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Identification and Characterization of a Bidirectional Promoter from the Intergenic Region between the Human DDX13 and RD Genes

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The human DDX13 gene encodes a putative RNA helicase of the DExH-box family. In an earlier report we showed that the human DDX13 and RD genes were arranged head-to-head in the class III MHC complex and their ATG start codons were separated by 745 base pairs. We have now analyzed the common 745 bp intergenic region in detail and characterized their promoters. Northern blot analysis revealed that DDX13 and RD exhibit distinct patterns of steady-state expression among multiple human tissues. The promoter regions for DDX13 and RD genes were identified by deletion analysis from 740 bp to 176 bp of the intergenic region fused to a chloramphenicol acetyltransferase (CAT) reporter gene using transient transfection assays. Results indicated that a promoter sequence as small as 176 bp is sufficient for basal expression of both genes in HeLa and HepG2 cells. Functional analysis using a bidirectional reporter system demonstrates that the sequence 262 bp proximal to the DDX13 gene is sufficient for concurrent expression in both directions. However, the common 740 bp intergenic region showed promoter activity in DDX13 only, suggesting the presence of a negatively acting region for the RD gene within the region −267 to −744. It appears that RD expression is controlled by a complex system of positively and negatively acting elements present on distant portions of both genes.

Keywords: Bidirectional Promoter; DDX13; Intergenic Region; RD.

Introduction

The DDX13 translation product of 1,245 amino acid is a human homologue of yeast SKI2 and belongs to the DEAD/DExH protein family of putative helicases (Dangel *et al*., 1995; Lee *et al*., 1995). The members of DEAD/DExH box protein family share seven highly conserved helicase motifs including the characteristic Asp-Glu-Ala-Asp (DEAD)/Asp-Glu-x-His (DExH) motif. They are implicated in a variety of cellular processes including ribosome assembly, RNA splicing, translation initiation, RNA decay, and development of cell growth (Carpousis *et al*., 1999; Gorbalenya *et al*., 1989; for a review, see Wassarman and Steitz, 1991). Both yeast and mammals have large families of DEAD/DExH-box proteins, many of unknown function. The yeast Ski2p appears to be involved in the antiviral response by blocking the expression of viral mRNA through its role in the degradation of mRNA (Anderson and Parker, 1998; Masison *et al*., 1995; Widner and Wickner, 1993). The human DDX13 protein has been shown by immunofluorescence studies to be localized to the nucleoli and the cytoplasm (Qu *et al*., 1998). However, its cellular function has not been determined.

DDX13 was localized in the class III region of the human MHC between the genes coding for two other nuclear proteins, RD and RP1 (Albertella *et al*., 1996; Dangel *et al*., 1995; Lee *et al*., 1995). Recently, another new gene, DOM3Z, was discovered between DDX13 and RP1 (Yang *et al*., 1998). RD-DDX13 and DOM3Z-RP1 are arranged as two head-to-head oriented gene pairs and share the common features of being ubiquitously expressed. The

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Abbreviations: β-Gal, β-galactosidase; CAT, chloramphenicol acetyltransferase; DEAD/DExH, conserved aa sequence motif; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MHC, major histocompatibility complex; PCR, polymerase chain reaction; UTR, untranslated region; x, any aa.

translation start codons of DDX13 and RD are separated by 745 bp, and their first exons overlapped. The biochemical properties or functions of the RD genes are not known (Cheng *et al*.,1993; Surowy *et al*., 1990), however its deduced amino acid suggests that it is a nuclear RNA-binding protein. Furthermore, a leucine zipper motif and a RNA binding motif were present in both the DDX13 and RD proteins, suggesting their possible role in regulation of RNA function. Whether the DDX13 and RD genes are involved in related cellular processes awaits further investigation. However, the close proximity of the DDX13 and RD genes in reverse orientation suggested that these two housekeeping genes might be coordinately regulated by mechanisms such as the sharing of regulatory elements, antisense regulation, and/or promoter occlusion. The 745 bp intergenic region appeared to represent housekeeping gene promoters as indicated by a high GC content, the existence of typical sequence motifs including SP-1 and AP-2 consensus sequence, and the lack of TATA and CCAAT boxes (Lee and Song, 1997). To delineate the mechanisms of expression and regulation of RD and DDX13, we have focused on a functional analysis of the common 745 bp intergenic region.

Materials and Methods

Primer extension analysis To prepare Poly A^+ RNAs from the human cell line EJ and HeLa, the Oligotex Direct mRNA Mini kit (Qiagen, USA) was used according to the manufacturer's instructions. Primer extension was performed essentially as described previously (Lee and Song, 1997) with a 25 mer oligonucleotide (NC42, 5′-CAGAGCCTCCTCTTCCTC GCTCAGT-3′) complementary to +21 to +45 nucleotides downstream of the translation start site of the human RD. The product was analyzed on a 6% polyacrylamide/7 M urea gel along with appropriate dideoxy sequencing samples as size markers.

Northern blot analysis Northern blot analysis was carried out with Human Multiple Tissue Northern (MTN) blots (MTN blots I and II; Clontech, La Jolla CA) according to the manufacturer's instructions. For the expression of DDX13, a PCR product of 140 bp was obtained with primers derived from the nucleotide sequences between 91 to 108 (sense primer; 5′-CTGCTGAACTTGCCTGGA-3′) and 206 to 230 (antisense primer; 5′-GCTGAGTGCTCCACACCATGCAGAG-3′) relative to its translation initiation codon. For RD, the probe was obtained by cloning the RT-PCR product of 144 bp with primers derived from the nucleotide sequences between 830 and 849 (sense primer; 5′-GTCGGATTCATTCCCTGAAC-3′), 954 and 973 (antisense primer; 5′-TTTCTGGGTGGGTCCATGGA-3′) from the cDNA sequence published by Cheng *et al*. (1993). Hybridization with a labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed to compare RNA loading.

Generation of a construct with two reporter genes All the plasmids were constructed using standard recombinant DNA techniques. Sequence-position numbering in the DDX13 gene is relative to its translation initiation codon (Lee and Song, 1997). Vectors pCAT-Enhancer [containing the simian virus 40 (SV40) enhancer], pCAT-Control (containing SV40 promoter and enhancer), and pSV-β-Galactosidase were purchased from Promega. pCAT-Enhancer contains a chloramphenicol acetyltransferase (CAT) reporter gene located immediately downstream from a polylinker for analysis of promoter activity of cloned fragments. The pCAT-Control plasmid was used as a positive control. For monitoring transfection efficiency pSV-β-Galactosidase was used as a control vector. To construct a plasmid with two reporter genes, the coding region of β-Gal gene was PCR amplified using two 30-mer oligonucleotides. The sense primer had *Sal*I linker (5′-TCGACGTCGACTTGGGATC-TCTATAATCTC-3′) and the antisense primer had *Hin*dIII linker (5′-AGCTTAAGCTTCTAGAGGATCCAGACATGA-3′). The amplified product of 3,766 bp was cloned into *Sal*I-*Hin*dIII linearized pCAT-Enhancer (pCeG).

Reporter gene constructs for DDX13 A 740 bp *Bst*XI fragment between the ATG codons of RD and DDX13 was isolated from a gel, blunted with T4 DNA polymerase, and ligated with a pCAT-Enhancer and pCeG that was digested with *Sal*I and blunted with the Klenow fragment of DNA polymerase I. The orientation of the inserted fragment was confirmed by restriction enzyme cleavage patterns. The 740 bp DDX13/RD intergenic region was inserted in both orientations (CAT in the DDX13 direction; peD740 or pCeG-D740, CAT in the RD direction; peR740 or pCeG-R740).

Deletion constructs are schematically depicted in Fig. 3. Integrity and orientation of the insert were confirmed by sequencing both strands. Construct names reflect the portion of intergenic region retained in the construct and are numbered relative to the DDX13 ATG codon. To construct peD508 and peD419, peD740 was digested by *Kpn*I + *Pst*I, *Sac*II + *Pst*I, respectively, made blunt with T4 DNA polymerase, then self-ligated and transformed. To construct peD482 and peD262, peD740 was linearized by *Xma*III + *Xba*I, *Xma*III + *Hin*dIII, respectively, and the 5′ overhangs were filled in with the Klenow fragment followed by self-ligation and transformation. To construct peD176, peD740 was linearized by *Bss*HII + *Hin*dIII, and the 5′ overhangs were filled in with the Klenow fragment followed by self-ligation and transformation. peD350 was generated by digesting peD740 with *Ava*I followed by self-ligation. To construct peD247, peD419 was digested with *Bss*HII + *Xba*I, blunted with the Klenow fragment followed by self-ligation. To construct peD319, peD740 was digested with *Sac*II + *Sal*I, blunted with T4 DNA polymerase and the Klenow fragment followed by self-ligation.

Reporter gene constructs for RD To construct peR628 and peR362, a *Pst*I fragment of 1,154 bp, between 43 bp downstream from the RD ATG codon and 162 bp of intron 2 of DDX13, was cloned into *Pst*I linearized pCAT-Enhancer vector in the RD direction (peR1154). Then, peR628 was generated by digesting peR1154 with *Xma*III + *Xba*I, blunted with the Klenow fragment followed by self-ligation. peR362 was generated by digesting peR1154 with *Bst*XI + *Sal*I, blunted with T4 DNA polymerase and the Klenow fragment followed by self-ligation. To construct peR262, peR740 was digested with *Xma*III + *Xba*I, blunted with the Klenow fragment followed by self-ligation. To construct

peR176, peR740 was linearized by *Bss*HII + *Xba*I, and the 5′ overhangs were filled in with the Klenow fragment followed by self-ligation. To construct pCeG-R262, β-Gal gene (*Hin*dIII − *Sal*I fragment of pCeG) was cloned into *Hin*dIII + *Sal*I linearized peR262.

For construction and transfection experiments, plasmid DNA was isolated using standard procedures including two cycles of purification by centrifugation in CsCl gradients.

Transient transfection of HeLa and HepG2 cells HeLa or HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin and 100 units/ml streptomycin at 37°C in 5% CO_2.

All transfections were performed with 1 μg of the reporter plasmid and 1 μg of pSV-β-Galactosidase plasmid using lipofectAMINE^{TM} reagent (GibcoBRL) according to the manufacturer's instruction. To minimize sample variability, duplicate DNA precipitates were mixed and half was then added to each plate. Briefly, cells were plated in 35 mm dishes at a density of 2.5×10^5 cells per dish 24 h prior to transfection. One μg of the reporter plasmid +1 μg of pSV-β-Galactosidase plasmid and lipofectAMINE (5 μl per each plate) were each diluted in 100 μl of serum- and antibiotic-free DMEM in polypropylene tubes. The solutions were combined and incubated for 45 min at room temperature. Then 800 μl of serum- and antibiotic-free DMEM was added and the solution was carefully dropped onto the cells. At 48 to 72 h post transfection, the cell lysates were analyzed for both β-Gal and CAT activities using assay kits from Promega. After normalization to the β-Gal control, the transactivation activity of each test construct was calculated relative to the pCAT-Control vector, the activity of which was arbitrarily defined as 1. Each construct was tested in 3–6 different transfection experiments and each experiment was performed in duplicate.

Results and Discussion

Identification of the transcription start sites for RD We have shown in previous works that transcription of DDX13 is initiated at multiple start sites (Lee and Song, 1997). To define the regulatory regions of the two head-to-head arranged genes, we further mapped the transcription start sites for the RD gene by primer extension analysis using an oligonucleotide derived from nucleotides +21 to +45 as primer and RNAs from either HeLa or EJ cells as templates. As shown in Fig. 1A, several start sites, 120, 162, 208, 225, and 273 bp upstream from the DDX13 ATG start codon, were found in HeLa cells. Similar start sites were also present in EJ cells (data not shown). The 5′ cap site at nt 225, which was approximately 86 bp upstream from the RD ATG start codon, gave the strongest signal. As was expected from the absence of identifiable TATA boxes at either end, both DDX13 and RD transcripts were initiated from multiple transcription start sites. Depending on the transcription start sites being used, the 5′ UTR of the DDX13 and RD transcripts would be complementary (Fig. 1B).

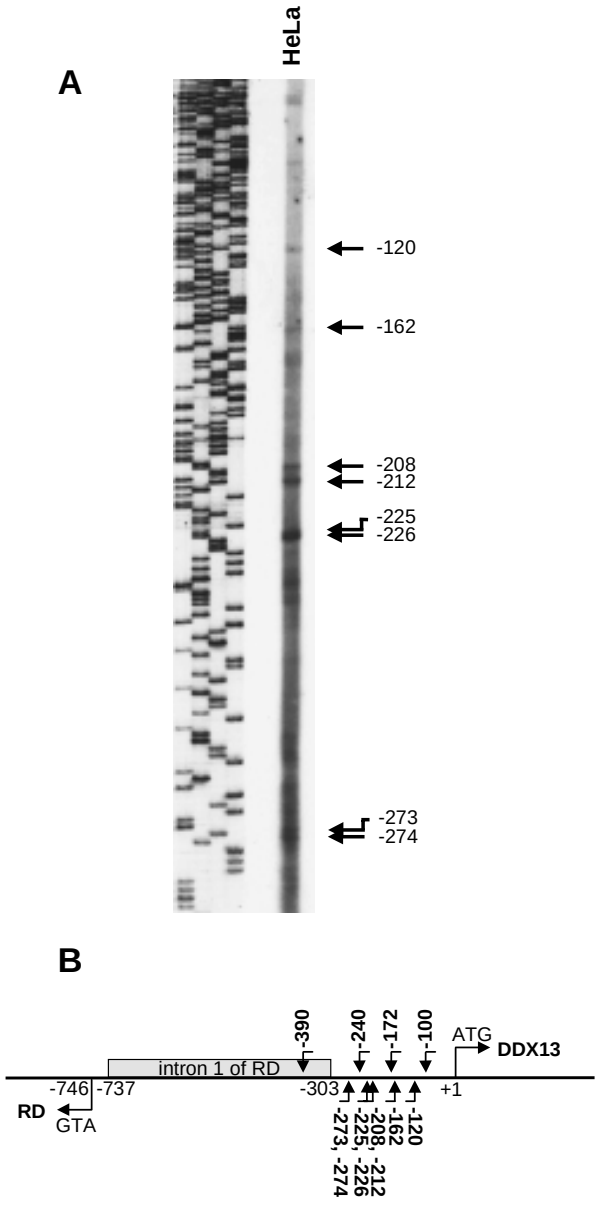


Fig. 1. A. Primer extension analysis to determine the transcription start sites for the RD gene. Lane 1, HeLa cell poly A^+ mRNAs primer extended from a RD specific primer (complementary to nt +45 to +21). Prominent bands consistent in all experiments are indicated by arrows, and the position is relative to the DDX13 ATG codon. Absolute product lengths were obtained by comparison to a sequencing ladder. Position within the promoter was assigned by use of a value obtained by subtracting the size of the primer size from the absolute product length. **B.** Schematic representation of the transcription start sites for the RD and DDX13 genes (14).

DDX13 and RD mRNA transcripts in human tissues Both DDX13 and RD were expressed in various cell lines (Dangel *et al*., 1995; Lee *et al*., 1995). We next examined the expression level of DDX13 and RD in specific cell types by Northern blot analysis using human

Multiple Tissue Northern (MTN) blots as described in Materials and Methods. As shown in Fig. 2, the tissue distribution patterns for DDX13 and RD transcripts varied depending on tissue after correcting for loading differences, suggesting tissue specific bidirectional transcription control of RD and DDX13. It appeared that most tissues produced more RD transcripts. The expression level for RD was especially high in the testis.

Promoter activity of the common 740 bp intergenic region So far, several human genes were found to be present in a head-to-head configuration. The examples include the α1 and α2 chains of type IV collagen gene (Poschl *et al*., 1988), WIT-1 and WIT-2 of the Wilms tumour locus (Campbell *et al*., 1994), the Surf1 and Surf2 genes of the Surfeit locus (Lennard *et al*.,1994), the histidyl-tRNA synthetase (HRS) and unknown cDNA HO3 (Tsui *et al*., 1993), dihydrofolate reductase and mismatch repair protein 1 (Shinya and Shimada, 1994), the human transporter associated with antigen processing 1 (TAP1) and low molecular mass polypeptide 2 (LMP2) genes (Wright *et al*., 1995), the genes for ataxia telangiectasia ATM and unknown cDNA E14 (Byrd *et al*., 1996), the NBR2 and BRCA1 genes (Xu *et al*., 1997), the translational inhibitor p14.5 gene and the gene for the human RNase P and RNase MRP protein subunit hPOP1 (Schmiedeknecht *et al*., 1997), and the theta-class glutathione S-transferase 2 (GSTT2) gene and the D-dopachrome tautomerase (DDCT) gene (Coggan *et al*., 1998). From all of these genes except the type IV collagen and GSTT2/DDCT genes, a bidirectional promoter with significant activity was identified in the intergenic region. A bidirectional promoter of the collagen type IV genes is controlled by a complex system of positively and negatively acting elements present on distant portions of both genes (Poschl *et al*., 1988).

The head-to-head configuration of DDX13 and RD in close proximity suggests that both genes are transcribed divergently from a bidirectional promoter. Since all the transcription start sites for the DDX13 and RD genes lie in the common 745 bp intergenic region, this region could serve as a bidirectional promoter. Previous sequence analysis of the intergenic region revealed a GC-rich promoter with several consensus sequences for transcription factors including SP-1 and TCF-1 in both directions (Lee and Song, 1997).

A promoter activity of the intergenic region was assayed by cloning the 740 bp *Bst*XI fragment into the pCAT-

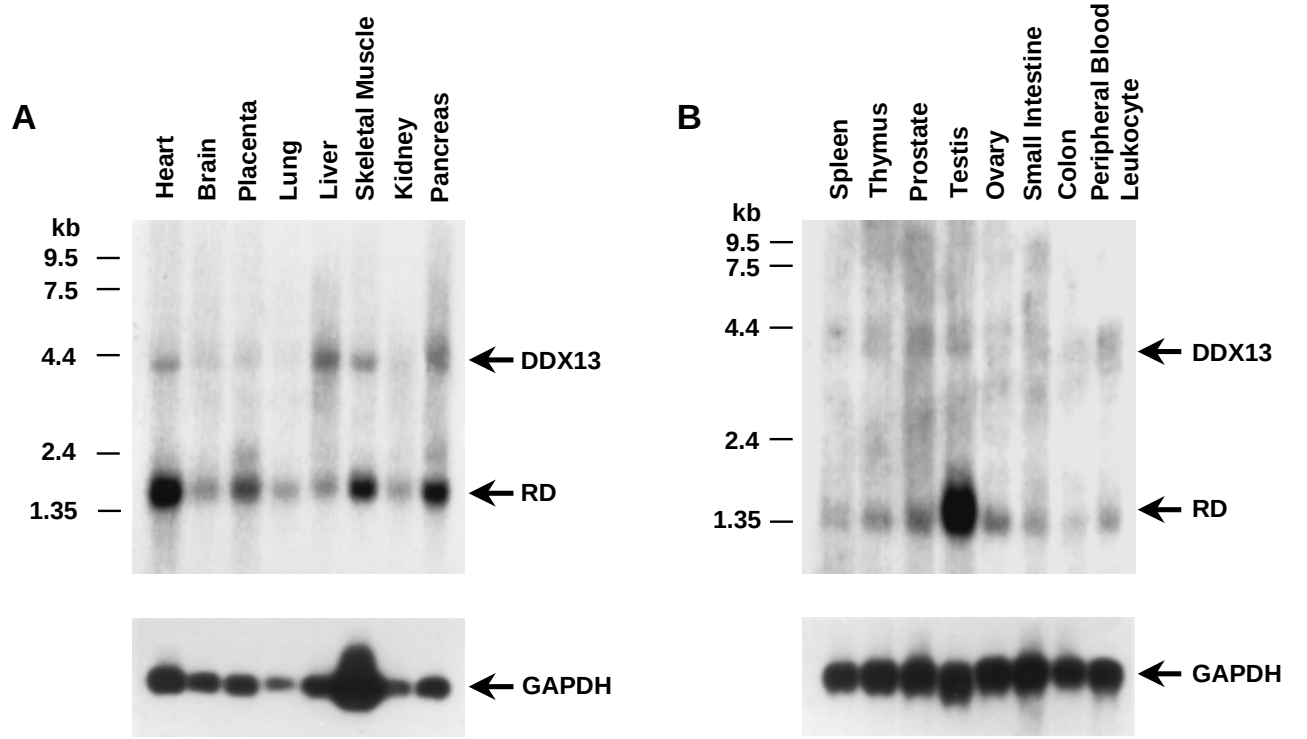


Fig. 2. Human tissue survey of DDX13 and RD mRNA expression. Messenger RNA blots representing multiple human tissues were hybridized with cDNA probes specific for DDX13 (a 140 bp fragment) and RD (a 144 bp fragment) as described in Materials and Methods. Although relative DDX13 and RD mRNA levels varied considerably among tissues, each was clearly present in all tissues as demonstrated by longer film exposures. The positions of RNA size markers (diagrammed at the left of each panel) were used to estimate the sizes of the DDX13 (4 kb) and RD (1.5 kb) transcripts.

Enhancer vector in either orientations. The resulting constructs were analyzed by transient transfection experiments in HeLa and HepG2 cells. In both cells, the 740 bp intergenic region showed significant promoter activity in the DDX13 direction only (Fig. 3). Compared to the SV40 promoter, the 740 bp fragments supported the reporter gene activity at the level of 178% in HeLa cells and 376% in HepG2 cells when cloned in the DDX13 orientation (peD740), but 27–28% when cloned in the RD orientation (peR740). This was rather surprising since our MTN Northern blot analysis revealed higher level of steady-state mRNA expression of RD than DDX13 in most tissues (Fig. 2). Thus, we focused on defining promoter regions for DDX13 and RD, respectively.

Functional analysis of the human DDX13 and RD promoters To define the promoter for each gene functionally, a series of deletion constructs were generated and analyzed by transient transfection experiments in HeLa and HepG2 cells (Fig. 3). All deletion points are presented relative to the DDX13 ATG codon. As shown in Fig. 3, maximal DDX13 promoter activity was observed with the construct containing sequences between −5 and −266 (deletion mutants, peD262) in HepG2 cells. In both cell types, other constructs containing the −5 to −266 region, peD740, peD508, peD419, and peD350 showed an over 2-fold increase in promoter activity relative to the SV40 promoter, whereas the ones without this region, peD482 and peD319, had essentially no promoter activity. Construct peD176 (between −5 and −180) produced relatively high activity in both cell types, albeit lower than the CAT activities displayed by transfection of peD262. Inspection of the sequences of the 176 and 262 bp regions revealed the presence of additional binding sites for the transcription factors AP-2 (nt −180) and GATA-1 (nt −242) in the latter (Lee and Song, 1997). The 176 bp region contains three binding sites for SP-1 and one for AP-2. It is not clear whether the presence of additional AP-2 binding site causes the differences of promoter activities between peD176 and peD262. These results suggested that the 176 bp region could be the minimal promoter for DDX13.

Transfection of peR740 showed essentially no promoter activity, indicating the necessity of additional elements for the expression of RD. However, transfection of the constructs peR628 and peR262 showed strong promoter activity. Since no activity was detected with peR362, it appeared that the RD promoter was located in the same 262 bp fragment that also displayed maximal promoter activity in the DDX13 direction. Since its promoter activity is potent in both directions (peD262 versus peR262), the 262 bp region appears to have a critical role in activating distal RD as well as the proximal DDX13 gene. Furthermore, the 176 bp region in the RD direction also produced moderately high activity in HeLa cells, suggesting that this region could be the minimal promoter

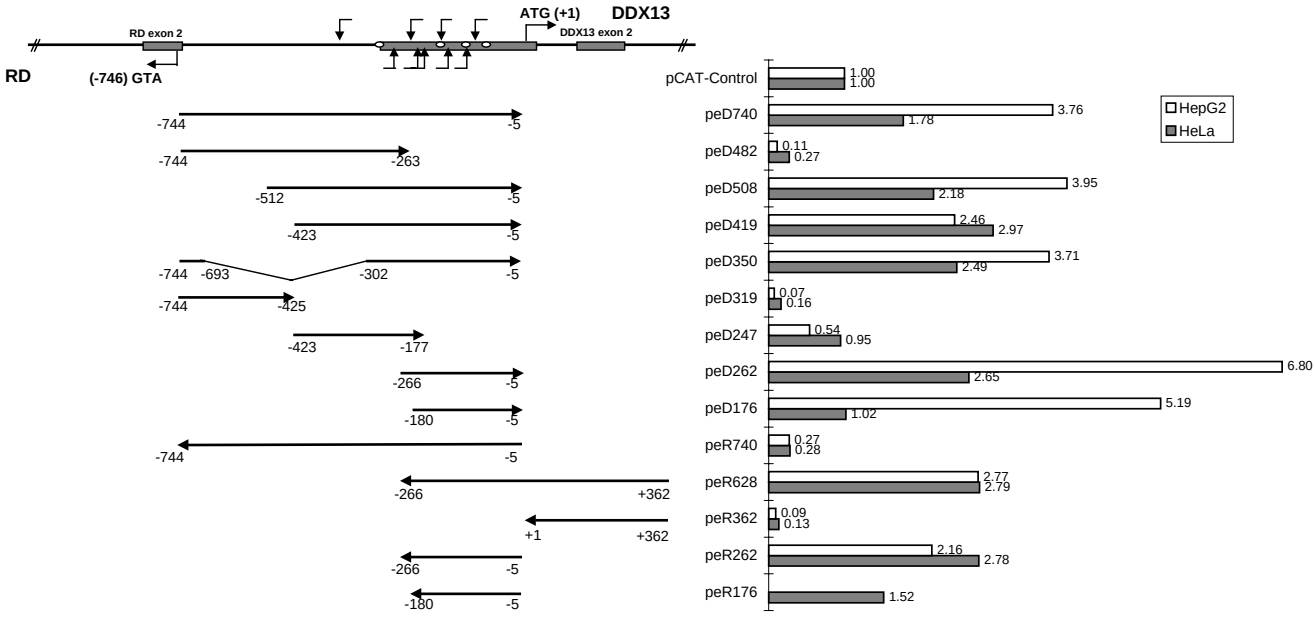
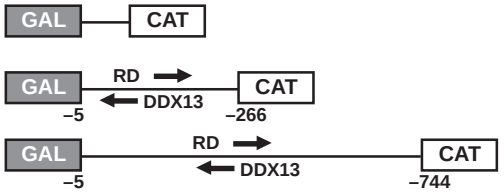


Fig. 3. Transient expression assays of chimeric CAT genes containing different segments of the intergenic region. Open circles indicate SP-1 binding sites; the transcription start sites are marked by the vertical arrows. On the left are schematics of the vector and reporter constructs used, indicating the orientations of the promoter fragments tested. The constructs were assayed in HeLa (gray bar) and HepG2 (white bar) cells as described in Materials and Methods. CAT activity for each construct was normalized for β-Gal activity. The relative CAT activity was calculated after defining the activity of pCAT-Control as 100%. Each value represents the average of at least five independent transfections.



	GAL activity (O.D.$_{420}$)	CAT activity (10^4 cpm)
pCeG	0.029 ± 0.027	0.103 ± 0.066
pCeG-R262	1.231 ± 0.171	1.247 ± 0.192
pCeG-R740	1.083 ± 0.208	0.089 ± 0.029

Fig. 4. The 262 bp fragment proximal to the DDX13 gene functions bidirectionally *in vivo*. Constructs (pCeG-R740 and pCeG-R262) used to assay promoter function in HeLa cells are illustrated schematically. The values are mean ± S.D. from three independent transfections.

for both DDX13 and RD. This result also suggested that the sequences from 2263 to 2744 might contain a silencer negatively regulating the expression of RD gene, which may explain the failure to detect promoter activity for peR740.

Identification of a bidirectional regulatory element The deletion studies suggested that a region of 262 bp encompassing the transcription start sites constitute a bidirectional promoter involved in coordinated regulation of DDX13 and RD expression. To confirm these results, the 262 bp region was cloned between two divergently oriented reporter genes, β-Gal and CAT. The resulting construct when transfected into cells allows the simultaneous measurement of transcriptional activity in both directions. Transfection of HeLa cells with the bidirectional reporter gene construct pCeG-R262 resulted in significant transcription in both the DDX13 and the RD direction compared with the promoterless control (pCeG) or pCeG-R740 (Fig. 4). Transfection with a bidirectional reporter construct containing the 740 bp intergenic region in both orientations, pCeG-R740, resulted in significant transcription in the DDX13 direction only (Fig. 4). A similar result was obtained with pCeG-D740 in both HeLa and HepG2 cells (data not shown). The relative promoter activity of the 262 bp in the RD direction was over 10-fold higher than that of the 740 bp intergenic region in the RD direction. These results indicated that the DDX13 and RD genes shared a common 262 bp promoter region, which was sufficient to promote bidirectional transcription of both genes.

Our MTN Northern blot data suggested that bidirectional transcription of DDX13 and RD might be regulated in a tissue-specific manner, and also revealed higher levels of steady-state mRNA expression of RD than DDX13 in most tissues. However, transient transfection experiments with HeLa, HepG2, and COS (data not shown) cells revealed a promoter activity of the 740 bp intergenic region in the DDX13 direction only. By Northern blot analysis, the expression levels of the endogenous DDX13 and RD in HeLa, HepG2, and COS cells were high (data not shown), thus it appeared that the reporter construct peR740 was not faithfully reproducing as the endogenous RD gene. Since the 262 bp DDX13 proximal region was also essential for RD transcription, it could be due to the presence of a silencer negatively regulating the expression of RD gene in the absence of additional elements outside the 740 bp intergenic region. The presence of a silencer showing a negative effect on transcription needs further analysis. It also remains to be seen whether this silencer has any effect on the expression of the endogenous genes in various tissues.

The 1.1 Mb class III region of the human MHC is known to be a gene dense area and to contain over 50 genes with relatively little intergenic DNA (Albertella *et al*., 1996; Campbell and Trowsdale, 1993). In addition to the DDX13 and RD genes in this region, the genes for the extracellular matrix protein tenascin-X, XA and XB overlap the 3′ ends of the adrenal steroid 21-hydroxylase genes CYP21A and CYP21B, respectively (Tee *et al*., 1995).

The juxtaposed DDX13 and RD genes provide us with an exceptional model to study the mechanisms of differential transcription control of two housekeeping genes. It has been suggested that genes sharing a bidirectional promoter might be involved in the same biochemical pathway, encoded interacting gene products, or unrelated by homology or function (Byrd *et al*., 1996; Poschl *et al*., 1988; Wright *et al*., 1995). At the moment, biological functions of DDX13 and RD are not known, thus these possibilities remain to be confirmed.

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