing liquid growth medium. Liquid cultures were incubated overnight, with shaking, and were scored for growth before passing to template preparation. Template DNA was extracted from liquid bacterial culture using a procedure based upon the alkaline lysis miniprep method (*173*) adapted for high throughput processing in 384-well microtiter plates. Bacterial cells were lysed; cell debris was removed by centrifugation; and plasmid DNA was recovered by isopropanol precipitation and resuspended in 10 mM tris-HCl buffer. Reagent dispensing operations were accomplished using Titertek MAP 8 liquid dispensing systems. Plate-to-plate liquid transfers were performed using Tomtec Quadra 384 Model 320 pipetting robots. All plates were tracked throughout processing by unique plate barcodes. Mated sequencing reads from opposite ends of each clone insert were obtained by preparing two 384-well cycle sequencing reaction plates from each plate of plasmid template DNA using ABI-PRISM BigDye Terminator chemistry (Applied Biosystems) and standard M13 forward and reverse primers. Sequencing reactions were prepared using the Tomtec Quadra 384-320 pipetting robot. Parent-child plate relationships and, by extension, forward-reverse sequence mate pairs were established by automated plate barcode reading by the onboard barcode reader and were recorded by direct LIMS communication. Sequencing reaction products were purified by alcohol precipitation and were dried, sealed, and stored at 4°C in the dark until needed for sequencing, at which time the reaction products were resuspended in deionized formamide and sealed immediately to prevent degradation. All sequence data were generated using a single sequencing platform, the ABI PRISM 3700 DNA Analyzer. Sample sheets were created at load time using a Java-based application that facilitates barcode scanning of the sequencing plate barcode, retrieves sample information from the central LIMS, and reserves unique trace identifiers. The application permitted a single sample sheet file in the linking directory and deleted previously created sample sheet files immediately upon scanning of a