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Genomic structure and cloned cDNAs predict that four variants in the kinase domain of serine/threonine kinase receptors arise by alternative splicing and poly(A) addition

(transforming growth factor β receptor/activin/hepatoma cells/signal transduction/dimerization)

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ABSTRACT **Heterodimers of types I and II serine/threonine kinase receptor monomers compose the active receptor complex for ligands of the transforming growth factor β family. Here we show that the genomic organization of coding sequences for the intracellular domain of a widely expressed type I serine/threonine kinase receptor is similar to that of the activin type II receptor gene. The genomic structure and cDNA clones indicate that poly(A) addition to alternative exons at each of three carboxyl-terminal coding exon–intron junctions may be a common feature of both type I and II receptor genes. The predicted products are monomers truncated at kinase subdomains VII, IX, and X which vary in kinase activity and potential serine, threonine, and tyrosine phosphorylation sites. These results suggest that combinations of variants that affect the signal-transducing intracellular kinase domain of both type I and II receptor monomers within the transforming growth factor β ligand family may add to the heterogeneity of biological effects of individual ligands in the family.**

Transmembrane serine (Ser)/threonine (Thr) protein kinases participate in oligomeric receptor complexes that mediate the activity of ligands within the transforming growth factor β (TGFβ) superfamily of cytokines (1–4). Two distinct subbranches of Ser/Thr kinase receptors constitute distinct TGFβ and activin type I and type II binding sites which can be distinguished by size of the covalently crosslinked ligand–receptor complexes. In mammals, five genetically distinct Ser/Thr kinase receptors (SKRs) have been described which are candidates for type I receptors (2–7), while type II receptors for activin (ActRII and ActRIIB) and TGFβ1 (TGFβRII) have been characterized (8, 9). The TGFβRII and ActRII receptor kinases are specific for TGFβ and activin, respectively, while some type I SKRs appear to crossreact with both TGFβ and activin dependent on the type II receptor with which they form a complex (refs. 2–4, 8, and 9; unpublished results).

In tyrosine kinase transmembrane receptors, ligand-enhanced stabilization of dimerization of noncatalytic extracellular domains facilitates intermolecular transphosphorylation of tyrosines within the substrate-binding and catalytic intracellular domains (10). Transphosphorylation of specific tyrosines is obligatory for binding, phosphorylation, or activation of specific substrates (10–14). Kinase- and phosphorylation site-deficient monomers with an intact extracellular dimerization domain act as dominant negative inhibitors of function by competition with the formation of active homodimeric complexes (14). In the fibroblast growth factor receptor (FGFR) kinase family, alternative splicing results in monomers from the same gene with altered kinase activity and tyrosine phosphorylation sites (14–16). An alternative 5′ donor-site splice results in a kinase- and phosphorylation site-defective isoform which combines with a dimerization-competent extracellular domain with low affinity for ligand (14, 15). Exon skipping results in a largely intact intracellular kinase domain with a deletion of the specific tyrosine in the carboxyl terminus that, when phosphorylated, is required for binding of phospholipase Cγ1 (16). In addition to mRNA coding for the complete 11 subdomains of the ActRII Ser/Thr kinase, an isoform is expressed in *Xenopus* embryos which exhibits only subdomains I–VII (17). The variant arises by addition of a poly(A) tract to an alternatively spliced extension of the 3′ end of the homolog of exon 8 in the mouse ActRII gene (18). Here we report variant mRNAs in coding sequence for the intracellular kinase domain of a widely expressed human type I Ser/Thr kinase, SKR2. Cloning and sequence analysis of the human SKR2 gene revealed a similar genomic structure† coding for the intracellular kinase domain and that alternative polyadenylylation and alternative splicing at the 3′ end of exons 8, 9, and 10 may be a general feature of receptors for the TGFβ ligand superfamily.

MATERIALS AND METHODS

Analysis of cDNAs. A 156-bp SKR2 cDNA was cloned from a PCR using cDNA template from human hepatoma cell (Hep G2) poly(A)$^+$ RNA (5). Rapid amplification of cDNA ends (RACE)–PCR (19) was carried out to identify the 5′ end of the SKR2 cDNA, using the SKR2-specific 3′ primer P10 (5′-TATAGAATTCTATGGCACACATGCCATTT-3′).

To determine the complete coding sequence of the SKR2 cDNA, cDNAs were isolated from a λgt11 phage library of Hep G2 cell cDNAs (Clontech) by screening by both the PCR method described previously (5) and with partial SKR2 cDNAs.

Analysis of Genomic DNA. A 1269-bp genomic DNA fragment was generated and cloned by using paired primers P22 and P5 and a DNA template from a phage library prepared from human leukocyte genomic DNA (Clontech) (5). The DNA was used as a probe to identify two clones from about 10^6 phage. Both clones contained inserts of about 15 kb which

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Abbreviations: SKR2, serine/threonine kinase receptor; TGFβ, transforming growth factor β; TGFβRII, TGFβ receptor type II; ActRII, activin receptor type II; FGFR, fibroblast growth factor receptor; RACE, rapid amplification of cDNA ends; RPA, RNase protection.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L31848).

exhibited identical restriction patterns when subjected to *Xho* I, *Bam*HI, *Eco*RI, and *Xba* I (see Fig. 2). DNA fragments of 1.8 kb (*Bam*HI–*Bam*HI), 2.65 kb (*Bam*HI–*Xba* I), 5.1 kb (*Xba* I–*Eco*RI), and 2.2 kb (*Eco*RI–*Xho* I) were subcloned into pBluescript II SK vector (Stratagene). The sequence of each DNA fragment was determined by analysis of a nested set of shorter subclones that were generated with the Erase-a-Base kit (Promega). Exons were identified by overlap with cDNA sequences. Exon 11, which encodes kinase subdomain XI (see Figs. 1 and 2) was first identified by homology of the deduced amino acid sequences with human SKR1 (5) and rat SKR2 (6) and later was confirmed at the mRNA level by PCR.

Analysis of mRNA. Expression of the SKR2 gene was analyzed by RNase protection (RPA) with the MAXIscript and RPAII kits (Ambion, Austin, TX). Total RNA (20 μg) isolated by the RNAzol method (Cinna/Biotecx Laboratories, Friendswood, TX) from the indicated cells and tissues was used. A 240-nt RNA probe complementary to an invariant coding sequence in human SKR2 mRNA was transcribed by T7 polymerase from an SKR2 cDNA template in the pBluescript SK vector. The probe was complementary to a 178-bp coding sequence which began in the SKR2 extracellular domain at an *Xmn* I site and extended through the middle of the transmembrane domain. The probe also contained 62 bp from the vector. For analysis of rat SKR2 mRNA, a rat SKR2 cDNA fragment spanning coding sequence for the SKR2 extracellular and transmembrane domains was generated by PCR with human primers P18 and P19 using rat prostate cDNA template. The resulting 469-bp cDNA fragment was subcloned into the *Eco*RI site of pBluescript SK and the sequence was determined. The cDNA was used to generate a 295-nt cRNA probe with T3 polymerase within which was a 201-nt rat sequence between *Xmn* I restriction sites complementary to SKR2 coding sequence extending from a point in the extracellular domain through the transmembrane domain. The probe also contained 20 nt of human sequence from primer P19 and 74 nt from the vector. Human and mouse β-actin RNA probes were used as positive controls for human and rat RPA, respectively. Yeast RNA was used as a negative control.

Poly(A)$^+$ RNA from Hep G2 cells was purified on oligo(dT)-cellulose (GIBCO/BRL) and used for both reverse transcription for PCR and RPA. The SKR2-1 mRNA, which encodes the full-length carboxyl terminus of SKR2, was detected by paired 5′ primer P9 (5′-AATGTCGACGTCATGATGCAGTCACTGA-3′) and 3′ primer P60 (5′-TAGGATCCACAGAGGGCTGGGCAGAAAC-3′). P9 was complementary to coding sequence within kinase subdomain VII. P60 was complementary to a 3′ noncoding sequence of SKR2-1 which was 85–105 bp downstream of the translation stop codon within exon 11. A 5′ primer, P28 (5′-CTCGAAGATGCAATTCTGGA-3′), which was complementary to sequence coding for subdomain IX was combined with an SKR2-2 isoform-specific 3′ primer, P30 (5′-TATGAATTCGTAGAGATGGGGGTCTCCCCACA-3′), or an SKR2-3 isoform-specific 3′ primer, P29 (5′-TATGAATTCTTAACAATGAAACAGCAGGGTT-3′), to detect mRNA encoding the SKR2-2 and SKR2-3 variants, respectively. P30 and P29 were complementary to nucleotide sequence just upstream of the poly(A) tracts of the SKR2-2 and SKR2-3 cDNAs, respectively.

DNA templates for making ^{32}P-labeled cRNA probes to analyze SKR2 variants by RPA were generated by PCR with 5′ primer P28 and SKR2-2-specific 3′ primer P34 (5′-CGCGTAATACGACTCACTATA*GGGCGAATTG*GCAGGAGGCCAGCTTCTTACCTCATA-3′) or SKR2-3-specific 3′ primer P35 (5′-CGCGTAATACGACTCACTATA*GGGCGAATTG*CACAGCTACCTCCACACCTTTGATG-3′). A T7 promoter sequence (underlined) and an unrelated 10 nt (italics) were incorporated upstream of the sequences com-

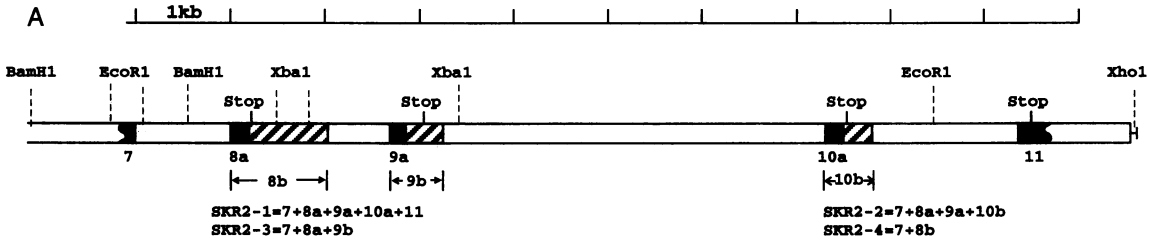
FIG. 1. Deduced sequences of human SKR2 variants. The amino acid sequences of SKR2-1, SKR2-2, and SKR2-3 were deduced from the nucleotide sequences described in the text. Residues conserved among human SKR2 (GenBank nos. L10125 and L10126), SKR1 (ref. 5; GenBank no. L02911), and TGFβRII (ref. 9; GenBank no. M85079) are shown in reverse print. Conserved cysteine residues in the extracellular domain (solid boxes), the transmembrane domain (boxed), intracellular Ser/Thr kinase subdomains (I–XI), conserved potential Ser/Thr (open circles) and tyrosine (solid circles) phosphorylation sites, and the sites where the sequence of SKR2-2 (open triangle) and SKR2-3 (closed triangle) and SKR2-4 (open diamond) diverge from SKR2-1 are indicated. The location and direction of nucleotide primers used in the study are also indicated.

plementary to SKR2 in the 3′ primers. A 182-nt cRNA probe was transcribed with T7 RNA polymerase from the PCR-derived template sequence between paired P28 and P34. The cRNA probe consisted of a 153-nt sequence common to SKR2-1 and SKR2-2 followed by 19 nt of SKR2-2-specific sequence plus the unrelated 10-nt sequence in the template. A 192-nt cRNA probe was transcribed with T7 polymerase from the PCR-derived template resulting with P28 and P35. The probe was composed of 22 nt of common SKR2 sequence followed by 160 nt of SKR2-3-specific sequence and the unrelated 10-nt sequence from the template. The unrelated 10-nt sequence was incorporated in both templates to distinguish protected RNA bands from the probe (see Fig. 4 *D* and *E*).

RESULTS

Identification and Characterization of the SKR2 cDNAs. Application of the PCR and sequence analysis of a 156-bp DNA fragment generated between degenerate primers complementary to conserved subdomains VIB and VIII of mouse ActRII and *Caenorhabditis elegans daf-1* genes revealed a distinct Ser/Thr kinase homologue, SKR2, of previously reported SKR1 (5) from Hep G2 cells (Fig. 1). Application of 5′-RACE-PCR using 3′ primer P10, which was complementary to coding sequence for kinase subdomain VII, yielded a 1061-bp cDNA beginning 7 bp upstream of the translational initiation site. cDNA clones derived from a Hep G2 cDNA phage library overlapped with and confirmed authenticity of the cDNA generated by 5′-RACE-PCR. The phage clones were used to deduce the carboxyl-terminal sequence of SKR2 variants downstream of kinase subdomain VIII. The amino acid sequence deduced from overlapping 871-bp and 947-bp cDNAs which exhibited poly(A) tails diverged beginning at the end of kinase subdomains IX (SKR2-3) and X (SKR2-2), respectively (Fig. 1). The 1795-bp SKR2-2 cDNA exhibited an open reading frame encoding 476 aa extending through kinase subdomain X followed by a 12-aa carboxyl-terminal tail. The SKR2-3 cDNA exhibited 1301 bp in common with SKR2-2 and a reading frame of 421 aa which extended through kinase subdomain IX followed by a 66-aa carboxyl-terminal tail (Fig. 1). Kinase subdomain XI and the carboxyl-terminal tail of the full-length SKR2 homolog (SKR2-1) (Figs. 1 and 2) were deduced from cloned SKR2 genomic DNAs containing exon 11 as described below. While this study was in progress and after the SKR2-2 and SKR2-3 sequences reported here were released by the GenBank data base (February 1993), He *et al.* (6) and ten Dijke *et al.* (7) reported the cloning of cDNAs for the rat and human SKR2-1 (ALK4) isoform which extends through kinase subdomain XI and the carboxyl-terminal tail.

Structure of the SKR2 Gene Coding for the Variant Carboxyl-Terminal Domains. The presence of significant poly(A) tracts in cDNAs coding for the SKR2-2 and SKR2-3 variants suggested that both may arise by alternative splicing and poly(A) addition. To determine whether this hypothesis was compatible with the structure of the SKR2 genome, genomic DNA fragments spanning the coding sequence for the variant SKR2 carboxyl-terminal domains were cloned from a library of human leukocyte genomic DNAs. Preliminary restriction and sequence (Fig. 2) analyses of two independent clones with inserts of 15 kb suggested that exon–intron boundaries were similar to those of the mouse ActRII gene (18), the only member of the Thr/Ser kinase receptor family for which the



B

EXON	(bp)	EXON 3'	INTRON	(bp)	EXON 5'
exon 7	(?)	CAAG gtgagtggactagcg...intron 7		(1026)...ccctgtttttttctctgccag	GGAAGC
		Q G			K P
exon 8a	(157)	AAACG gtaggagggcctggg...intron 8		(1471)...atgtttgttttgctattgcag	ATACAT
		K R			Y M
exon 9a	(127)	GGAG gtacctttctttttt...intron 9		(4555)...agatactttcttttctcccag	GAGTCC
		G G			V H
exon 10a	(121)	TATGAG gtaagaagctggcct...intron 10		(1930)...ggctcctgggtctctgcacag	GCACTG
		Y E			A L
exon 11	(>230)				

C

ALTERNATE EXON	(bp)	JUNCTION	POTENTIAL POLY(A) ADDITION SIGNAL	POLY(A) ADDITION SITE
8b	(>954)	AAACG / GTAGGA ...785 bp.....AATAAA		.....(?)
		K R *	(potential)	
9b	(532)	GGAG / GTACCT ...240 bp...TATAAACAAACAAA		...139 bp...TTGTTA(aaaaaa...)
		G G T F,		
10b	(471)	TATGAG / GTAAGA ...204 bp.....AAGAAA		.....128 bp...TCTACA(aaaaaa...)
		Y E V R		

FIG. 2. Genomic arrangement of coding sequences for the carboxyl-terminal domain of the SKR2 gene. (*A*) Schematic showing restriction enzyme sites and the exon (solid and hatched) and intron (unshaded) relationships. The exons and introns were numbered according to the system used for the ActRII gene (18). Alternative exons resulting in truncated variants are hatched. Stop codons for each variant are indicated. Exon combinations for four potential SKR2 variants are indicated. (*B*) Sequence at exon–intron junctions of exons coding for the full-length carboxyl terminus of SKR2-1. Exon and intron sequences are indicated in uppercase and lowercase letters, respectively. Lengths of exons and introns are indicated in base pairs. (*C*) Alternative terminal coding exons for truncated SKR2 variants. The length of alternative exons and between the indicated sequences is shown in base pairs. Poly(A) tails (parentheses) on the cloned cDNAs for SKR2-2 and SKR2-3 are indicated.

genomic structure has been reported. The 11-kb 3′ sequence covered five exons designated exons 7, 8a, 9a, 10a, and 11 (Fig. 2) which were counterparts of exons 7–11 of the ActRII gene. Exons 7, 8a, 9a, 10a, and 11 encode the full-length amino acid sequence of the SKR2-1 kinase, which includes subdomains VI-A, VI-B, VII, VIII, IX, X, XI, and the carboxyl-terminal tail. The SKR2-3 variant consisting of exons 7, 8a, and alternative exon 9b apparently arises by cleavage within intron 9 and poly(A) addition to alternative exon 9b in response to a signal within intron 9. A reading-frame shift beginning at the 3′ end of exon 9a followed by an in-frame downstream stop codon results in the unique 66-aa carboxyl terminus lacking kinase subdomains X and XI which characterizes the SKR2-3 isoform. Similarly, the SKR2-2 variant apparently arises by cleavage within intron 10 and poly(A) addition to alternative exon 10b. A reading-frame shift at the 3′ end of exon 10a and a subsequent stop codon results in the unique 12-aa carboxyl terminus of the SKR2-2 isoform which lacks kinase subdomain XI. Although it has not yet been confirmed by cDNA sequence or mRNA expression analysis, the sequence analysis of the SKR2 genomic DNA indicates the potential for generation of an additional variant tentatively designated SKR2-4 that arises by cleavage and RNA chain termination within intron 8 and poly(A) addition to resultant alternative exon 8b (Fig. 2). The predicted translation product is a variant consisting of an intracellular domain of only intact kinase subdomains I–VII as a consequence of the presence of an in-frame stop codon in resultant alternative exon 8b, just past the 3′ end of exon 8a (Fig. 2*C*). Potential alternative exon 8b exhibits a perfect consensus poly(A) addition signal 800 bp past the 3′ end of exon 8a.

Expression of SKR2. Nuclease protection analysis of total RNA with a probe containing sequence complementary to common sequences in all SKR2 isoforms revealed that the SKR2 mRNA was widely expressed in cells and tissues from human (Fig. 3*A*) and rat (Fig. 3*B*).

Expression of the SKR2-1, SKR2-2, and SKR2-3 variants in Hep G2 cells was confirmed by analysis of purified mRNA preparations [poly(A)$^+$ RNA] with isoform-specific primers in the PCR and RPA with variant-specific cRNA probes. Primer pair P9/P60 revealed the 565-bp product indicative of the presence of the SKR2-1 mRNA (Fig. 4*A*). Primer pairs P28/P30 and P28/P29 revealed the expected 507-bp SKR2-2 and 434-bp SKR2-3 bands which specifically hybridized with ^{32}P-labeled SKR2-2 and SKR2-3 cDNAs, respectively (Fig. 4 *B* and *C*). RPA further confirmed the expression of the SKR2 isoforms in Hep G2 cells (Fig. 4 *D* and *E*). Protection of 153- and 172-nt fragments within a 182-nt cRNA probe designed to detect SKR2-1 and SKR2-2 isoforms confirmed the presence of both transcripts in mRNA preparations from Hep G2 cells (Fig. 4*D*). Expression of the transcript for the SKR2-3 variant was confirmed by protection of the expected (182-nt sequence) within a 192-nt cRNA probe designed to detect specifically the SKR2-3 variant mRNA (Fig. 4*E*). The results suggest that SKR2-1, -2-2, and -2-3 are expressed in Hep G2 cells from which their cDNAs were identified by PCR and conventional cloning. The amount of SKR2-1 mRNA was highest and that of SKR2-2 mRNA was lowest in Hep G2 cells. The SKR2-1 and SKR2-2, but not SKR2-3, mRNAs have also been detected in human umbilical vein endothelial cells (data not shown).

DISCUSSION

Here we show that the genomic structure coding for the carboxyl-terminal intracellular domain of a widely expressed type I SKR, SKR2, is similar to that reported for the type II kinase receptor ActRII gene (18). Alternative splicing and cleavage and poly(A) addition within introns at the 3′ end of

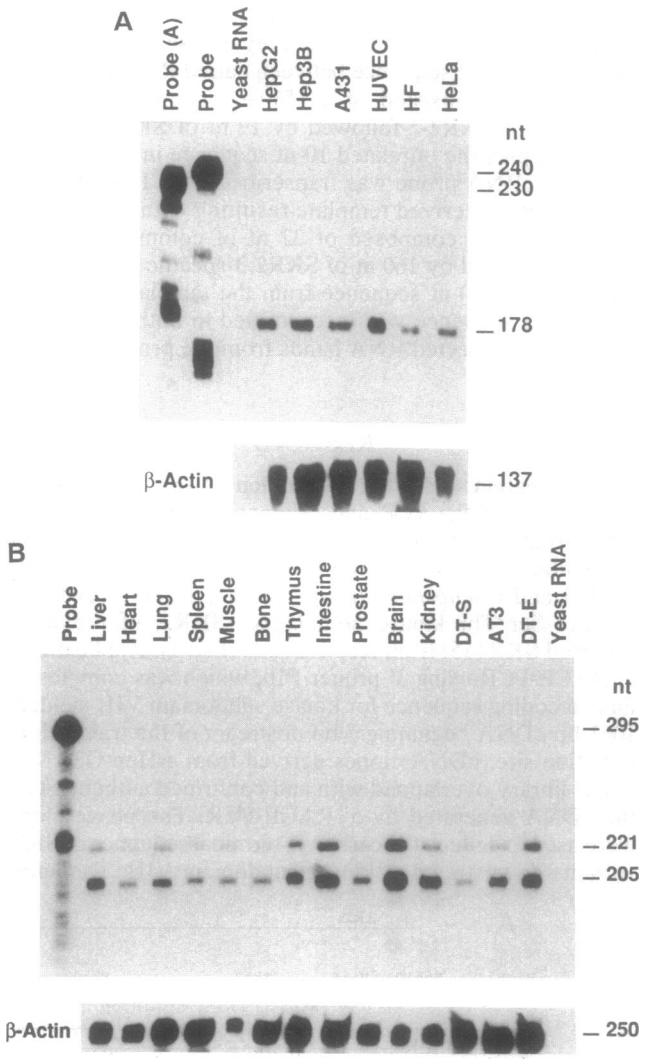


FIG. 3. Expression of SKR2 mRNA. (*A*) Human cells. Total RNA (20 μg) from the indicated cells was analyzed by the RNase protection method. "Probe (A)" and "probe" indicate the labeled β-actin (230-nt) and SKR2 (240-nt) cRNA probes, respectively, in the absence of RNA. Probe (A) contained 137 nt of the human β-actin sequence. Hep G2 and Hep 3B, well-differentiated hepatocyte-like hepatoma cells; A431, cervical carcinoma epithelial cells; HUVEC, human umbilical vein endothelial cells; HF, human fibroblasts (Flow 2000) derived from lung; HeLa, cervical carcinoma epithelial cells. (*B*) Rat tissues and rat prostate tumor cells. RNA from the indicated tissues and cells were analyzed with a 295-nt cRNA probe. The probe contained 20 nt of human SKR2 sequence in addition to the 201-nt rat SKR2 sequence (*Materials and Methods*), which resulted in variable levels of protected 221-nt band due to mismatch between the human and rat sequences. RNA loads were normalized with a 300-nt mouse β-actin cRNA probe which crossreacts with rat β-actin mRNA to protect a 250-nt fragment.

the last two coding exons (exons 9a and 10a) for the full-length carboxyl terminus of SKR2 results in variants predicted to be devoid of kinase subdomains XI (SKR2-2) and X and XI (SKR2-3), respectively. Although expression of an mRNA has not been confirmed, the presence of a perfect poly(A) addition signal within intron 8 downstream of exon 8a suggests that an SKR2 mRNA may arise similar to that reported for the ActRII gene, which lacks the coding sequence for kinase subdomains VIII–XI (17).

Tyrosine kinase receptors are composed of homodimers in which one or more tyrosines within a monomer are obligatorily substrates of the adjacent kinase within the homodimer (10–14). In the FGFR tyrosine kinase family, kinase- and

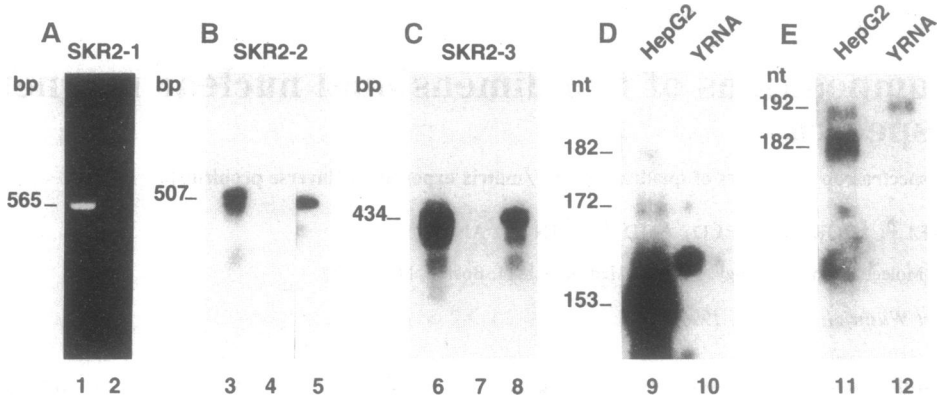


FIG. 4. Expression of SKR2 variants in Hep G2 cells. (*A–C*) Expression of the indicated variants analyzed by PCR with isoform-specific primer pairs (Fig. 1 and *Materials and Methods*) P9/P60 (*A*), P28/P30 (*B*), and P28/P29 (*C*). Lanes 1, 5, and 8, cDNA template prepared by reverse transcription of poly(A)$^+$ RNA; lanes 3 and 6, positive controls (cloned SKR2-2 and SKR2-3 cDNA templates, respectively); lanes 2, 4, and 7, negative controls (no reverse transcription product). *A* is an agarose gel stained with ethidium bromide. *B* and *C* are autoradiographs of Southern blots hybridized with ^{32}P-labeled SKR2-2 and SKR2-3 cDNAs, respectively. (*D* and *E*) RPA of SKR2 variants. Lane 9, a 182-nt cRNA probe containing 153 nt complementary to sequences common to SKR2-1 and SKR2-2 and 19 nt complementary to the unique SKR2-2 sequence; lane 11, a 192-nt cRNA probe consisting of 22 nt complementary to common SKR2 sequence and 160 nt complementary to specific SKR2-3 sequence; lanes 10 and 12, yeast RNA (YRNA) instead of poly(A)$^+$ RNA (5 μg). Results in *D* and *E* were obtained with the same mRNA preparation hybridized with probes of similar specific activity and are from the same film exposed for the same period.

phosphotyrosine site-defective monomers that arise by alternative splicing depress signal transduction by dimerization with monomers with intact kinase activity and phosphotyrosine sites (11, 14–16). In contrast, receptors for the TGFβ family are composed of heterodimers of two genetically distinct Ser/Thr kinases (types II and I) both of which are obligatory for biological activity (1–4). The type II component is specific for individual members of the TGFβ ligand family, whereas some type I components are shared among multiple members of the ligand family (refs. 1–4, 8, and 9; unpublished results). Our results, together with those reported for the ActRII gene (17), suggest that monomers with four variant intracellular domains of both type I and specific type II components potentially add to the heterogeneity of the heterodimeric receptor complex for a single member of the TGFβ ligand family. Expression of artificial constructions of the TGFβRII and ActRII receptors which are devoid of the complete intracellular kinase domain appear to depress ligand-induced activities in *Xenopus* embryos (20) or transfected cells (21, 22). However, the truncated isoform of the ActRII receptor exhibiting kinase domains I–VII which arises as a result of alternative splicing of intron 8 and poly(A) addition exhibits a positive activity that exceeds that of intact ActRII in *Xenopus* embryos (17). Truncated forms of ActRII and SKR2 (SKR2-2 and SKR2-3) are devoid of domains demonstrated to be essential in other kinases and are unlikely to exhibit full kinase activity. What then might be the function of sequentially truncated kinase-defective monomeric receptor isoforms? The intrinsic kinase activity of epidermal growth factor receptor kinase monomers appears to be unessential for activation of the MAP-2 kinase pathway, provided that tyrosine sites essential for binding of substrates which initiate the pathway are phosphorylated by a receptor monomer with intact kinase activity (23). Truncated kinase-inactive type I and type II monomeric receptors for the TGFβ ligands may play a role as asymmetric substrates of an active kinase monomer within type II–type I heterodimers. Truncated monomers predicted from cDNA differ by potential serine, threonine, or tyrosine phosphorylation sites around which different substrates may assemble (Fig. 1).

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