A Combined Computational and Experimental Study on the Structure-Regulation Relationships of Putative Mammalian DNA Replication Initiator GINS

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GINS, a heterotetramer of SLD5, PSF1, PSF2, and PSF3 proteins, is an emerging chromatin factor recognized to be involved in the initiation and elongation step of DNA replication. Although the yeast and *Xenopus GINS* genes are well documented, their orthologous genes in higher eukaryotes are not fully characterized. In this study, we report the genomic structure and transcriptional regulation of mammalian *GINS* genes. Serum stimulation increased the *GINS* mRNA levels in human cells. Reporter gene assay using putative *GINS* promoter sequences revealed that the expression of mammalian *GINS* is regulated by 17β-Estradiol-stimulated estrogen receptor α, and human *PSF3* acts as a gene responsive to transcription factor E2F1. The goal of this study is to present the current data so as to encourage further work in the field of *GINS* gene regulation and functions in mammalian cells.

Key words: DNA replication initiation, bioinformatics, gene structure, gene regulation, 17β-Estradiol, transcription factor E2F1

Introduction

In eukaryotic cells, chromosomal DNA replication requires the formation of a prereplicative complex (pre-RC) on origins, and assembly of other replication proteins in the S phase of the cell cycle to load the DNA polymerases to initiate DNA synthesis (*1*). The Cdc45 protein is crucial for the latter step of DNA replication initiation, as it activates the pre-RC that contains the MCM helicase. In addition to these proteins, GINS, a heterotetramer consisting of SLD5, PSF1 (partner of Sld five 1), PSF2, and PSF3 proteins, was recently identified as a loading factor for DNA polymerases, and as being required to separate the DNA strands at the replication fork in yeast and *Xenopus* egg extracts (*2–6*). Although GINS is supposed to play a significant role in eukaryotic cells, the biological functions of its orthologous genes in higher eukaryotes are not fully characterized. A comparative genomic approach for the study of the *GINS* orthologous genes in higher eukaryotes could help in the understanding of a common regulatory mechanism for mammalian *GINS* genes.

In this study, we characterized the human and mouse *GINS* orthologous genes by using bioinformatics techniques. The gene structures, chromosomal localization, protein homology, and expression profiles are discussed. In addition, transcriptional regulations of human and mouse *GINS* genes are characterized. This is the first report of the identification of *GINS* up-regulation by serum stimulation and 17β-Estradiol (E2)-stimulated estrogen receptor α (ERα), and also is the first report that the putative promoter region of human *PSF3* is responsive to transcription factor E2F1.

Results

Characterization of the *GINS* orthologous genes in higher eukaryotes

To investigate the functional importance of the GINS proteins in higher eukaryotes, we initially compared the amino acid sequences among human, chimpanzee, orangutan, dog, cow, mouse, and rat GINS proteins. As summarized in Table 1, human PSF1, PSF2, PSF3, and SLD5 proteins showed 92%–94%, 86%–100%, 89%–99%, and 84%–88% total amino acid iden-

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tity, respectively, with the mammalian orthologous genes. Next, we sought to determine the gene structures of the human and mouse *GINS* genes. For this purpose, we searched for a genome database using human and mouse *GINS* cDNAs as query sequences in the BLAST program. Consequently, human (mouse) *PSF1*, *PSF2*, *PSF3*, and *SLD5* gene fragments were identified within the genome sequences NT_011387 (AL808125), NT_010498 (AC103360), NT_086851 (AC113951), and NT_086740 (AC126038), respectively. The gene structures, especially the exon length and location of the human and mouse *GINS* genes

were well conserved. Human (mouse) *PSF1*, *PSF2*, *PSF3*, and *SLD5* genes had 7 (7), 5 (5), 3 (3), and 8 (8) exons, respectively (Tables 2-5). The precise exon-intron boundaries of the human and mouse *GINS* genes were determined based on the consensus sequence ("gt...ag" rule of intronic sequence) of exon-intron junctions and the codon usage. One exception was found in intron2 ("gg...ag") of the human *PSF3* gene (Table 4). The human (mouse) *PSF1*, *PSF2*, *PSF3*, and *SLD5* genes were mapped to 20p11.21 (2G3), 16q24.1 (8E1), 16q21 (8C5), and 8p11.21 (8A3), respectively (Table 6).

Table 1 Conservation of Amino Acids in the GINS Proteins*

Mammal	PSF1		PSF2		PSF3		SLD5	
	Identify (%)	ORF (aa)	Identify (%)	ORF (aa)	Identify (%)	ORF (aa)	Identify (%)	ORF (aa)
Human	100	196	100	185	100	216	100	223
Chimpanzee	92	268	100	185	99	216	–	–
Orangutan	–	–	–	–	99	216	–	–
Dog	94	196	94	185	94	216	88	292
Cow	92	196	86	340	93	216	84	489
Mouse	92	196	92	185	89	216	87	223
Rat	93	196	92	434	90	216	88	223

*The percentage conservation in mammals, including chimpanzee, orangutan, dog, cow, mouse, and rat proteins relative to the sequence of the human protein (taken as 100%) is presented. The lengths of the amino acid (aa) sequences are indicated. "–" indicates that the protein is not deposited in the databases.

Table 2 Exon-Intron Structures of the Human *PSF1* and Mouse *Psf1* Genes*

Exon No.	Nucleotide sequence around exon-intron boundaries of human *PSF1* gene	Nucleotide position of exon in human genome sequence	Exon (bp)	Intron (bp)
1	AGCGCG—TTCAAC gtgagg	25328321–25328531 of NT_011387	211	5,896
2	ttctag GAGGAT—TGATGT gtaagt	25334426–25334490 of NT_011387	65	3,251
3	tatcag GAATGA—ATACCT gtaagc	25337740–25337838 of NT_011387	99	904
4	tcctag GTATGA—GAAGAA gtgagt	25338741–25338831 of NT_011387	91	7,017
5	ttgcag ATGGAG—ATTGAA gtatgt	25345847–25345963 of NT_011387	117	16,376
6	tttcag GTCCGG—AGCCAG gtattt	25362338–25362412 of NT_011387	75	4,148
7	cctcag CACTTT—ATGGAG	25366559–25367702 of NT_011387	1,144	

Exon No.	Nucleotide sequence around exon-intron boundaries of mouse *Psf1* gene	Nucleotide position of exon in mouse genome sequence	Exon (bp)	Intron (bp)
1	GGAGCT—TTTAAT gtgagg	76468–76660 of AL808125	193	2,989
2	tttcag GAGGAC—TGATGT gtaagt	79648–79712 of AL808125	65	3,292
3	tctcag GAATGA—ATACCT gtgagt	83003–82101 of AL808125	99	1,636
4	tcctag GTATGA—GAAGAA gtaagt	84736–84826 of AL808125	91	7,924
5	tcacag ACGGAG—ATTGAA gtatgt	92749–92865 of AL808125	117	2,045
6	tttcag GTGCGG—AGTCAG gtagtt	94909–94983 of AL808125	75	2,726
7	ttgtag CACTTT—TTTCAC	97708–98145 of AL808125	438	

*The nucleotide sequences around the exon-intron boundaries are shown in upper-case (exon) and lower-case letters (intron). The exon and intron lengths (bp) are shown.

Table 3 Exon-Intron Structures of the Human *PSF2* and Mouse *Psf2* Genes

Exon No.	Nucleotide sequence around exon-intron boundaries of human *PSF2* gene	Nucleotide position of exon in human genome sequence	Exon (bp)	Intron (bp)
1	GCGGCC—ATCGGG gtgagg	39336779–39336614 of NT_010498	116	1,236
2	tggcag GGGGAC—ATGTAG gtaagg	39335379–39335265 of NT_010498	115	5,780
3	ttttag AAAAGT—AAATCA gtaagt	39329486–39329387 of NT_010498	100	2,917
4	ctttag TGCTTC—GCCAAG gtaggt	39326471–39326345 of NT_010498	127	204
5	ctttag CTGGAT—AACTCA	39326142–39325479 of NT_010498	664	
Exon No.	**Nucleotide sequence around exon-intron boundaries of mouse *Psf2* gene**	**Nucleotide position of exon in mouse genome sequence**	**Exon (bp)**	**Intron (bp)**
1	GGGAAA—ATCGGG gtgagc	111624–111788 of AC103360	165	88
2	ttgcag GGGGAC—ATGTGG gtgagc	111875–111989 of AC103360	115	2,534
3	tcagag AGAAAC—GAATCA gtgagt	114522–114621 of AC103360	100	3,886
4	cttcag TGCTTC—GCCAAG gtaggc	118506–118632 of AC103360	127	272
5	tttcag CTGGAC—TATACT	118903–122081 of AC103360	3,179	

Table 4 Exon-Intron Structures of the Human *PSF3* and Mouse *Psf3* Genes

Exon No.	Nucleotide sequence around exon-intron boundaries of human *PSF3* gene	Nucleotide position of exon in human genome sequence	Exon (bp)	Intron (bp)
1	CCGCTT—CCACAG gtgagc	2010301–2010569 of NT_086851	269	9,759
2	ctgcag GGTTCC—CTGCAG ggcaag	2020327–2020560 of NT_086851	234	1,169
3	tcccag ACTTTT—TTAGCA	2021728–2023378 of NT_086851	1,651	
Exon No.	**Nucleotide sequence around exon-intron boundaries of mouse *Psf3* gene**	**Nucleotide position of exon in mouse genome sequence**	**Exon (bp)**	**Intron (bp)**
1	GAGTTT—CCTCAG gtgagg	126499–126244 of AC113951	256	4,028
2	acgcag GGTACA—CTGAAG gtaagt	122217–121984 of AC113951	234	5,061
3	cctcag ACTTTT—GCCTGC	116924–115517 of AC113951	1,407	

Table 5 Exon-Intron Structures of the Human *SLD5* and Mouse *Sld5* Genes

Exon No.	Nucleotide sequence around exon-intron boundaries of human *SLD5* gene	Nucleotide position of exon in human genome sequence	Exon (bp)	Intron (bp)
1	GCGACT—CCCCGA gtgagc	28874824–28874993 of NT_086740	170	789
2	cattag GTTCCT—GAGCAG gtaagc	28875781–28875895 of NT_086740	115	6,063
3	ttttag GCCTGG—CACATG gtaaga	28881957–28882043 of NT_086740	87	735
4	taatag GAAGAA—ATGAAG gtttga	28882777–28882890 of NT_086740	114	2,436
5	tcacag ATAGAG—CAGAGA gtgagt	28885325–28885422 of NT_086740	98	142
6	tttcag GTTCAT—GGGCAG gtaaac	28885563–28885651 of NT_086740	89	1,851
7	tggcag TTCCCA—GCAGAG gtgagt	28887501–28887591 of NT_086740	91	93
8	tttcag GGACTA—AAAAAA	28887683–28888085 of NT_086740	403	
Exon No.	**Nucleotide sequence around exon-intron boundaries of mouse *Sld5* gene**	**Nucleotide position of exon in mouse genome sequence**	**Exon (bp)**	**Intron (bp)**
1	GTGTTT—TGCGAG gtgagc	79323–79134 of AC126038	190	372
2	tttcag GTGTAG—GAGCAG gtaagc	78763–78644 of AC126038	120	2,141
3	ttttag GCCTGG—CACATG gtaaga	76504–76418 of AC126038	87	2,037
4	taatag GAAGAA—ATGAAG gtttga	74382–74269 of AC126038	114	2,903
5	tcacag ATAGAG—CAAAGA gtaagt	71367–71270 of AC126038	98	103
6	ttccag GTATAT—GGGCAG gtaaga	71168–71080 of AC126038	89	2,078
7	tggcag TTCCCA—GCAGAG gtgagt	69003–68913 of AC126038	91	75
8	tttcag AGACTA—AAGCTT	68839–68289 of AC126038	501	

Table 6 Chromosomal Localization of the Human and Mouse *GINS* Genes

	PSF1	*PSF2*	*PSF3*	*SLD5*
Human	20p11.21	16q24.1	16q21	8p11.21
Mouse	2G3	8E1	8C5	8A3

Expression profiles of human and mouse *GINS* mRNAs

In silico analysis revealed that the expression patterns of the *GINS* mRNA in human/mouse tissues and organs were quite different (Figure 1A). On the other hand, during the developmental stages, the *GINS* mRNA expression patterns were quite similar: highest in the embryo and juvenile stages, lower in adults, and no expression in the neonatal stage (Figure 1B). Next, we attempted a search for *GINS* mRNA in public databases. The Gene Expression Omnibus (GEO) database search revealed that the *SLD5* gene is down-regulated under two growth arrest conditions, namely, serum deprivation and contact inhibition, in T98G cancer cells (Accession No. GDS911), and *PSF1* is an estrogen target in human breast carcinoma MCF7 cells (Accession No. GDS118). It is of interest that *GINS* is regulated by serum stimulation, because the mRNA level of a known DNA replication initiator is reported to be induced when cells enter the G1/S phase of the cell cycle (7, 8). To substantiate this evidence, growth arrest of Saos-2 cells was induced by incubation in serum-free medium for three days, then the cells were re-entered into the cell cycle with the re-addition of 20% fetal bovine serum (FBS). Twenty-four hours after the serum re-addition, the *GINS* mRNA level was found to be up-regulated, while the *GAPDH* mRNA level remained unchanged (Figure 1C). At this time point, cyclin A protein accumulation was clearly observed as the cells re-entered the cell cycle (data not shown).

Transcriptional regulation of the human and mouse *GINS* genes

To gain more insights into the functions of the human and mouse *GINS* genes, we searched the proximal region of the transcription start site for potential *cis*-elements using the Transfac software (ver. 4.0, cut-off 85). The E2F- and/or NF-Y-binding consensus sequences were identified to be surrounded by GC-rich sequences in the putative promoter region of the human and mouse *GINS* genes (Figure 2A). In addition to that by serum stimulation, many genes involved in DNA replication initiation were found to be regulated by transcription factor E2F1 (7, 8). To demonstrate the importance of the putative E2F-binding element in the basal promoter activity, we generated human *PSF1*, mouse *Psf2*, human *PSF3*, and mouse *Sld5* promoter-luciferase constructs. As shown in Figure 2B, human *PSF1*, mouse *Psf2*, human *PSF3*, and mouse *Sld5* promoter constructs showed approximately 265-, 37-, 0.6-, and 82-fold increases in activity, respectively, as determined by measuring the relative luciferase activities, taking the activity in the control luciferase vector pGL3-Basic as 1. Exogenous coexpression of E2F1 caused up to approximately 1-, 0.2-, 8.3-, and 0.9-fold increases in the human *PSF1*, mouse *Psf2*, human *PSF3*, and mouse *Sld5* promoter activities, respectively, as compared to that of the pcDNA3 control vector (Figure 2C). These results suggest that the E2F-binding motif plays critical roles in the E2F1-mediated human *PSF3* promoter activity. This is the first report of the E2F1-dependent regulation of the *PSF3* gene.

To determine whether E2 also regulates *GINS* promoter activity, HeLa cells were cultured with E2, and the effect of cotransfection with ERα was measured. Interestingly, the initial transient transfection studies with pGL3-*hPSF1*, -*mPsf2*, -*hPSF3*, and -*mSld5* reporter vector constructs showed that treatment with 100 nM of E2 resulted in 1.2-, 0.8-, 1.3-, and 0.9-fold increases in the reporter gene activities, respectively, as compared to that after DMSO treatment (Figure 2D). However, following cotransfection with ERα, there was an enhanced (11~24-fold) induction of reporter gene activity after treatment with 100 nM of E2 as compared to that in the pcDNA3 control vector (Figure 2D). These results suggest that the *GINS* genes are regulated by E2 via ERα in cultured cells.

Discussion

In the present study, we attempted to characterize the gene structure and transcriptional regulation of the human and mouse *GINS* genes. Studies using bioinformatics techniques revealed that the GINS proteins are well conserved among several mammals. In addi-
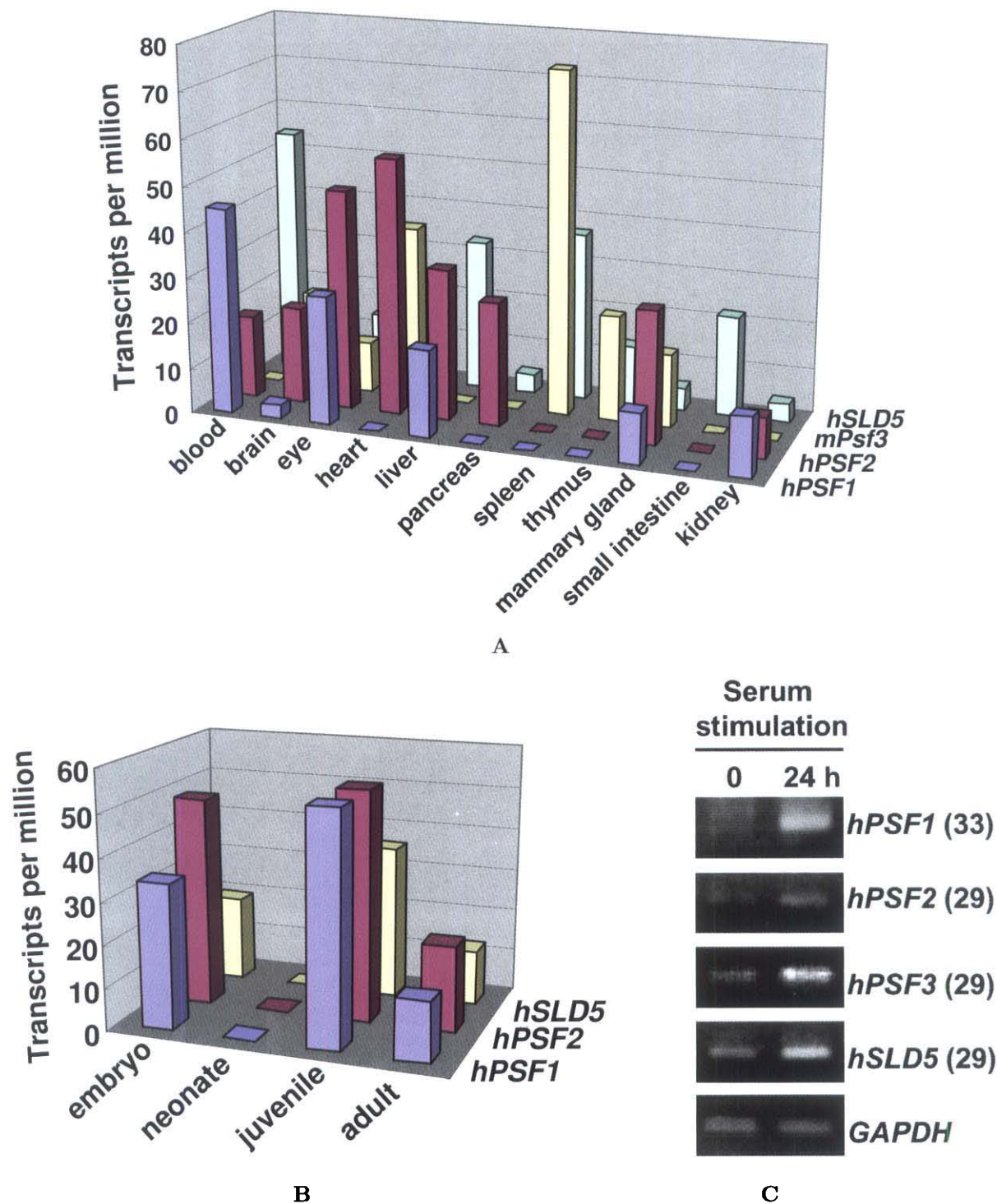
Fig. 1 Expression profiles of *GINS* genes. A search was conducted based on the EST counts in human/mouse tissues (**A**) and during the developmental stages (**B**) in the UniGene database (EST Profile Viewer, NCBI). The datasets used for *PSF1*, *PSF2*, *Psf3*, and *SLD5* genes were Hs. 360033, Hs. 433180, Mm. 35546, and Hs. 521557, respectively. The number of transcripts per million was calculated from the gene EST/total EST in the pool. **C**. Semi-quantitative RT-PCR analysis of the transcripts of *GINS* genes in Saos-2 cells after serum stimulation for 24 h. The number of PCR cycles is shown in parentheses. The housekeeping gene, *GAPDH*, was used as the control.
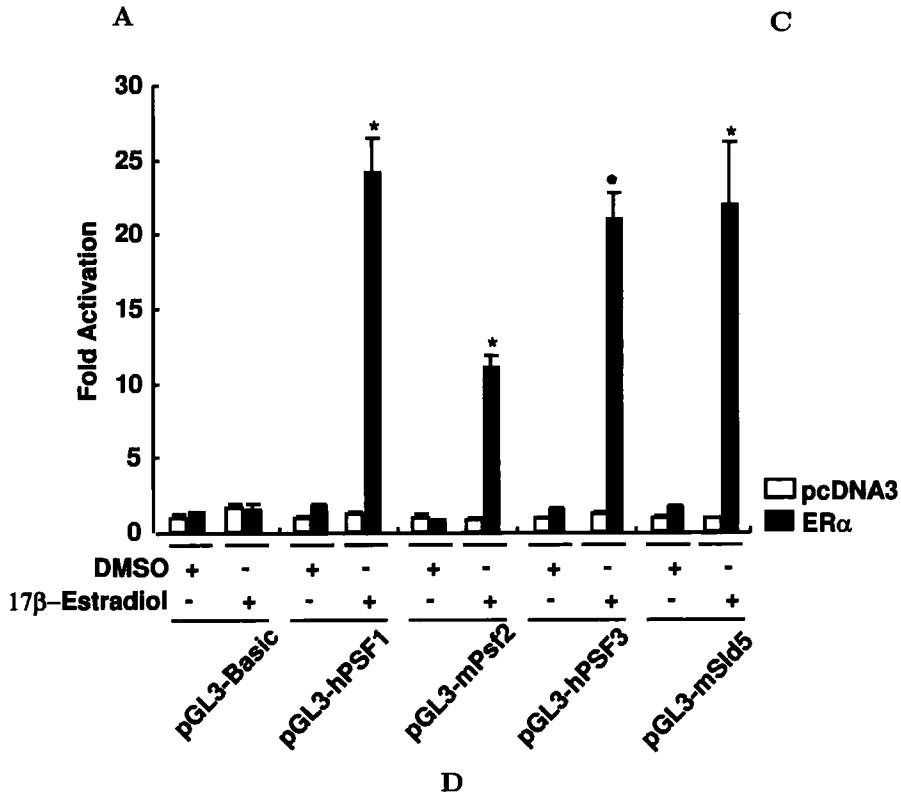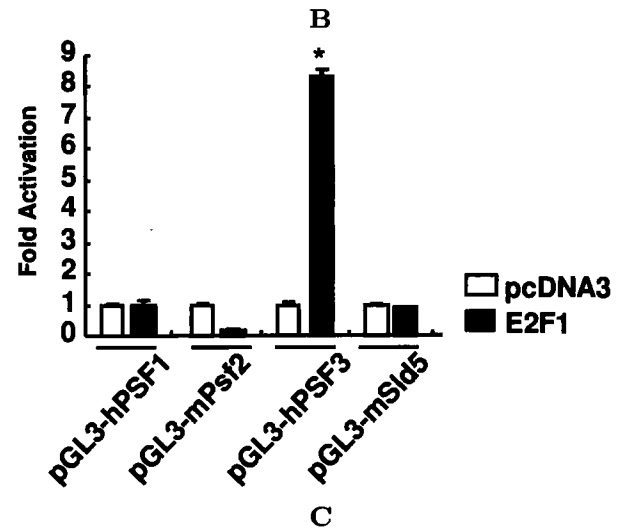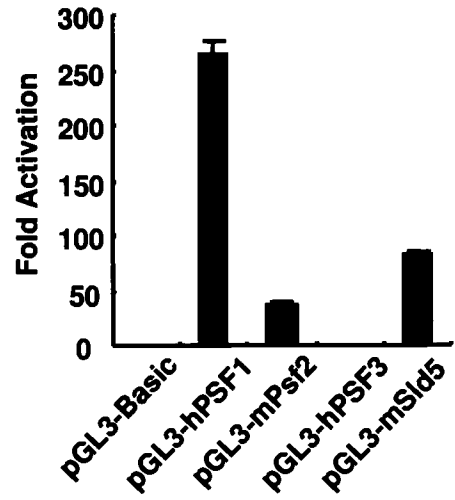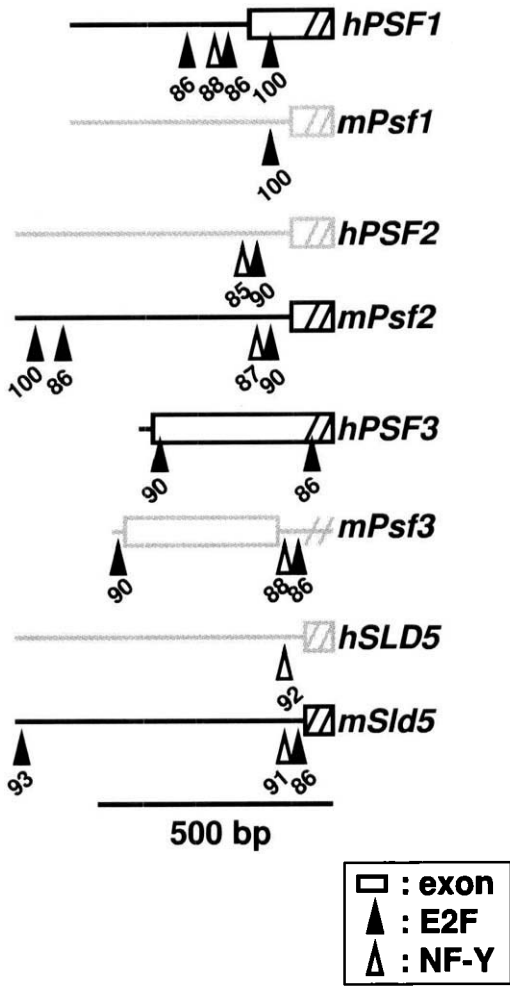
Fig. 2 Transcriptional regulation of the human and mouse *GINS* genes. **A.** Schematic representation of the vicinity of the transcription start sites of the human and mouse *GINS* genes. The putative transcription factor E2F- (closed isosceles triangles) and NF-Y-binding sites (open isosceles triangles) are indicated with their scores (maximum score 100) calculated by the Transfac program. Exon 1 (open boxes) is indicated. Scale bar equals 500 bp. **B.** Human and mouse *GINS* promoter activities in asynchronously growing human cells. HeLa cells were transfected with 200 ng of reporter constructs and 400 ng of the expression vector for E2F1, together with 0.6 ng of pRL-TK. The pcDNA3 vector was used as the negative control. At 48 h after the transfection, the cells were harvested, and extracts were prepared to measure the firefly and *Renilla* luciferase activities. Values are represented as relative luciferase activities, with that of pGL3-Basic being taken as 1. **C.** The E2F-binding motif of the *PSF3* promoter is sufficient to confer responses to ectopic E2F expression. The experiment was performed as described in panel B. Values are represented as relative luciferase activities, with that of the control vector pcDNA3 being taken as 1. Statistically significant ($P < 0.05$) induction is indicated by an asterisk. **D.** E2-induced activation of *GINS* promoter constructs in HeLa cells. Cells were transfected with the indicated reporter plasmids and pcDNA3 or pSG5-ERα expression vector, and the effects of E2 or DMSO on the luciferase activities were determined. Results are expressed as mean±S.D. for at least three triplicate determinations for each treatment group. Statistically significant ($P < 0.05$) induction is indicated by an asterisk.

tion, conservation of the gene structure, including the putative promoter region between human and mouse *GINS* genes, suggests that their transcriptional regulation might also be similar. We succeeded in identifying *PSF3* as an E2F1-regulated gene, and that the human and mouse *GINS* genes are regulated by growth stimuli, including serum addition and E2 treatment. In contrast to the rapid progress of research on the yeast and *Xenopus GINS*, only a few reports have been published regarding mammalian *GINS*. For example, mice homozygous for the *PSF1* gene died *in utero* around the time of implantation, indicating that PSF1 protein may be required during early embryogenesis (*9*). cDNA microarray analyses revealed that the *PSF2* gene is frequently up-regulated in cholangiocarcinoma, suggesting that PSF2 protein may play an important role in the development of this type of liver cancer (*10*).

The action of E2 on its target cells is mediated via ERα or ERβ, which function as ligand-activated transcription factors regulating gene expression. E2 has been shown to play a central role in breast cancer development; therefore it is important to find out the critical downstream targets of E2/ERα action. The majority of E2 target genes are characterized by imperfect estrogen-responsive element (ERE: AGGTCANNNTGACCT) sequences or complex organization with the presence of half an ERE site. Interestingly, many E2-responsive genes, which do not contain ERE but do contain other *cis*-elements, such as Sp1, have also been described. ERs can regulate transcription by direct protein-protein interaction with Sp1 (*11*). Indeed, the *GINS* promoter constructs used in this study contained GC-rich sequences, but no ERE-like sequences. The mitogenic effect of ERα is me-

diated at the regulation level of kinases that govern the transition from the G0/G1 to S phase of the cell cycle, such as under the influence of cyclin D-Cdk4/6 and cyclin E-Cdk2, to increase phosphorylation of the retinoblastoma protein (pRb) (*12*). pRb physically interacts with E2F1 to yield a transcriptionally repressed complex. Phosphorylated pRb results in dissociation of the pRb-E2F1 complex and subsequent up-regulation of E2F1-dependent genes (*13-15*). Although the regulation of E2F1-dependent transactivation is closely linked to pRb, E2 is also known to induce E2F1 mRNA and protein in human cultured cells (*16*). This could mean that the E2F1-dependent regulation of *PSF3* expression may be partially mediated by E2. More detailed studies are needed to clearly elucidate the mechanism by which GINS affects the development of breast cancer.

In conclusion, our study clearly demonstrated that a comparative genomic approach for the *GINS* orthologous genes in higher eukaryotes could help in the understanding of common or unique regulatory mechanisms for important genes. We identified the conserved gene structure and for the first time characterized the promoter region of the human and mouse *GINS* genes. This is the first report of the identification of the induction of *GINS* by serum stimulation and E2/ERα-induced genes, and is also the first report that human *PSF3* gene is regulated by transcription factor E2F1.

Materials and Methods

Bioinformatics

Bioinformatics techniques were employed as previously described (*17, 18*). A search for *GINS*

orthologous genes was conducted using the HomoloGene or BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/). The Transfac software (http://motif.genome.jp/) was used to determine the transcription factor binding elements. The search was conducted based on the expression profile suggested by analysis of the EST (expressed sequence tag) counts in human/mouse tissues and organs in the UniGene database (EST Profile Viewer, NCBI). The datasets used for *PSF1*, *PSF2*, *Psf3*, and *SLD5* genes were Hs. 360033, Hs. 433180, Mm. 35546, and Hs. 521557, respectively. The number of transcripts per million was calculated based on the gene EST/total EST in the pool, and this value was exported to an Excel file. The expression profile suggested by analysis of Affymetrix GeneChip or SAGE (serial analysis of gene expression) was confirmed by using the GEO database search at NCBI.

Plasmids

The human and mouse *GINS* promoter fragments were generated by polymerase chain reaction (PCR) from genomic DNA and ligated into the pGL3-Basic vector (Promega, Madison, USA). Initial PCR primers were designed to amplify 548 bp (−427/+121), 638 bp (−613/+25), 315 bp (−14/+301), and 499 bp (−409/+90) of human *PSF1* (GenBank Accession No. AL353812), mouse *Psf2* (GenBank Accession No. AC103360), human *PSF3* (GenBank Accession No. AC009118), and mouse *Sld5* (GenBank Accession No. AC147247) promoter sequences, respectively, which are numbered relative to the transcription initiation site at +1 described in the NCBI UniGene database. The forward (F) and reverse (R) PCR primers for the promoter constructs were: 5′-gcagtccctaccagcactag-3′ (−427F), 5′-cggccttgccaaccacca-3′ (+121R), 5′-acccggagctgactttga-3′ (−613F), 5′-gcagcgcctcaggcactt-3′ (+25R), 5′-tcctgtctgatgcgtccc-3′ (−14F), 5′-tgcgctccgctccaggaa-3′ (+301R), 5′-tagagaacaactctgcgc-3′ (−409F), and 5′-agcggtgggcgccggaga-3′ (+90R), respectively. The forward/reverse PCR primers added *Bgl*II/*Hind*III (for *PSF1* and *Sld5*) or *Kpn*I/*Bgl*II (for *Psf2* and *PSF3*) sites to facilitate subcloning. The pcDNA3-HA-E2F1 expression plasmid was a generous gift from Dr. Joseph R. Nevins (Duke University, Durham, USA). The pSG5-ERα expression plasmid was kindly provided by Dr. Gary H. Perdew (The Pennsylvania State University, Univeristy Park, USA).

Cell culture and reporter assay

HeLa and Saos-2 cells were cultured in Earle's modified Eagle's medium (MEM) supplemented with 10% FBS and penicillin/streptomycin. To measure the growth-dependent induction of the human *GINS* gene expression, growth of the Saos-2 cells was arrested in the G0 phase by incubation in the absence of FBS for 3 d, and the cells were reintroduced into the cell cycle by culturing with 20% FBS. Total RNA and cell lysates were recovered at 0 and 24 h after the serum stimulation. To measure promoter activity, the cells were transfected with FuGENE6 (Roche, Indianapolis, USA) according to the manufacturer's instruction. Briefly, 400 ng of expression plasmid, 200 ng of firefly luciferase reporter plasmid (pGL3, Promega), and 0.6 ng of *Renilla* luciferase reporter plasmid (pRL-TK, Promega) per 24-well dish were used in each transfection. After 24 h, cells were treated with 100 nM of E2 (Sigma, St. Louis, USA) or DMSO for 34–40 h. The cells were harvested, and luciferase assay was performed using the Dual-Luciferase Reporter Assay System following the manufacturer's protocol (Promega). Experiments were done at least in triplicate, and the relative activities and S.D. values were determined. To control for transfection efficiency, firefly luciferase values were normalized to the values for *Renilla* luciferase.

RT-PCR

Total cellular RNA was extracted from Saos-2 cells using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction. RT-PCR was performed with the SuperScript RT-PCR system according to the manufacturer's instruction (Invitrogen). PCR was performed as follows: denaturation at 94°C for 2 min, followed by multiple cycles at 94°C for 15 s, 55°C for 30 s, and 68°C for 30 s. The following primers were used: PSF1 (GenBank Accession No. NM_021067), 5′-tgagatcacaggcgtgac-3′ and 5′-ctgtccccttccaaagtg-3′; PSF2 (GenBank Accession No. NM_016095), 5′-cagcctctggagagtact-3′ and 5′-cacctctgtgagagagtc-3′; PSF3 (GenBank Accession No. NM_022770), 5′-ctgcctgcagggagttac-3′ and 5′-agagggccccttcatagtc-3′; SLD5 (GenBank Accession No. NM_032336), 5′-gcctctcgccggaagagt-3′ and 5′-cctgacctcatgatccgc-3′. As a control, a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primer set was used (R&D Systems, Minneapolis, USA). The

amplified products were separated on 1% agarose gels and visualized under ultraviolet transillumination.

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Authors' contributions

RH, TA, MT, and YG carried out the reporter assays and performed the statistical analysis. KY conceived of the study, participated in its design, and completed the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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