In vivo study of *Drosophila*-raf protein-protein interactions by yeast two-hybrid analysis

by

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ABSTRACT

Proper development of the *Drosophila* embryo requires four developmental systems: dorsal-ventral, anterior, posterior, and terminal class systems. The terminal class system also known as the Torso signal transduction pathway controls the formation of the anterior (acron) and posterior (telson) structures of the *Drosophila* embryo during early development. *Drosophila*-raf is a serine/threonine protein kinase, homologous to mammalian Raf-1, and has been shown to be an essential component for proper function of the Torso pathway. Although previous experiments had demonstrated the importance of D-raf in the Torso pathway, the mechanism for D-raf activation and regulation remain unclear. To investigate D-raf activation and regulation we used the yeast two-hybrid system as an *in vivo* model to study D-raf/D-raf interaction as well as D-raf interaction with D-Ras and 14-3-3ζ. A series of fusion proteins containing full-length D-raf or conserved regulatory regions of D-raf were created that contain the binding or activation domain of the yeast GAL4 protein. Different combinations of fusion constructs were introduced into yeast and the ability of these fusion proteins to interact was determined by yeast two-hybrid analysis. We were not able to detect D-raf/D-raf interaction, but we did observe interaction between D-raf and the two signaling molecules D-Ras and 14-3-3ζ. Our data also indicates that the N-terminus of D-raf acts as a regulatory module to block the ability of D-Ras and 14-3-3ζ to interact with D-raf.

INTRODUCTION

D-raf is a *Drosophila* serine/threonine protein kinase, homologous to the human Raf1 proto-oncogene and is required in the Torso signal transduction pathway (Ambrosio *et al.*, 1989; Nishida *et al.*, 1988; Perrimon *et al.*, 1985). The Torso pathway is activated during early embryogenesis in *Drosophila* and is responsible for controlling pattern formation at the terminal ends of the embryo (Sprenger *et al.*, 1989). After activation of the Torso receptor tyrosine kinase by a ligand an intracellular signal transduction pathway is initiated at the embryonic poles (Casanova *et al.*, 1995; Casanova and Struhl, 1989). This cascade of events is responsible for cellular determination through activation of the gap genes, *tailless* and *huckebein,* to promote formation of the head and tail in the *Drosophila* embryo (Strecker *et al.*, 1986; Strecker *et al.*, 1988; Weigel *et al.*, 1990). Based on structural and functional comparisons between D-raf and human Raf1, as a member of the Torso pathway it is likely that D-raf participates in a complex with other proteins, including D-Ras, 14-3-3 and D-MEK (Sternberg and Alberola-Ila, 1998). Recent studies indicate that the activation and further regulation of Raf1 is not due to a single event but rather to a more complex mechanism (Morrison and Cutler, 1997). We believe that like its mammalian counterpart D-raf's activation and/or regulation may also be controlled by more than one mechanism, which may include protein-protein interaction, phosphorylation, oligomerization and/or conformation change(s).

In the present model the activation of D-raf is facilitated by D-Ras, which localizes D-raf to the membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Once at the membrane D-raf becomes activated by an unknown component(s), which then gives rise to D-MEK activation and transmission of the Torso signal. Because the Torso pathway makes use of a conserved cassette of signaling proteins, which include D-Ras, D-raf, D-MEK and MAPK for transduction of extracellular signals, identification of the mechanisms that regulate D-raf and its activation in the Torso pathway will lead to a more complete model and a better understanding of how activation is achieved for Raf family members.

Project objective

The objective of this project was to investigate possible mechanisms of D-Raf's regulation by looking at the interaction of D-raf, or portions of the D-raf protein, with themselves and with other signaling proteins using the yeast two-hybrid system. First, we looked at the ability of D-raf molecules to form dimers. This was done by looking at the interaction between full-length D-raf proteins in the yeast two-hybrid system. Interaction between full-length D-raf molecules in the yeast two-hybrid system may indicate a similar interaction in the *Drosophila* embryo. Second, we investigated the existence of an intramolecular interaction in which one portion of a D-raf molecule may interact with another portion of the same molecule or another D-raf molecule. For example, we looked at the interaction between the CR2 region of D-raf and the CR3 region of D-raf. Third, we looked

at the interaction of the signaling molecules D-Ras and 14-3-3 with full-length D-raf as well as with individual regions of D-raf.

Our yeast two-hybrid study demonstrated the complexity of the D-raf molecule and how different regions of the molecule can affect D-raf's ability to interact with other components of the pathway. Our study showed that interaction of D-raf with D-Ras and 14-3-3ζ could be blocked by the presence of the N-terminus region (aa 1-176). This result suggests that amino acids 1-176 of D-raf could be part of an autoregulatory mechanism to control D-raf's activity level. In order to validate the accuracy of this hypothesis these findings will be tested *in vivo* using the *Drosophila* embryo. It has already been shown that deletion of amino acids 1-176 of D-raf gives rise to a more active molecule in *Drosophila* embryos (Baek *et. al.* 1996). Based on our results, it is likely that this increase in D-raf's activity can be attributed to the lack of negative regulation due to the loss of the N-terminal aa 1-176. This truncated form of D-raf is free to interact with D-Ras or 14-3-3ζ, which allows for the formation of more active complexes. The next step is to determine if mutations at the N-terminus of D-raf may abolish its negative regulation. If our hypothesis is correct, embryos expressing these mutated forms of D-raf will show an increase in Torso pathway activity similar to the embryos expressing the truncated forms.

The Torso pathway is an interesting and useful model system for the study of signal transduction because it controls more than one biological response, such as expression of *tailless* at 0-15% egg length and expression of *huckebein* at 8-21% egg length (Furriols *et al.*, 1996; Melnick *et al.*, 1993). Also, the Torso pathway utilizes the *Drosophila* Ras, Raf,

MEK, and MAPK proteins, a conserved cassette, which also transmits extracellular signals in more complex organisms. *Drosophila* allows the use of genetics and molecular biology making the study of signaling molecules like D-raf easier. The information we learn concerning how individual members of this conserved cassette function in the Torso signal transduction pathway of *Drosophila* will help us understand how their homologues work in other organisms.

The *Drosophila* Torso signal transduction pathway

In the early *Drosophila* embryo cellular determination is under the control of the following four developmental systems: dorsal-ventral, anterior, posterior, and terminal class systems (Nusslein-Volhard, 1991). The terminal system or the Torso signal transduction pathway controls the development of cells at the anterior (acron) and posterior (telson) poles of the developing embryo. The Torso pathway is active at 2-3 hours after fertilization and gives rise to zygotic expression of the gap genes *tailless* and *huckebein* at the embryonic termini (Pignoni *et al.*, 1990; Sprenger and Nusslein-Volhard, 1992; Weigel *et al.*, 1990). Torso is a receptor tyrosine kinase uniformly distributed along the membrane of the early *Drosophila* embryo. Activation of Torso is due to binding of a diffusible ligand, which is located in the vitelline fluid that surrounds the embryo (Casanova *et al.*, 1995; Casanova and Struhl, 1989; Sprenger and Nusslein-Volhard, 1992). The ligand is restricted to the embryonic poles by binding to the Torso receptor. After ligand activation the Torso receptor initiates a series of events to regulate the transmission of a signal to the nuclei at the poles. The

mechanism by which the signal is transmitted from the embryo's membrane to the nucleus is through a phosphorylation cascade.

A working model for the Torso signal transduction pathway

The genetic and biochemical information collected from both mammalian and *Drosophila* systems indicate that D-raf participates in a protein complex which includes Drk, D-Ras, D-MEK, and possibly Hsp 90, 14-3-3, and KSR. In the present model Torso a receptor tyrosine kinase, is activated by a ligand (see Figure 1) (Casanova *et al.*, 1995; Sprenger *et al.*, 1989). After activation by the ligand the receptor tyrosine kinase dimerizes and undergoes transphosphorylation (Heldin, 1995). Drk, an adapter molecule, recognizes the phosphorylated tyrosine residues on the activated Torso receptor through an SH2 domain (Src homology domain) (Raabe *et al.*, 1995). Drk contains one SH2 domain and two SH3 domains. Its SH3 domain is recognized by the proline rich region of Sos, a guanine nucleotide exchange factor to form a complex with Drk. Then Sos induces the activation of D-Ras by catalyzing the exchange of GDP (inactive GDP-Ras) for GTP (active GTP-Ras). The activated form of Ras (GTP-Ras) helps recruit D-raf to the membrane where it becomes activated by an unknown mechanism. Activation of D-raf initiates the phosphorylation cascade of D-MEK and MAPK. Activated MAPK is then translocated into the nucleus where it interacts with transcription factors that control the expression of the *tailless* and *huckebein* genes.

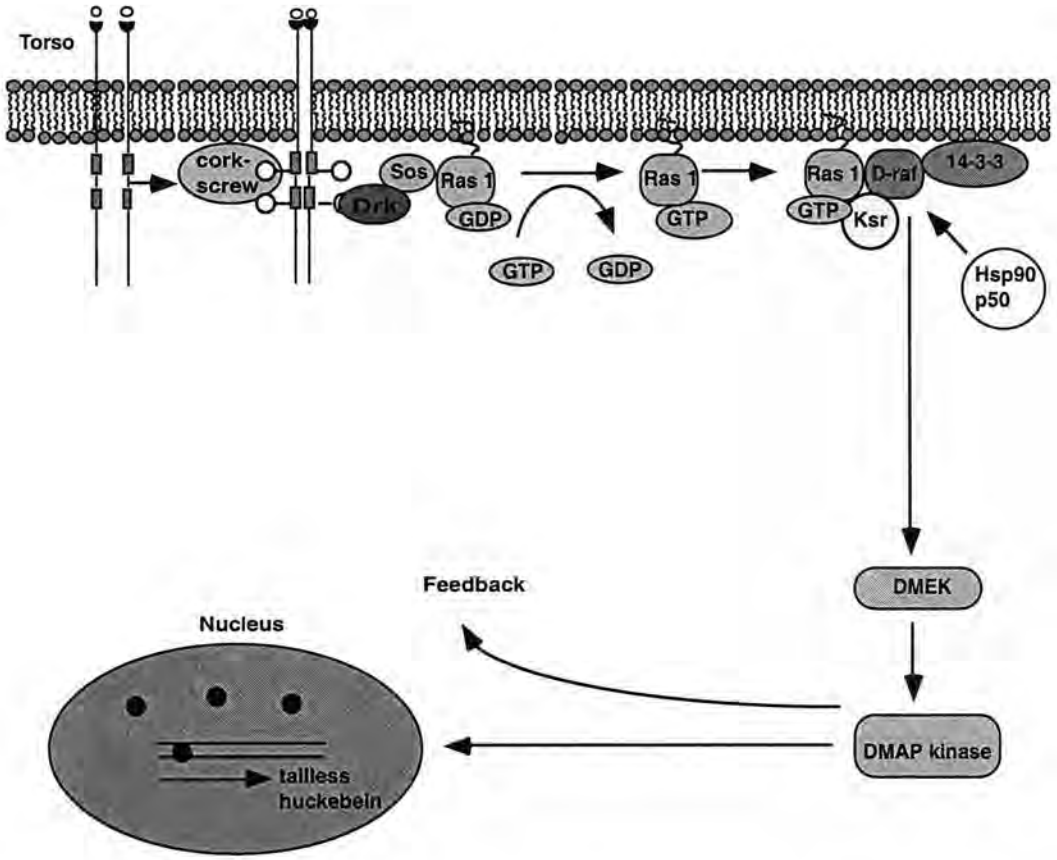
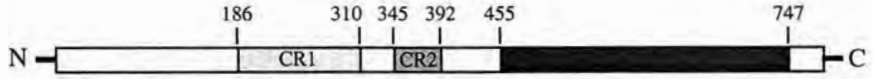


Figure 1. Model of the *Drosophila* Torso signal transduction pathway. After ligand activation the receptor tyrosine kinase dimerizes and undergoes transphosphorylation. Drk, an adapter molecule, recognizes the phosphorylated tyrosine residues on the activated Torso receptor through an SH2 domain (Src homology domain). Drk contains one SH2 domain and two SH3 domains. Its SH3 domain is recognized by the proline rich region of Sos, a guanine nucleotide exchange factor to form a complex with Drk. Sos induces the activation of D-Ras by catalyzing the exchange of GDP (inactive GDP-Ras) for GTP (active GTP-Ras). The activated form of Ras (GTP-Ras) helps recruit D-raf to the membrane where it becomes activated by an unknown mechanism. Activation of D-raf initiates a phosphorylation cascade that includes D-MEK and MAPK. Activated MAPK is then translocated into the nucleus where it interacts with transcription factors that control the expression of the *tailless* and *huckebein* genes.

There are other components that work to regulate the Torso signal transduction pathway but their involvement is less understood. One of such components is corkscrew (csw), a tyrosine phosphatase, the *Drosophila* homolog of mammalian PTP1 (Perkins *et al.*, 1992). It is thought that csw acts in conjunction with D-raf as a positive regulator of the Torso receptor by dephosphorylating the pY918 regulatory site (Cleghon *et al.*, 1998; Perkins *et al.*, 1992). Other components in the Torso pathway are KSR, a protein kinase that functions upstream or in parallel to D-raf, and 14-3-3 which has been shown to associate with Raf1 (Fantl *et al.*, 1994; Therrien *et al.*, 1995). These and other components of the pathway may work to regulate and add specificity to the Torso pathway.

The D-raf serine/threonine protein kinase

D-raf and its human homologue, Raf1, contain three conserved regions CR1, CR2, and CR3 (see Figure 2). The CR1 region contains the Ras interaction domain and a cysteine rich motif. The CR1 region appears to be important for a specific protein-protein interaction that regulates D-raf's activation (Ghosh and Bell, 1994; Ghosh *et al.*, 1994; Vojtek *et al.*, 1993). The CR2 region is a serine rich region and contains a 14-3-3 binding motif identified as RSxSxP (Muslin *et al.*, 1996). An important residue of the 14-3-3 binding motif in D-raf, is the phospho serine at position 388. Previous experiments showed that a mutation of S388 to alanine results in a D-raf molecule with 30 fold more *in vivo* activity than wild-type D-raf (Baek *et al.*, 1996). This data suggests that binding of 14-3-3 to the CR2 region of D-raf may play play a role in the negative regulation of the molecule, but the mechanism by which this



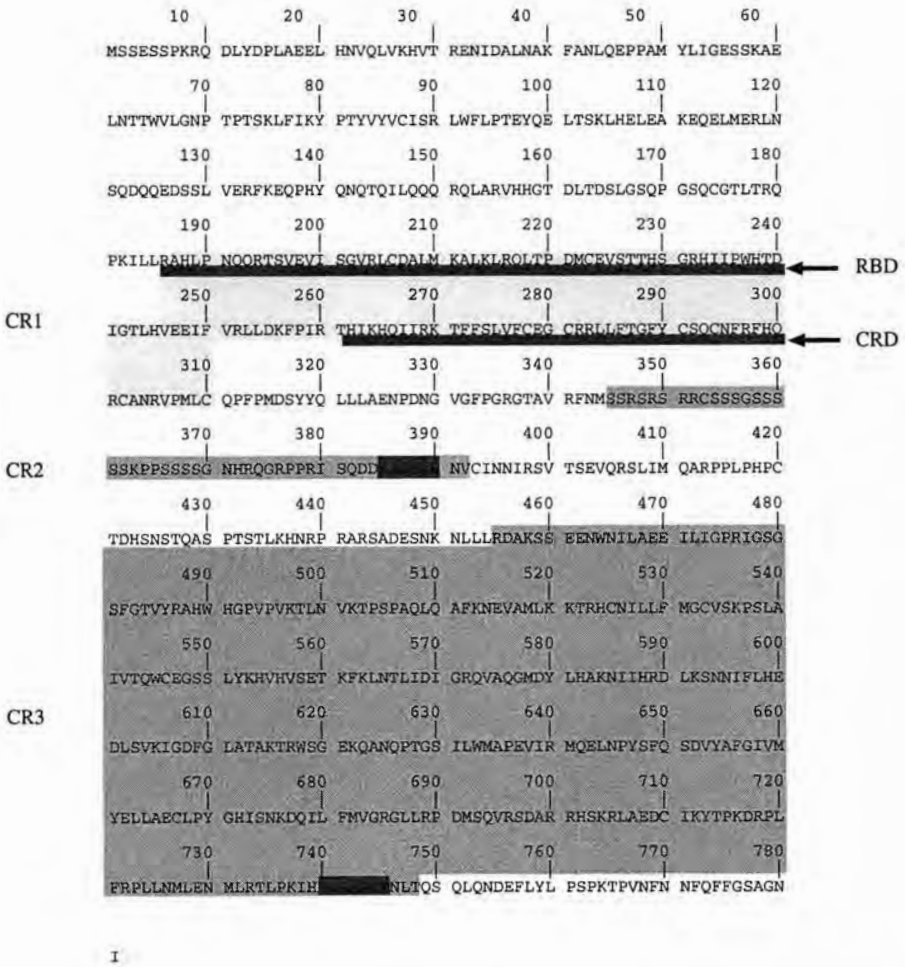


Figure 2. Linear representation and amino acid sequence of D-raf. D-raf contains three conserved regions CR1, CR2, and CR3 represented by the shaded portions. The CR1 region contains the Ras binding domain (RBD) and a cysteine rich domain (CRD) marked by the underlined regions. The CR2 region is a serine rich region, and contains a 14-3-3 binding motif RSxSxP. The CR3 region represents the kinase domain of the protein and also contains a second 14-3-3 binding motif (RSxSxP). The dark boxes within the CR2 and CR3 regions indicate the location of the 14-3-3 binding motifs.

regulation occurs is not clear. In vivo activity experiments have also shown that the N-terminus of D-raf/Raf1 works as a negative regulator, truncated forms of D-raf containing only CR3 region of the molecule showed twice the level of activity when compared to wild-type proteins (Baek *et al.*, 1996). To help understand the regulation and activation of D-raf/Raf1 we need to understand the mechanisms by which the N-terminus of D-raf/Raf1 work to regulate the protein. In this study we took a molecular approach to further investigate the role on D-raf's amino half (aa 1-396) and its effect on D-raf's ability to interact with the signaling molecules D-Ras and 14-3-3.

Possible roles of D-Ras and 14-3-3 on D-raf regulation

It was demonstrated that interaction between Raf1 and Ras is essential but not sufficient for Raf1's activation (Egan *et al.*, 1993; Moodie *et al.*, 1993). Although a lot is known about Raf1 and Ras interaction, the mechanism by which this interaction occurs and its role on Raf1's activation and/or regulation is still unclear. In the present model, during transmission of the extracellular signal, the inactive form of Ras (GDP-Ras) is converted to the active form (GTP-Ras) by a guanine nucleotide exchange factor. GTP-Ras then interacts with Raf1 through the Ras binding domain (RBD residues 55-131). The initial interaction between Ras and Raf1's RBD facilitates Ras interaction with the cysteine rich domain (CRD residues 139-184) and helps localize Raf1 to the membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Recent findings indicate that members of the 14-3-3 family of proteins may also be involved in the mechanism that regulates Ras binding to Raf1.

14-3-3 proteins are a highly conserved group of signaling molecules found in a diverse group of organisms and are involved in many cellular processes including signal transduction. 14-3-3 proteins have been shown to interact with Raf1 at positions 259 (CR2) and 671 (CR3) and have also been shown to associate with both the active and inactive forms of the molecule (Freed *et al.*, 1994). It is believed that binding of Ras to the RBD displaces 14-3-3 from the CR2 region allowing Ras to interact with the CRD region of Raf1 as reviewed by Morrison and Cutler, 1997. These series of events results in conformational changes in the Raf1 molecule exposing the kinase domain and allowing transmission of the signal. Biochemical analysis has shown that interaction of 14-3-3 at position 621 is a stronger interaction than at position 259 of Raf1, but the importance of 14-3-3 interaction at position 621 is not clear (Muslin *et. al., 1996)*. It has been postulated that interaction of 14-3-3 at position 621 helps stabilize the active form of Raf1.

Because of the similarities and differences between Raf1 and D-Raf we investigated D-Raf's interaction with the signaling molecules 14-3-3ζ and D-Ras. D-raf shows a 42% homology to the mammalian Raf1 protein (see Figure 3). The majority of the conserved residues are located in the regions CR1, CR2, CR3. The major difference between D-raf and Raf1 is in their size. D-raf consists of 781 amino acids while Raf1 consists of only 627 amino acids and based on sequence analysis the extra amino acids of D-raf can be attributed to a longer N terminus.

While the overall homology between D-raf and Raf1 is 47.8% this homology encompasses only 582 residues. The highest homology between Raf1 and D-raf is 60%

found in the kinase domain and a 40% homology is found in the CR1 region of the proteins. When comparing the N-terminus of D-raf and Raf1, amino acids 1-185 of D-Raf and amino acids 1-62 of Raf1, no significant homology (>25%) is observed (see Figure 3). The significance of the longer N-terminus found in D-raf is not well understood, but biochemical and genetic studies indicate that the N-terminus of Raf1 and D-raf are regulatory regions (Baek *et al.*, 1996; Daum *et al.*, 1994; Heidecker *et al.*, 1990; Stanton *et al.*, 1989). Is also important to notice that although no significant homology is found among the area between the CR1 and CR3 regions of the protein, the distance between these conserved regions is maintained.

D-raf also has a 48.8% homology to B-Raf. B-raf is a member of the Raf family of serine/threonine protein kinases and is found mainly in neuronal tissues. Alternative splicing of the *B-raf* gene gives rise to multiple isoform, which are believed to introduce a higher level of regulation (Barnier *et al.*, 1995; Eyechene *et al.*, 1995). Like D-raf, B-Raf is a bigger molecule than Raf1 and based on sequence similarity it can be concluded that B-Raf also contains a longer N-terminus. Because of the higher level of homology found between D-raf and B-Raf than between D-raf and Raf1, we believe that D-raf regulation may be closer to B-raf regulation than Raf1.

Our study of D-raf's interaction with the signaling molecules 14-3-3 and D-Ras supports a model for D-raf autoregulation in which the N-terminus (aa 1-176) of D-raf acts as a negative regulator. These findings also suggest a possible mechanism for this negative regulation whereby the intramolecular interaction between a 12 amino acid motif (aa 12-23) of

D-raf blocks the interaction of 14-3-3ζ with the CR2 domain. Our data also indicates that aa 12-23 and the CRD region of D-raf may be working together to form a binding site for 14-3-3. In or experiments when this to regions together were able to interact with 14-3-3 but each region along was not. This data is supported by interaction studies, which have shown 14-3-3 to interact with molecules that do not contain a 14-3-3 binding motif (RSxSxP) but do contain a CRD.

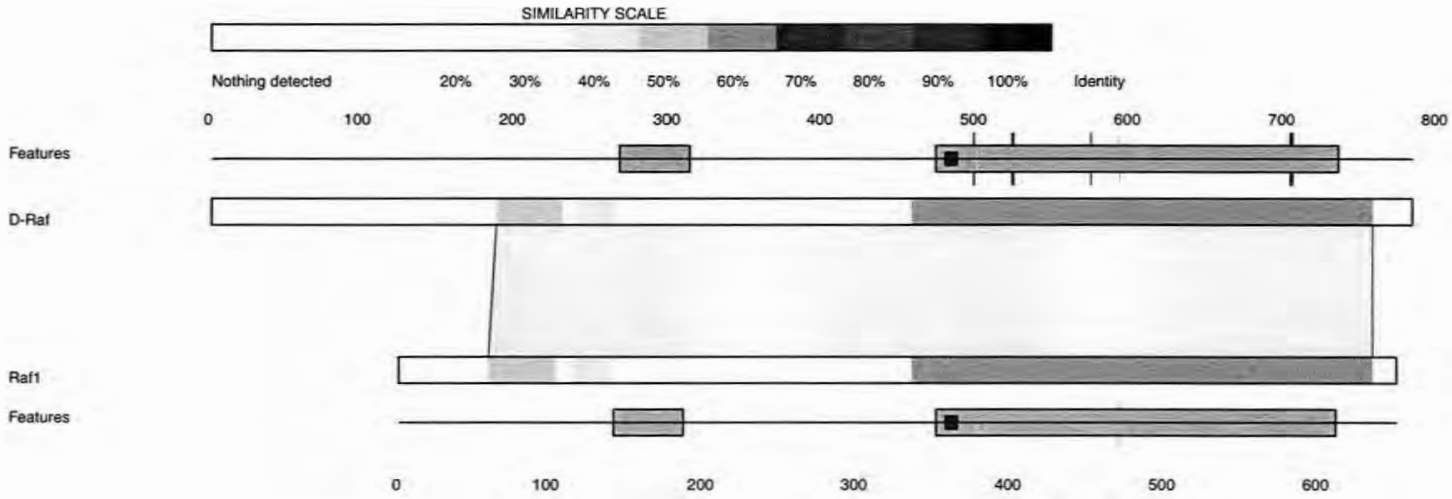


Figure 3. Graphical representation of the percent homology between D-raf and Raf1. Figure 3 shows the areas of homology between D-raf and Raf1. The level of homology, between D-raf and Raf1, is represented by the shaded regions. The highest level of homology (60%) is found in the CR3 regions of the proteins, which constitute the kinase domain. Another significant level of homology (40-30%) is found within the CR1 region, which contains the Ras binding domain and a cysteine rich motif.

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47.8% identity in 582 residues overlap; Score: 1249.0; Gap frequency: 2.9%

Raf1          58 IRVFLPNKQRTVVNVRNGMSLHDCLMKALKVRGLQPECCAVFRLLHEHKGKKARLDWNTD
D-raf        185 LRAHLPNQQRTSVEVISGVRLCDALMKALKLRQLTPDMCEVSTT---HSGRHI-IPWHTD
                  *  *** *** * *  *  * * ****** * * *  * *      * *      * **

Raf1         118 AASLIGEELQVDFLDHVPLTTH---NFARKTFLKLAFCDICQKFLLNGFRCQTCGYKFHE
D-raf        241 IGTLHVEEIFVRLLDKFPIRTHIKHQIIRKTFFSLVFCEGCRRLLFTGFYCSQCNFRFHQ
                   *  **  *  **  *  **      ****  * **  *   *  ** * *   **

Raf1         175 HCSTKVPTMCVDW--SNIRQLLLFPNSTIGDSGVPALPSLTMRRMRESVSRMPVSSQHRY
D-raf        301 RCANRVPMLCQPFPMDSYYQLLLAENPDNGVGFPGRGTAVRFNMSSRSRSRRCSSSGSSS
                  *   **  *         ****  *   *                  * **   **

Raf1         233 ST-PHAFTFNTSSPSSEGSLSQRQRSTSTPNVHMVSTTLPVDSRMIEDAIRSHSESASPS
D-raf        361 SSKPPSSSSGNHRQGRPPRISQDDRSNSAPNV-CINNIRSVTSE-VQRSLIMQARPPLPH
                 *  *                **  ** * ***        * *               *

Raf1         292 ALSSSPNNLSPTGWSQPKTPVPAQRERAPVSGTQEKNKIRPRGQRDSSYYWEIEASEVML
D-raf        419 PCTDHSNSTQ----ASPTSTLKHNRPRARSADESNKNLLL-RDAKSSEENWNILAEEILI
                       *         *       * **       **    *    *   * * * *

Raf1         352 STRIGSGSFGTVYKGKWHGDVAVKILKVVDPTPEQFQAFRNEVAVLRKTRHVNILLFMGY
D-raf        474 GPRIGSGSFGTVYRAHWHGPVPVKTLNVKTPSPAQLQAFKNEVAMLKKTRHCNILLFMGC
                   ***********   *** * ** * *  * * * *** **** * **** *******

Raf1         412 MTKDNLAIVTQWCEGSSLYKHLHVQETKFQMFQLIDIARQTAQGMDYLHAKNIIHRDMKS
D-raf        534 VSKPSLAIVTQWCEGSSLYKHVHVSETKFKLNTLIDIGRQVAQGMDYLHAKNIIHRDLKS
                   *  ***************** ** ****    **** ** *************** **

Raf1         472 NNIFLHEGLTVKIGDFGLATVKSRWSGSQQVEQPTGSVLWMAPEVIRMQDNNPFSFQSDV
D-raf        594 NNIFLHEDLSVKIGDFGLATAKTRWSGEKQANQPTGSILWMAPEVIRMQELNPYSFQSDV
                 ******* * ********** * ****  *  ***** ***********  ** ******

Raf1         532 YSYGIVLYELMTGELPYSHINNRDQIIFMVGRGYASPDLSKLYKNCPKAMKRLVADCVKK
D-raf        654 YAFGIVMYELLAECLPYGHISNKDQILFMVGRGLLRPDMSQVRSDARRHSKRLAEDCIKY
                 *  *** ***    *** ** * *** ******   ** *          ***  ** *

Raf1         592 VKEERPLFPQILSSIELLQHSLPKINRSASEPSLHRAAHTED
D-raf        714 TPKDRPLFRPLLNMLENMLRTLPKIHRSASEPNLTQSQLQND
                     ****   *   *     **** ****** *       *
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Figure 1. (continued)

MATERIALS AND METHODS

Cloning of D-raf molecules into yeast two-hybrid expression vectors

In order to study protein/protein interactions using the yeast two-hybrid system we first cloned a series of D-raf constructs into the yeast two-hybrid vectors pBDGal4 Cam and pADGal4-2.1 from *Stratagene* (see Figures 4, and appendix A). 10 D-raf constructs were isolated from D-raf ^{wt} (vector pGEM7 Glo mp156) by PCR. The primers used for PCR also introduced an EcoRI site at both the 5' and 3' ends of the amplified regions (see Table 1). After cloning into the appropriate vectors the new constructs were sequenced to verify that no mutations occurred during the PCR or cloning process. Cloning of the D-raf constructs into the pADGAL4-2.1 and pBDGAL4 Cam vectors allowed for the creation of fusion proteins between the AD (aa 761-881 of GAL4) or BD (aa 1-147 of GAL4) of the N terminus of D-raf.

Analysis of protein interactions by yeast two-hybrid analysis

To determine protein/protein interactions the desired plasmid combination encoding a fusion protein to the Gal4BD and Gal4AD were cotransformed into the yeast strain YRG2 (*Stratagene*) using a lithium acetate method (Schiestl and Gietz, 1989). Both vectors pBDGal4 Cam and pADGal4-2.1 contain selection markers for yeast, pBDGal4 Cam (TRP) and pADGal4-2.1 (Leu). These selection markers were under the control of the ADH1 promoter.

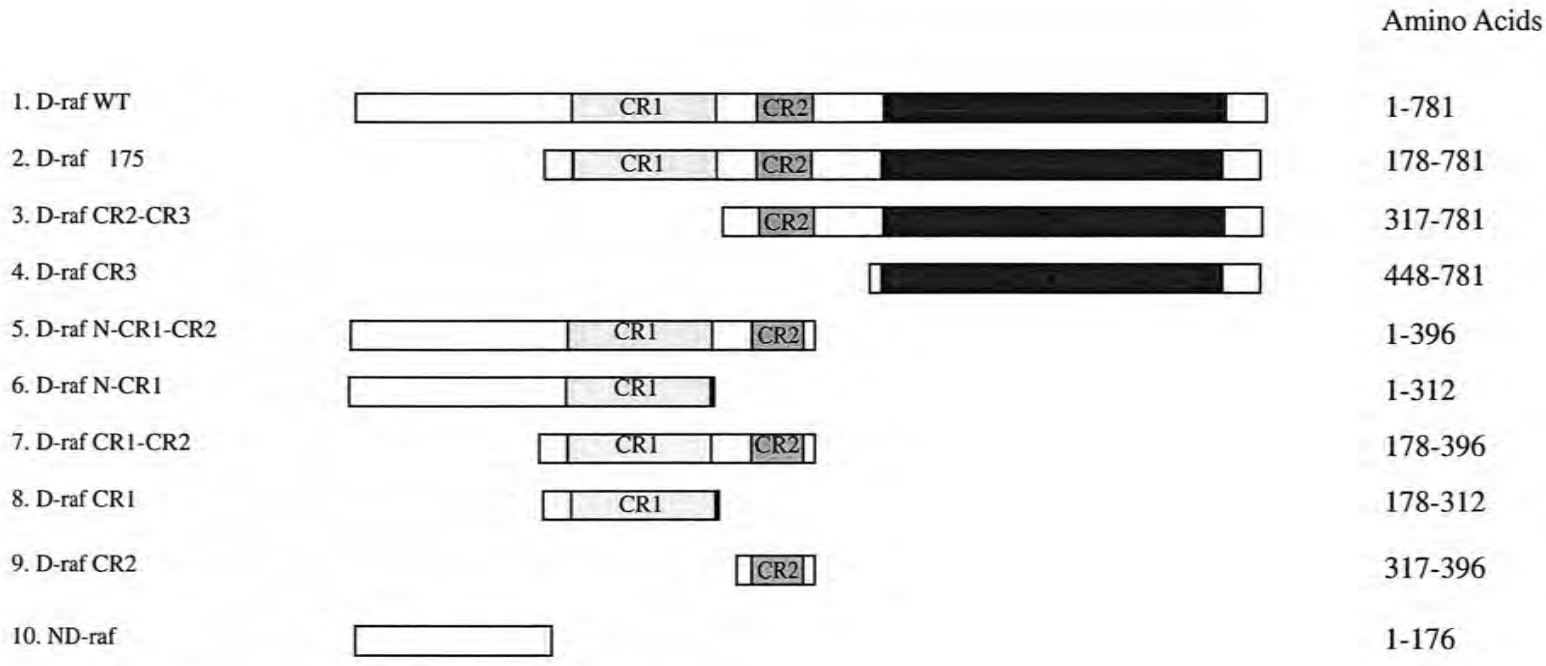

Figure 4. Graphical representation of D-raf constructs used for cloning into yeast two-hybrid vectors. This figure shows 10 D-raf constructs isolated by PCR and cloned into the vectors pADGAL4-2.1 and pBDGAL4 cam. Constructs were cloned as EcoRI fragments. The D-raf constructs were fused at the N-terminus with the activation or binding domains of GAL4.

Table 1. D-raf constructs and primers

D-raf Construct	Primers used for PCR
D-raf ^{WT}	5' GATACCGAATTCAGCGAGTTCTCC 3'
	5' AGGTAGAATTCGCTGTCTAGATATTC 3'
D-raf $^{CR1\text{-}CR2\text{-}CR3}$	5' AGC CAATGTGAATTCTTGACCCGTCAGC 3'
	5' AGGTAGAATTCGCTGTCTAGATATTC
D-raf $^{CR2\text{-}CR3}$	5' TGCCAGCCCTTTGAATTCGATAGCTACTAT 3'
	5' AGGTAGAATTCGCTGTCTAGATATTC
D-raf ^{CR3}	5' GGCCAGGTCCGAATTCGAGAGCAATA 3'
	5' AGGTAGAATTCGCTGTCTAGATATTC
D-raf ^{NCR1}	5' GATACCGAATTCAGCGAGTTCTCC 3'
	5' CTATCCATGGGAATTCGCTGGCACAG 3'
D-raf ^{CR1}	5' AGC CAATGTGAATTCTTGACCCGTCAGC 3'
	5' CTATCCATGGGAATTCGCTGGCACAG 3'
D-raf $^{NCR1\text{-}CR2}$	5' GATACCGAATTCAGCGAGTTCTCC 3'
	5' CCGATCGAATTCTGTTGATGCACAC 3'
D-raf $^{CR1\text{-}CR2}$	5' AGC CAATGTGAATTCTTGACCCGTCAGC 3'
	5' CCGATCGAATTCTGTTGATGCACAC 3'
D-raf ^{CR2}	5' GTTGAATTCCCCGGCAGAGGCACTCC 3'
	5' CCGATCGAATTCTGTTGATGCACAC 3'
D-raf 1-176	5' ATACCGAATTCAGCGAGTTCTCC 3'
	5' CTGACGGGTCAGAATTCCACATTGGC 3'

Protein/protein interaction was determined by expression of the reporter genes *his* and *lacZ*. After cotransformation of YRG2, cells were plated on solid SD media lacking Trp and Leu. Transformants were allowed to grow for five days at which point four different transformants were picked and plated for isolation in SD plates lacking Trp and Leu. This step was done to ensure the analysis of 4 different transformants. After 4 days of growth transformants were picked and plated in SD plates lacking Trp, Leu, and His. Plates were incubated at 30°C for seven days. Transformants were scored for growth at 3, 5 and 7 days.

Transformants that showed growth at 3 days were scored with a (+++), at 5 days with a (++) and at seven days with a (+). Transformants that after seven days did not show any growth or showed growth similar to the negative control were scored as (-). A qualitative analysis was also performed by determining expression of ß-galactosidase activity using a filter assay on colonies plated on SD plates lacking Trp and Leu (Schiestl and Gietz, 1989).

Computer analysis and sequence alignment

Protein alignments were done by SIM - Alignment Tool for protein sequences (http://www.expasy.ch/tools/sim-prot.html) (Miller, 1991). For our protein analysis we used a gap open penalty of 12 and a gap extension penalty of 4. The comparison matrix used was BLOSUM62.

RESULTS AND DISCUSSION

Study of D-raf/D-raf molecular interaction by yeast two-hybrid analysis

Oligomerization of D-raf molecules has been proposed as one of many possible mechanisms involved in the regulation of Raf1 activity (Morrison and Cutler, 1997). The idea of oligomerization as a regulatory mechanism was supported by evidence which indicated that Raf1 activity can be increased by chemical dimerization (Farrar *et al.*, 1996; Luo *et al.*, 1996). However it was not clear if the observed increase in Raf1 activity required the presence of Ras. Two mechanisms have been proposed to explain the increase in Raf1 activity by oligomerization: oligomerization causes a conformational change that decreases the negative effect of the N-terminus on the activity of the molecule, oligomerization allows for transphosphorylation or autophosphorylation of Raf1 (Morrison and Cutler, 1997). Based on this and other evidence, we wanted to investigate the formation of D-raf oligomers using the yeast two-hybrid system. We hypothesized that positive interaction between two D-raf molecules in the two-hybrid system may indicate the occurrence of a similar interaction in the *Drosophila* embryo.

Recombinant molecules were created by fusing the GAL4 binding domain (amino acids 1-147) or the GAL4 activation domain (amino acids 761-881) to the N-terminus of full-length D-raf molecules (see Figure 4). The presence or absence of molecular interaction was determined by the expression of the reporter genes *lacZ* and *His*.

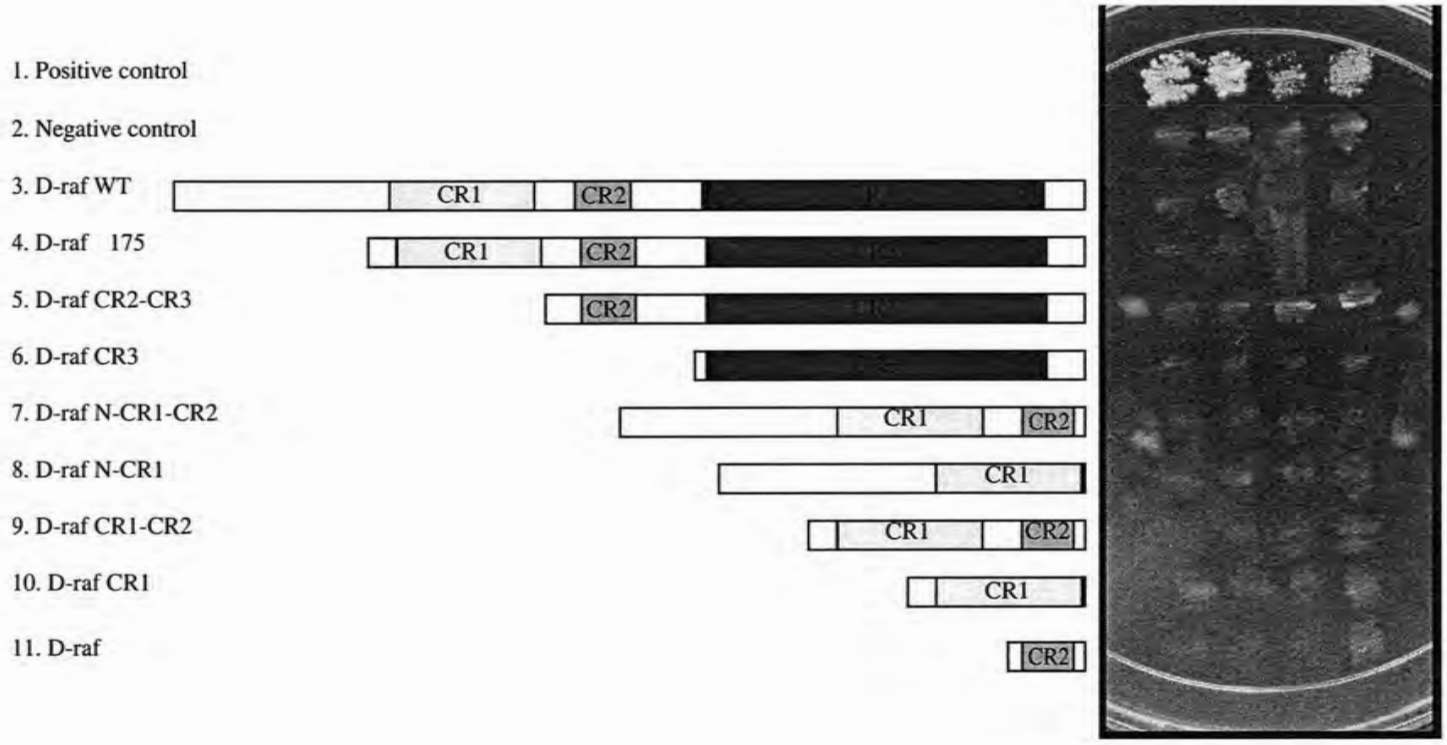

Figure 5. Protein-protein interaction of D-raf molecules by yeast two-hybrid analysis. Recombinant molecules were created by fusing the GAL4 binding domain (amino acids 1-147) or the GAL4 activation domain (amino acids 761-881) to the N-terminus of full-length D-raf molecules. The presence or absence of molecular interaction was determined by the expression of the reporter genes lacZ and His. Four different transformants were plated on SD plates lacking Trp, Leu and His and incubated at 30°C for five days. After five days cell growth was scored using a + or –.

In our study we did not detect interaction, or oligomerization, between full-length D-raf molecules (see Figure 5). Four different transformants expressing the fusion proteins GAL4AD-Draf^{WT} and GAL4AD-Draf^{WT} were plated on SD plates lacking Trp, Leu and His and incubated at 30°C for seven days. After seven days cell growth was scored using the (+) or (–) system as described in materials and methods (see Table 2). As shown in Figure 5 row #3 and summarized in Table 2, trasnsformants expressing both full-length D-raf recombinant molecules pADGAL4 D-raf ^{WT} and pBDGAL4 D-raf ^{WT} did not show any growth after seven days. The lack of growth indicated the absence of the *His* reporter gene expression. The positive control expressing the recombinant molecules p53 and SV40 (row #1) showed growth after 2 days at 30°C whereas the negative control expressing the recombinant molecules laminC and SV40 (row #2) showed no growth after 5 days (see Figure5). The negative control showed some growth after seven days, indicating the leaky expression of the *His* reporter gene. We interpreted the absence of growth as a lack of interaction between the recombinant molecules. This data was verified by a filter lift assay, which failed to detect expression of ß-galactosidase by transformats expressing both full-length D-raf fusion proteins.

Although the absence of growth by transformant yeast expressing both full-length D-raf recombinant molecules does not indicate the formation of D-raf/D-raf dimers, our results alone can not rule out this possibility. It is possible that D-raf molecules are able to interact in the *Drosophila* embryo, but this interaction is not detectable by yeast two-hybrid analysis due to the fact that we used recombinant molecules containing the AD or BD of GAL4.

Table 2. Protein-protein interaction of D-raf molecules by yeast two-hybrid analysis. The ability of nine different D-raf constructs was analyzed for their ability to interact with each other by yeast two-hybrid. Transformant yeast cells expressing two fusion proteins of D-raf were plated on media lacking TRP, LEU and HIS. Cells were incubated at 30°C for seven days and scored for growth. (-) Represents no growth and (+) represents growth.

D-raf costructs expressed as fusion proteins	His - Growth assay								
		CR1-CR2							
	WT	-CR3	CR2-CR3	CR3	NCR1-CR2	NCR1	CR1-CR2	CR1	CR2
 D-raf WT	-	-	-	-	-	-	-	-	-
 D-raf CR1-CR2-CR3	-	-	-	-	-	-	-	-	-
 D-raf CR2-CR3	-	-	-	-	-	-	-	-	-
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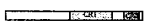
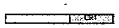
 D-raf NCR1-CR2	-	-	-	-	-	-	-	-	-
 D-raf NCR1	-	-	-	-	-	-	-	-	-
 D-raf CR1-CR2	-	-	-	-	-	-	-	-	-
 D-raf CR1	-	-	-	-	-	-	-	-	-
 D-raf CR2	-	-	-	-	-	-	-	-	-
 ND-raf 1-176	-	-	-	-	-	-	-	-	-

It is also possible that a third molecule(s) may be involved in the formation of D-raf/D-raf dimers by facilitating the interaction between two D-raf molecules. A possible candidate for this role is the signaling protein 14-3-3 which itself had been shown to dimers, and 14-3-3 dimers have also been shown to support Raf1 activation *in vitro* (Liu *et al.*, 1995).

To further investigate the possible role of 14-3-3 as a bridge between two D-raf molecules, I propose the following experiment. Two recombinant D-raf molecules, one fused to the GAL4 AD and the other to the BD, can be expressed in yeast together with 14-3-3. If 14-3-3 brings two D-raf molecules together a dimer may be formed bringing together the AD and BD and allowing for expression of the *His* reporter gene.

Study of D-raf/D-raf intramolecular interactions by yeast two-hybrid analysis

Based on our previous results, which indicated no interaction between two full-length D-raf molecules, we wanted to study the ability different regions of D-raf to interact with each other (Figure 5 and Table 2). A positive interaction between different regions of D-raf will suggest an intramolecular interaction as a possible mechanism of D-raf regulation. To screen for intramolecular interaction, selected regions of D-raf were fused to the GAL 4 BD or GAL4 AD and analyzed for interaction by expression of the *his* reporter gene. These fusion proteins represented the most conserved regions of D-raf. 81 combinations of the recombinant molecules were expressed in yeast and analyzed. (Table 2). After seven days of growth we were not able to detect interaction among any of the different regions of D-raf. Our results did not indicate the presence of intramolecular interaction but as in our previous

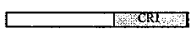
experiment this possibility can not be ruled out. It is possible that the interaction is present but too weak to be detected in the two-hybrid system. This can be due to the fact that we are working fusion proteins, or because the secondary structure of the recombinant molecules may not be the same in yeast as in the *Drosophila.*

Study of D-raf interaction with D-Ras

Although a lot has been learned about the interaction of Ras with Raf1, the mechanism by which this interaction occurs and the role this mechanism plays in the activation and regulation of D-raf still unclear. GTP bound Ras has been shown to be necessary but not essential for the activation of Raf 1 and the interaction of Ras with Raf1 has also been implicated in the localization of Raf1 to the member. In the current model GTPRas interacts with Raf1 through the RBD. After this first interaction GTPRas can then interact with the CRD of Raf1. Based on this information we wanted to investigate the interaction between D-raf and D-Ras. A fusion protein between the GAL4AD and D-Ras was created and tested for its ability to interact with a series of D-Raf constructs using the yeast hybrid system (Table 3).

Contrary to our predictions based on similar experiments done with Raf1, we were not able to detect interaction between full-length D-Raf and D-Ras. Other groups have also reported similar results. What does it mean that interaction can be detected between Raf1 and H-Ras by two-hybrid analysis but not between their *Drosophila* homologs?

Table 3. Protein-protein interaction of D-raf and DRas by yeast two-hybrid analysis. The ability of nine different D-raf constructs was analyzed for their ability to interact with D-Ras. Transformant yeast cells expressing both fusion proteins, D-ras and one of the D-raf constructs, were plated on media lacking TRP, LEU and HIS. Cells were incubated at 30°C for seven days and scored for growth. (-) Represents no growth and (+) represents growth.

	His - Growth assay
 D-raf WT	-
 D-raf CR1-CR2-CR3	-
 D-raf CR2-CR3	-
 D-raf CR3	-
 D-raf NCR1-CR2	-
 D-raf NCR1	-
 D-raf CR1-CR2	+
 D-raf CR1	+
 D-raf CR2	-

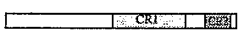
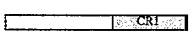
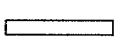
These results indicate a difference not only in the way these signaling molecules interact with each other but also in the way they may be regulated. Keep in mind that D-raf contains a longer N-terminus region than Raf1, and this may cause D-raf to fold differently from Raf1. We attributed the lack of interaction between D-raf and D-ras to molecular folding in which the RBD may be blocked by D-raf's N-terminus and not accessible to interact with D-Raf.

In our experiment we were able to detect D-Raf interaction with D-Ras only when the recombinant molecules D-raf CR1CR2 or D-rafCR1 were used. Our results indicate that the presence of either the N-terminus or kinase domain of D-Raf is sufficient to block interaction with D-Ras. Our results also suggest that interaction between D-raf and D-Ras may be facilitated by a conformational change in the D-Raf molecule.

Study of D-raf interaction with 14-3-3ζ

Biochemical as well as two-hybrid analysis have shown that 14-3-3 can physically interact with Raf1, which contains two 14-3-3 interaction sites. D-raf also contains two 14-3-3 interaction sites at positions 388 and 743 and recently Li and others demonstrated that the *Drosophila* 14-3-3 leonardo (Leo) is an essential component of the Torso pathway. They showed that over expression of 14-3-3 Leo activates D-raf in a Ras dependent manner (Li *et. al.*, 1991997). Based on this information we wanted to determine if we can detect physical interaction between 14-3-3ζ Leo and a series of D-raf constructs by yeast two hybrid analysis.

Table 4. Protein-protein interaction of D-raf and 14-3-3z by yeast two-hybrid analysis. Nine different D-raf constructs were analyzed for their ability to interact with 14-3-3z. Transformant yeast cells expressing both fusion proteins, 14-3-3 and one of the D-raf constructs, were plated on media lacking TRP, LEU and HIS. Cells were incubated at 30°C for seven days and scored for growth. (-) Represents no growth and (+) represents growth.

		His - Growth assay
	D-raf WT	-
	D-raf CR1-CR2-CR3	+ +
	D-raf CR2-CR3	+ +
	D-raf CR3	+
	D-raf NCR1-CR2	-
	D-raf NCR1	+
	D-raf CR1-CR2	-
	D-raf CR1	-
	D-raf CR2	-
	ND-raf1-176	-

As in our previous experiments we used 9 different D-raf constructs (see Figure 4) and analyzed their ability to interact with 14-3-3ζ (see Table 3). We were not able to detect interaction between full-length D-raf molecules and 14-3-3ζ. We speculated that the N-terminus of D-raf (aa 1-176) may be blocking the 14-3-3 interaction sites located in the CR2 and CR3 regions of the molecule and that by removing this residues we may remove the negative regulation.

To test our hypothesis we looked at the interaction between 14-3-3 and three D-raf constructs, D-raf Δ175, D-rafCR2CR3 and D-rafCR3. Our results supported our theory. When we removed the N-terminus of D-raf (aa 1-17) we were able to detect interaction with 14-3-3. First we saw interaction between 14-3-3 and D-rafΔ175 which contained all conserved regions of the protein (CR1, CR2 and CR3) including the two 14-3-3 binding sites. The observed growth was apparent after two days, which was similar to growth by the positive control. This result indicated that our failure to detect interaction when using full-length D-raf molecules was due to the presence of the N-terminal region. The fact that the observed rate of growth was similar to the positive control indicated a strong interaction. Based on information known about the interaction of Raf1 and 14-3-3 we concluded that the interaction between D-raf and 14-3-3ζ was through two predicted 14-3-3 binding sites RSxSxP. As expected similar results were obtained between D-raf CR2-CR3 and 14-3-3 when analyzed for interaction.

We also detected interaction between D-rafCR3 and 14-3-3, but unlike the previous results this interaction was weaker with no apparent growth after 3 days. Growth was

detected at day 5 at which point the negative control was not showing any growth. This data was confirmed by ß-galactosidase assay, in which we were able to detect a light blue color on transformants expressing the positive control or D-rafΔ175, D-rafCR2CR3 and 14-3-3ζ but not on transformants expressing DrafWT, DrafCR3 and 14-3-3ζ.

An unexpected result was the lack of detectable interaction between 14-3-3ζ and the CR2 region of D-raf, which contains a 14-3-3 binding site. Although unexpected this result was not surprising. The fact that the interaction between D-rafCR3 and 14-3-3 was weak and almost not detectable by our assay, suggests that a weaker interaction may not be detectable at all. This idea is supported by the fact that biochemical studies have shown that the interaction of 14-3-3 with the CR3 region of Raf1 is a stronger interaction that with the CR2 region of the molecule.

Another surprising result was the interaction between D-rafNCR1 and 14-3-3ζ. D-rafNCR1 does not contain a known 14-3-3 binding site, but researchers have suggested that 14-3-3 may also interact with the CRD region of Raf1. In our experiments we did not see interaction between 14-3-3 and DrafCR1CR2 or D-rafCR1, both of which contain the CRD region. Our results suggest that 14-3-3 may be interacting with and unidentified motif that resides in the N-terminus of D-raf or with its secondary structure.

To further investigate the possibility of a 14-3-3 binding site at the N-terminus of D-raf, we cloned amino acids 1-176 into pBDGAL4 Cam two-hybrid vector and tested for its ability to interact with 14-3-3ζ. We were not able to detect interaction of ND-raf (aa 1-176)

with 14-3-3ζ. This data indicates that the observed interaction between D-rafNCR1 and 14-3-3ζ is not due to a single interaction of 14-3-3 with a motif in the N-terminus or with the CRD but rather to interaction with both sites. We hypothesize that for us to detect interaction of D-raf with 14-3-3ζ a stable D-raf/14-3-3 complex needs to be formed. For example, the reason why we saw interaction when both the N-terminus and the CRD regions of D-raf were present (D-rafNCR1) is because for a stable complex to be formed 14-3-3ζ needs to interact with both sites. Removal of one of this two sites does not allow for the formation of an stable complex therefore interaction can not be detected.

Computer analysis of D-raf

Because our results indicated the presence of a possible 14-3-3ζ binding site within the N-terminus (aa 1-312) of D-raf we analyzed the primary and secondary structures of D-raf and compared the results to proteins in the Swiss-prot database by computational analysis. Our interaction analysis indicated that the N-terminus of D-raf was blocking the molecule's ability to interact with D-Ras and 14-3-3ζ. As previously described *in vivo* experiments indicated that the amino-half of D-raf (aa 1-396) works as a negative regulator. Also research shows that increase levels of 14-3-3ζ gives rise to a higher Torso signal (Li *et. al.,* 1997). Taken together these results suggest that the amino-half of D-raf may work as a negative regulator by blocking 14-3-3 interaction with the CR2 or CR3 regions of the molecule.

One possible component of this negative regulatory mechanism is the presence of a 14-3-3 like motif in the N-terminus region of D-raf. This 14-3-3 like motif may recognize the 14-3-3 binding sites in the CR2 or CR3 regions of the molecule and block the binding of 14-3-3 to those areas. To search for a possible 14-3-3 like domain we aligned 14-3-3 Leo and D-raf using the Swiss-prot SIM alignment tool (http://www.expasy.ch). These results allowed us to identify a 12 aa region (YDxxAxxxxxV) of homology between D-raf and 14-3-3 Leo.

Prediction analysis of the secondary structure of the region around the RDxxAxxxxxV domain in both D-raf and 14-3-3ζ indicated the formation of a helical structure. In 14-3-3 this region is part of B'-helix which is part of a three-helix bundle formed across the dimer interface of two 14-3-3 molecules (Xlao *et al.*, 1995; Liu *et. al.*, 1995). Our next step is to confirm the existence this domain (RdxxAxxxxxV) . Currently we are in the process of determining if interaction between the N-terminus of D-raf and 14-3-3 can be detected by two-hybrid analysis.

CONCLUSION

The *Drosophila*-raf (D-raf) serine/threonine protein kinase is an essential component for proper function of the Torso signal transduction pathway. The Torso signaling pathway controls the formation of the terminal structures of the *Drosophila* embryo by inducing activation of the gap genes *tailless* and *huckebein*. Activation of *tailless* and *huckebein* requires activation of a phosphorylation cascade of highly conserved molecules known as RAS signaling cassette. In the *Drosophila* embryo the Ras signaling cassette includes the molecules D-raf, DRas, DMEK and MAPK. Similar cassettes have been found on other organisms including humans. D-raf is 47.8% homologous the mammalian Raf1 serine/threonone protein kinase. Raf1 is also part of a Ras signaling cassette and is involved in a range of cellular processes including cell regulation and proliferation among others. The study of Raf1 and its involvement in the regulation of extracellular signals has become an important area of study. Because of the similarities between Raf1 and D-raf what we learn from one molecule will allow us to understand the other. Both Raf1 and D-raf contain three highly conserved region CR1, CR2, and CR3. The CR1 region has been shown to contain the Ras binding domain (RBD) and a Cysteine rich region. The CR2 region is a serine rich region and contains one of two 14-3-3 binding motifs. The CR3 region is composed by the kinase domain and also contains a 14-3-3 binding motif. The amino-half of the protein has been shown to be involved in negative regulation of the protein, but the mechanism by which this negative regulation may be occurring is not clear.

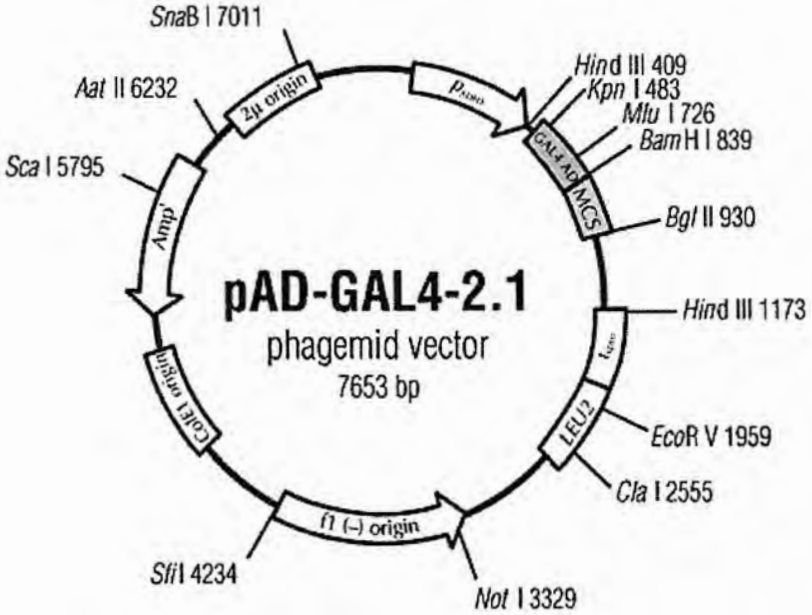
In our study we examine protein-protein interaction between selected regions of D-raf and the signaling molecules D-Ras and 14-3-3. Based on our results we propose a new model to help explain the mechanism by which amino acids 1-176 together with the CRD portion of D-raf may work to negatively regulate its own activity. There is substantial evidence showing the importance of D-Ras binding to D-raf and its role in transmission of the extracellular signaling. Evidence also shows that removal of N-terminal regions of D-raf (aa 1-176 or 1-396) gives rise to a more active protein, suggesting a negative regulatory role of the amino-half portion of the protein. Current models proposed by Morrison and Cutler in 1997 and by Tzivion and others in 1998 also suggest that 14-3-3 works as part of this mechanism by maintaining both the active and inactive form of the Raf1 molecule. In the current model 14-3-3 is bound to Raf1 at the 14-3-3 motifs located in the CR2 and CR3 regions of the molecule. The activated form of Ras (GTP-Ras) binds Raf/D-raf at the CRD region, this causes displacement of 14-3-3 from the CR2 region and allows Ras to interact with the Ras binding domain (RBD) at the CR1 region of the molecule.

In previous two-hybrid studies, done by Freed and others in 1994, it was determined that full-length Raf1 and Raf1 recombinant molecules, containing only the Raf1's N-terminus and CR1 region (aa 1-197), were able to interact with H-Ras, 14-3-3 ß and 14-3-3 ζ. Although, the interactions between Raf1 1-197 with 14-3-3ζ was weaker than with 14-3-3ß. Based on these results we expected to see interaction between full-length D-raf1 and D-Ras, but that was not the case (see Table 3).

The fact that we did not see interaction between full-length D-raf and D-Ras as expected indicates a difference in the way that D-raf interacts with D-Ras when compared to Raf1/H-Ras interaction. We attributed the lack of interaction to a longer N-terminus of D-raf. Based on our results we suggest that the N-terminus of D-raf including aa 13-23 and the CRD may be working as a negative regulator by blocking 14-3-3 interaction with D-raf. Without 14-3-3, D-raf may not be able to form an stable protein complex. Without a proper protein complex transmission of the signal will not occur.

It has been reported that B-Raf can from protein complex with the signaling proteins Ras and MEK (Papin *et. al.*, 1996). Two-hybrid studies showed that full-length B-Raf can interact with Ras and 14-3-3 proteins (Papin *et. al.*, 1996). In this two-hybrid study B-raf constructs containing B-raf (aa 1-433) were able to interact with both Ras and 14-3-3. B-raf aa 1-433 contains all of the protein N-terminus, including the CR1 and CR2 regions. This construct is similar to our D-raf NCR1-CR2 with which we were not able to detect interaction with D-Ras or 14-3-3ζ. Because in two-hybrid analysis B-raf behaved similar to Raf-1 and not D-raf, I believe that although B-raf also contains an elongated N-terminus, this N-terminus does not have the same properties as amino acids 1-176 of D-raf.

APPENDIX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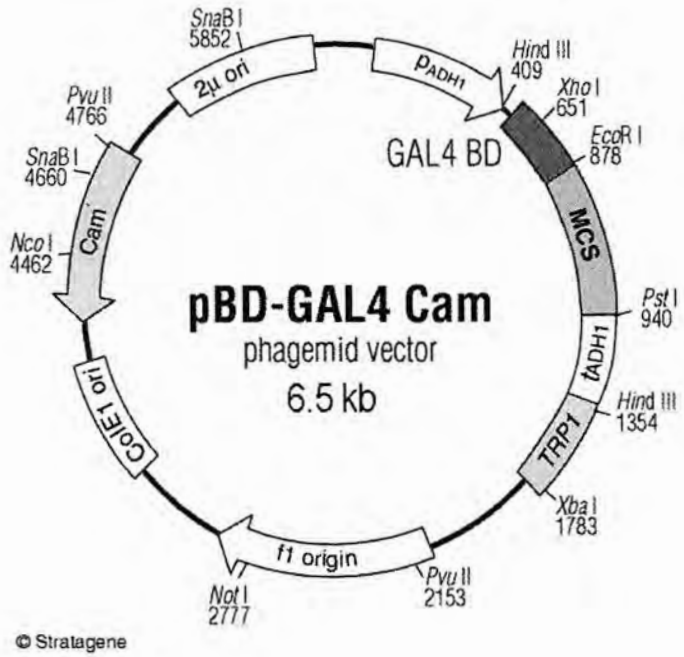
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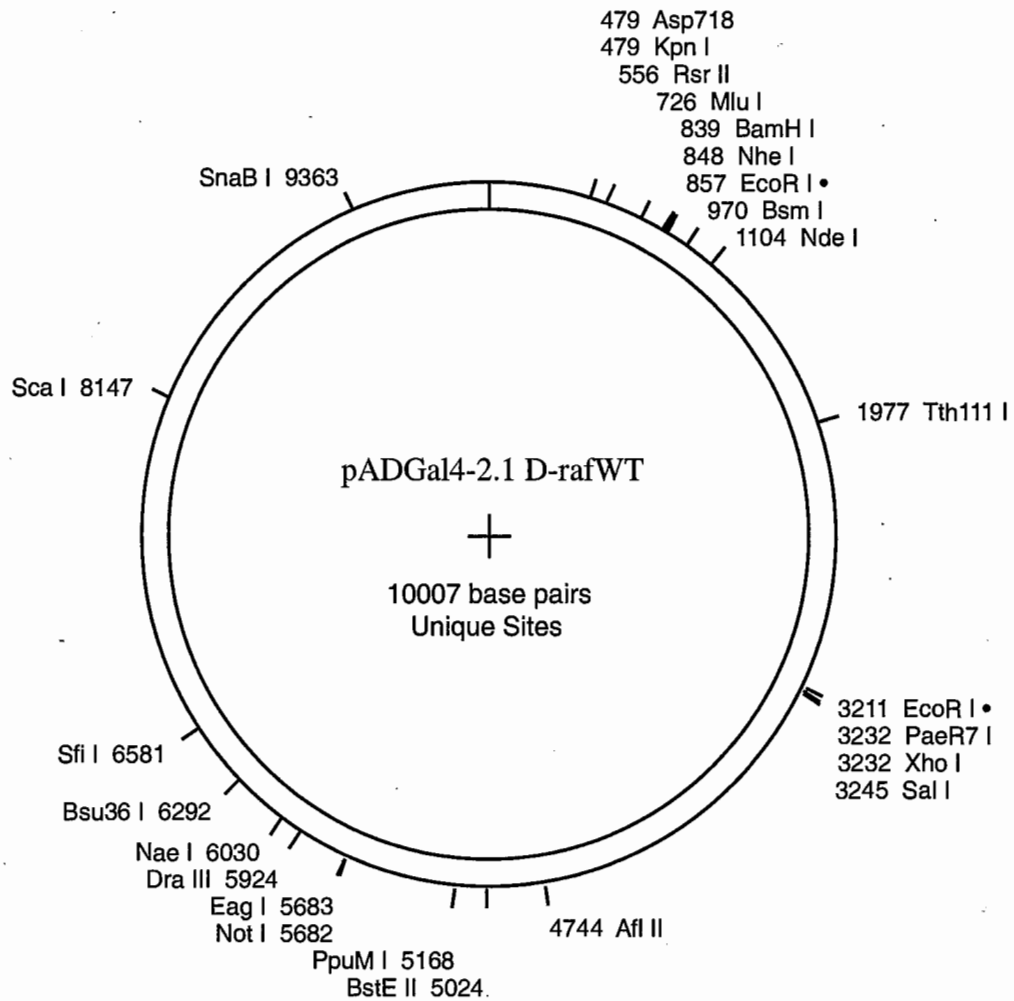
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3211 EcoR I •
3232 PaeR7 I
3232 Xho I
3245 Sal I
4744 Afl II
PpuM I 5168
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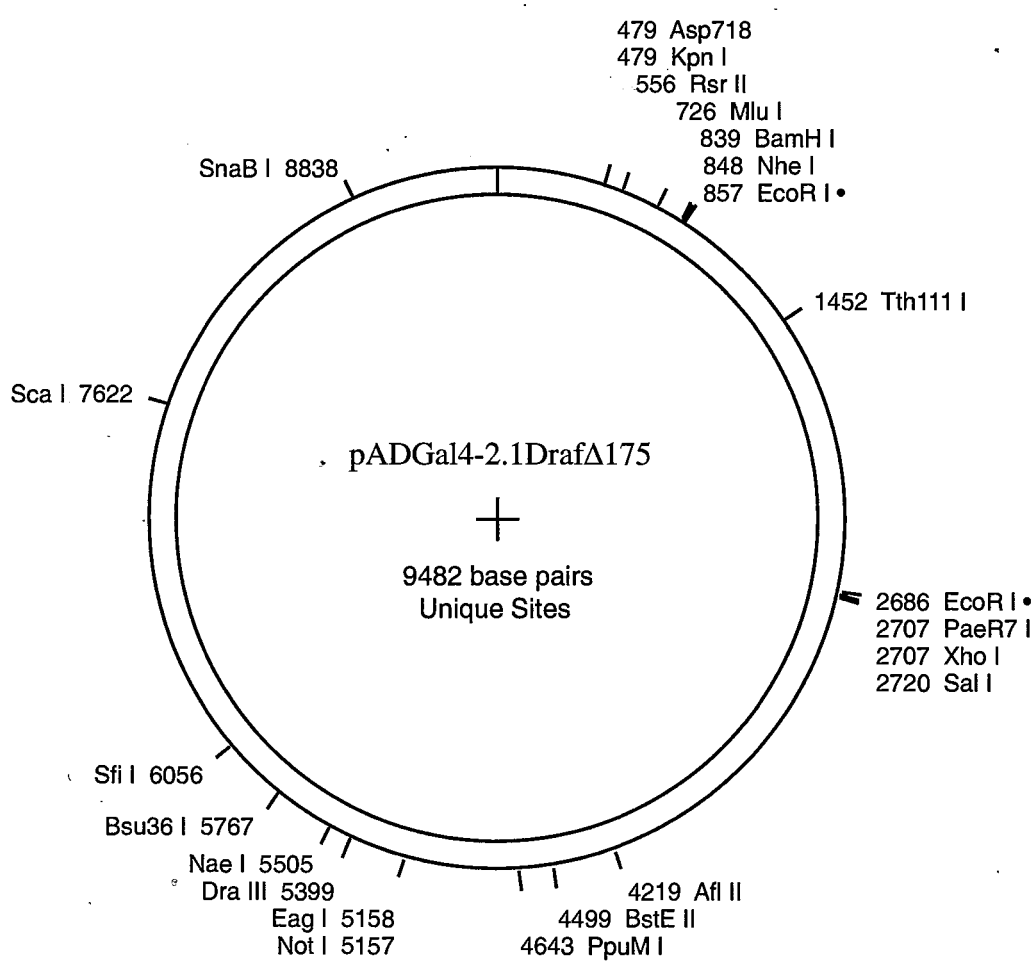
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2707 Xho I
2720 Sal I
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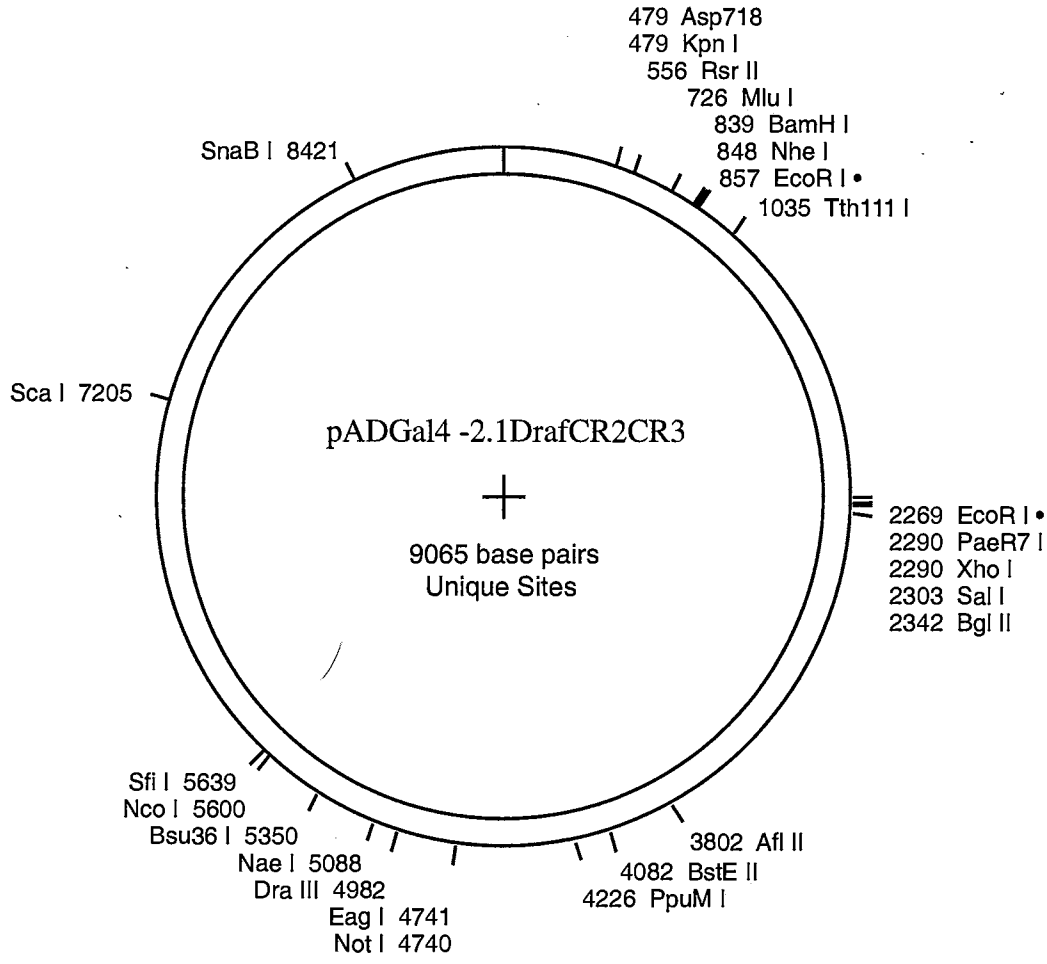
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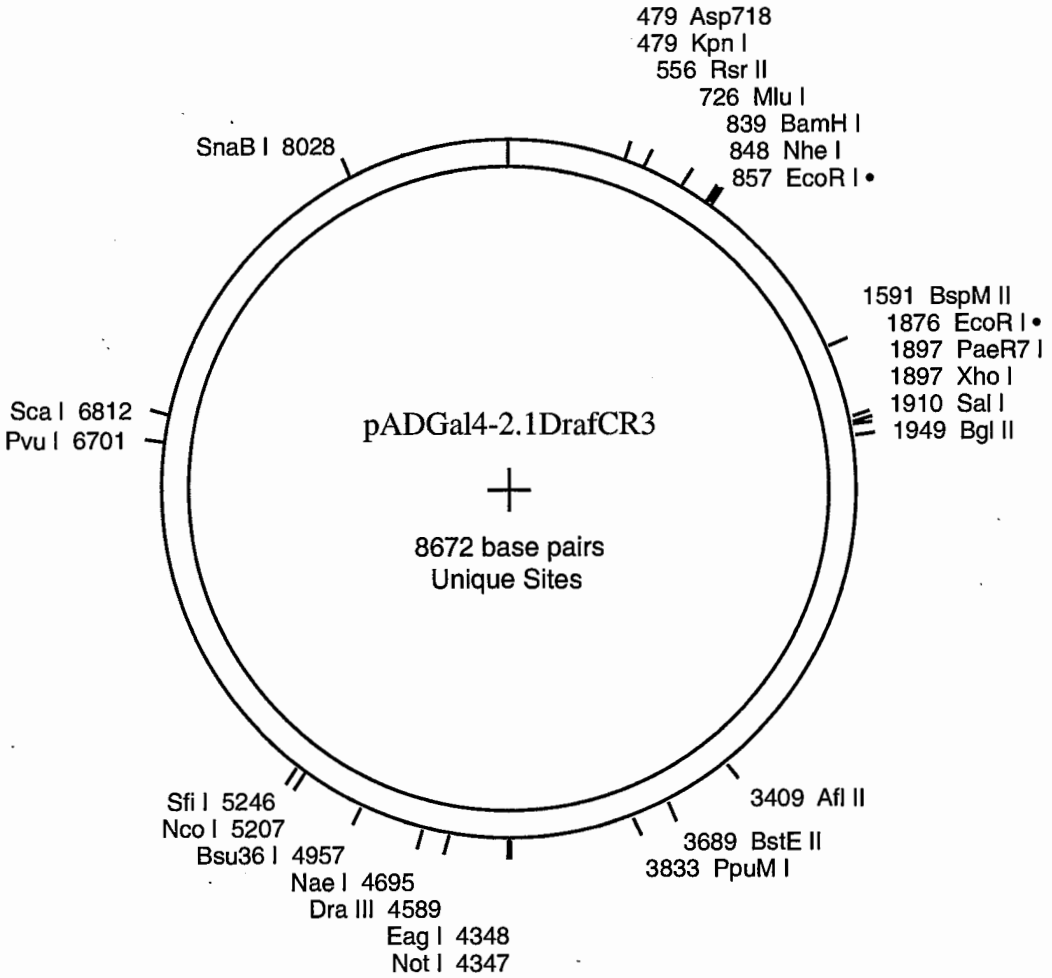
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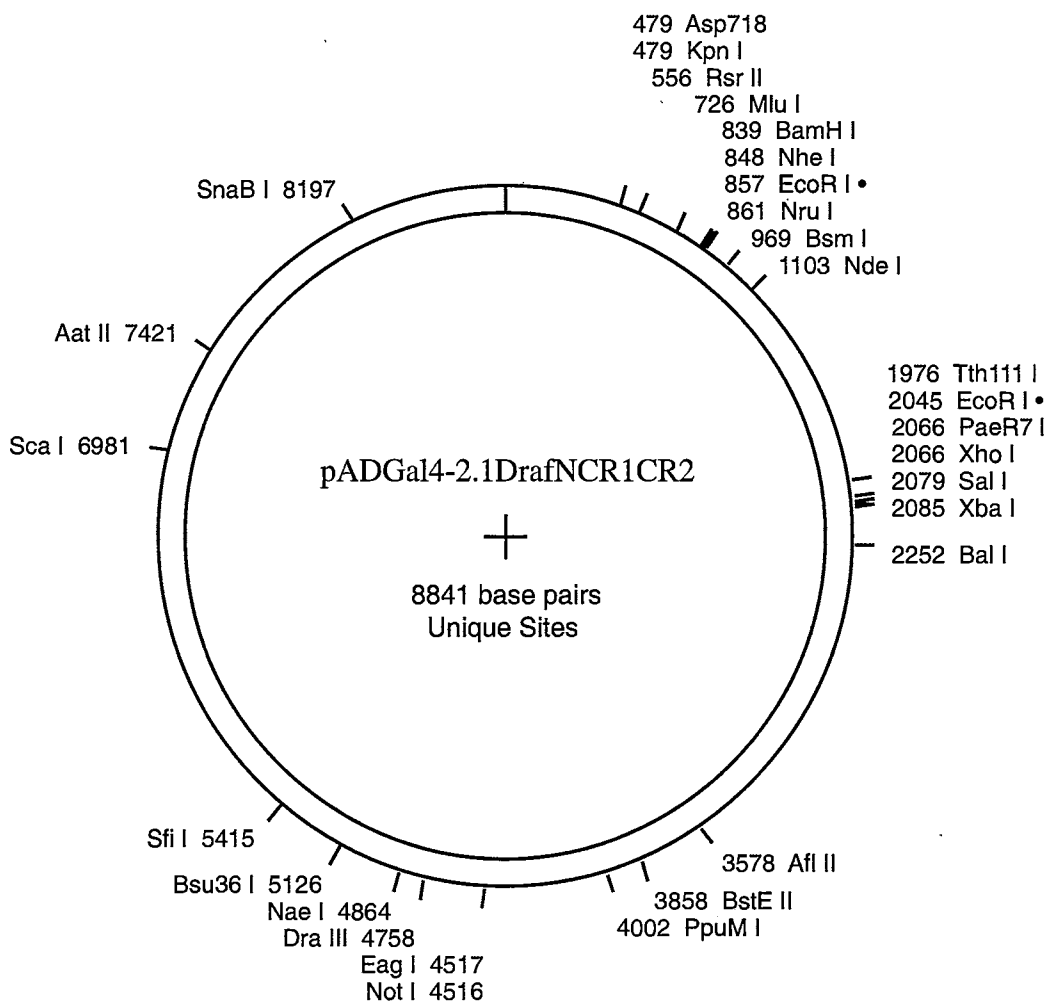
3409 Afl II
3689 BstE II
3833 PpuM I
Eag I 4348
Not I 4347
Dra III 4589
Nae I 4695
Bsu36 I 4957
Nco I 5207
Sfi I 5246
Sca I 6812
Pvu I 6701
SnaB I 8028
pADGal4-2.1DrafCR3
8672 base pairs
Unique Sites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1976 Tth111 I
2045 EcoR I •
2066 PaeR7 I
2066 Xho I
2079 Sal I
2085 Xba I
2252 Bal I
3578 Afl II
3858 BstE II
4002 PpuM I
Eag I 4517
Not I 4516
Dra III 4758
Nae I 4864
Bsu36 I 5126
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Sca I 6981
Aat II 7421
SnaB I 8197
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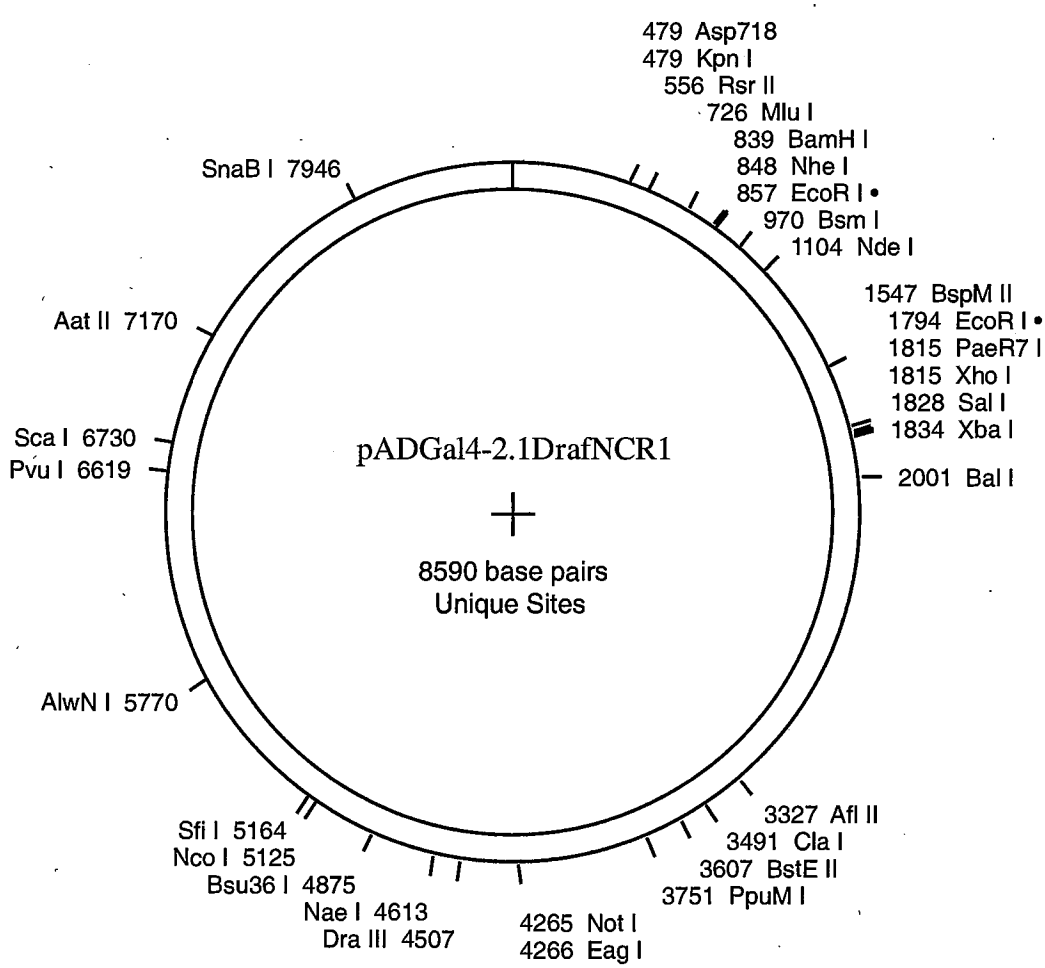
8841 base pairs
Unique Sites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1794 EcoR I •
1815 PaeR7 I
1815 Xho I
1828 Sal I
1834 Xba I
2001 Bal I
3327 Afl II
3491 Cla I
3607 BstE II
3751 PpuM I
4265 Not I
4266 Eag I
Nae I 4613
Dra III 4507
Bsu36 I 4875
Nco I 5125
Sfi I 5164
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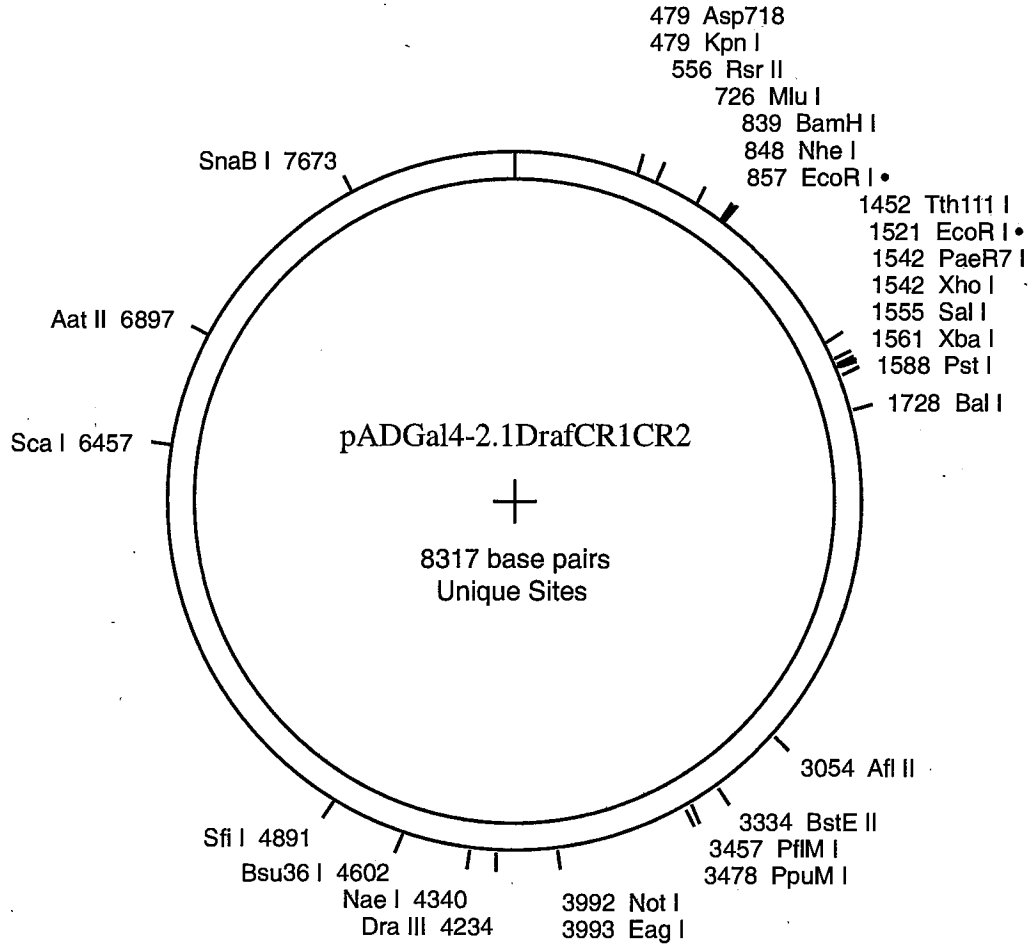
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1542 PaeR7 I
1542 Xho I
1555 Sal I
1561 Xba I
1588 Pst I
1728 Bal I
3054 Afl II
3334 BstE II
3457 PflM I
3478 PpuM I
3992 Not I
3993 Eag I
Dra III 4234
Nae I 4340
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Sca I 6457
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SnaB I 7673
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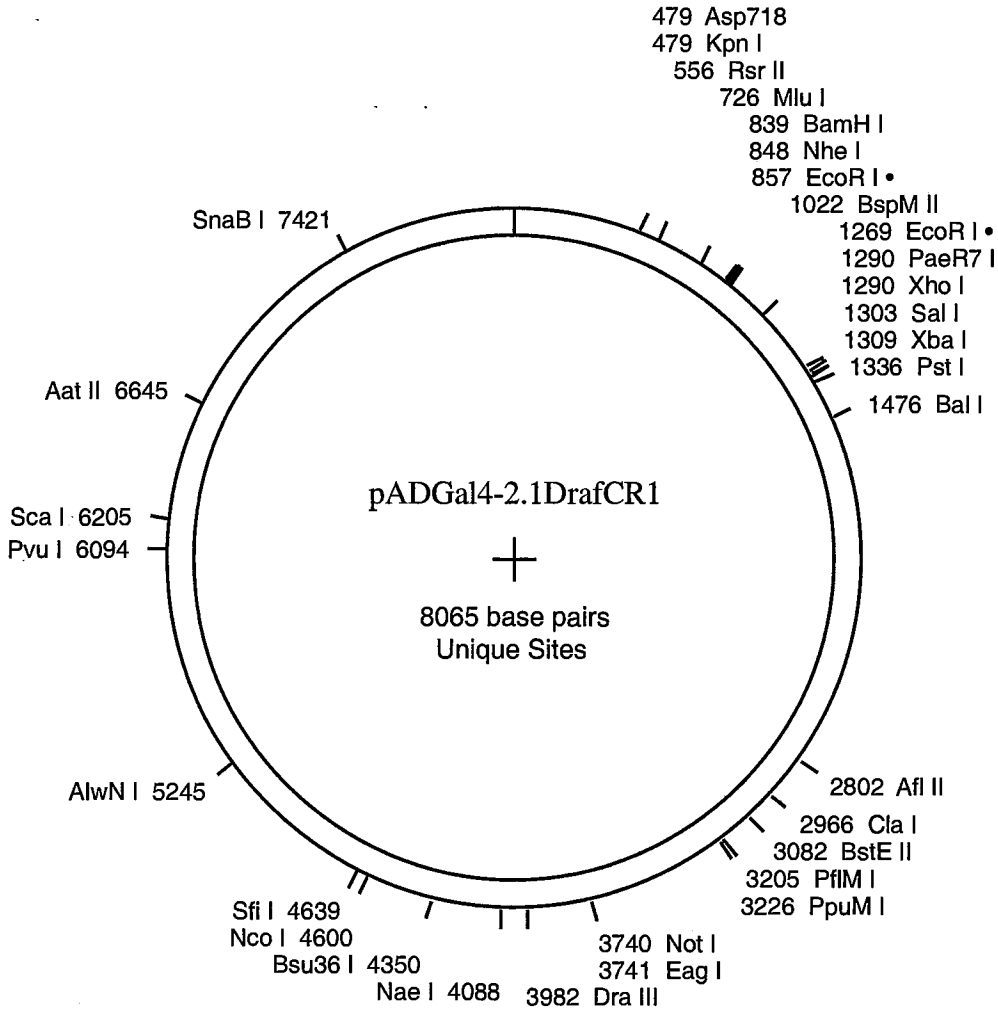
8317 base pairs
Unique Sites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1290 PaeR7 I
1290 Xho I
1303 Sal I
1309 Xba I
1336 Pst I
1476 Bal I
2802 Afl II
2966 Cla I
3082 BstE II
3205 PflM I
3226 PpuM I
3740 Not I
3741 Eag I
3982 Dra III
Nae I 4088
Bsu36 I 4350
Nco I 4600
Sfi I 4639
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Sca I 6205
Aat II 6645
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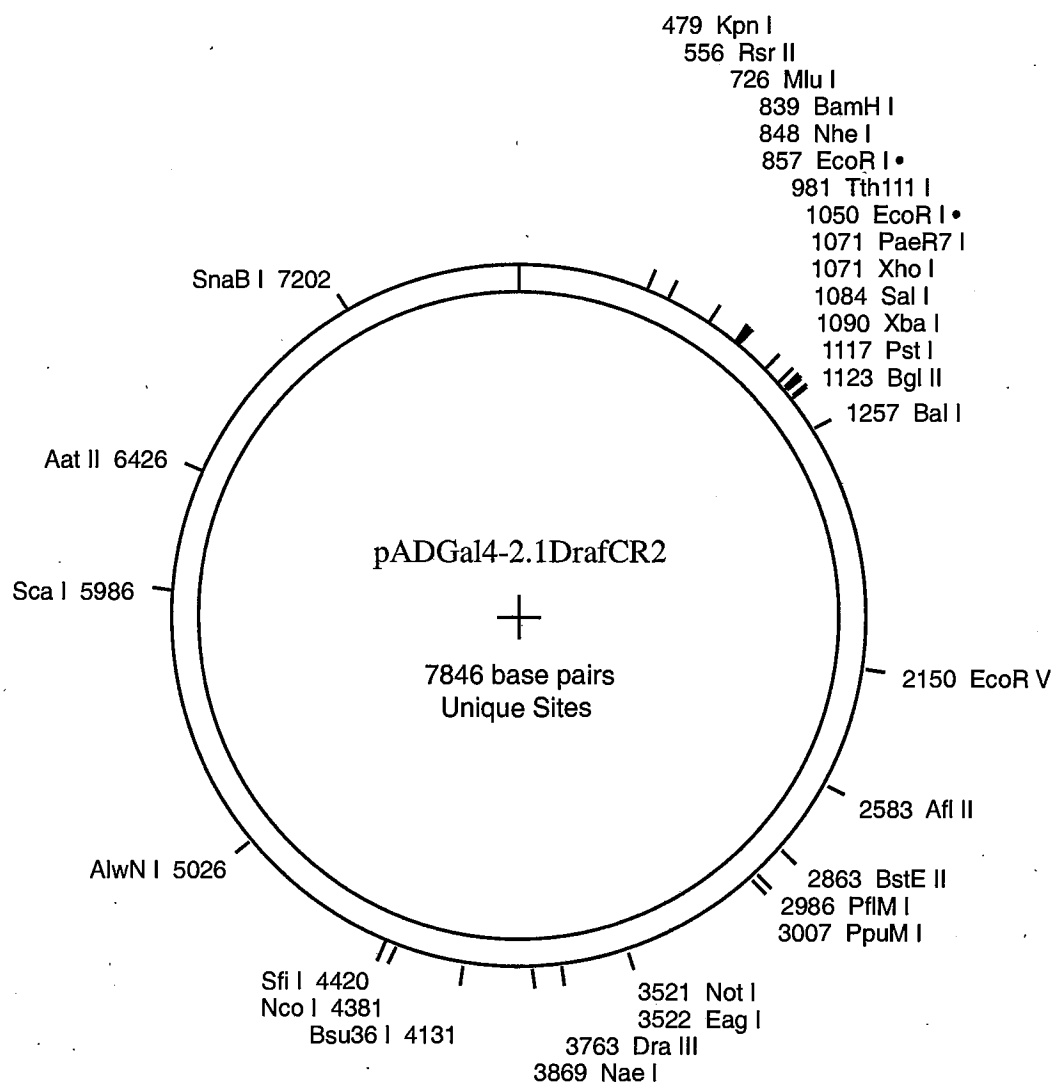
8065 base pairs
Unique Sites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1050 EcoR I •
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1071 Xho I
1084 Sal I
1090 Xba I
1117 Pst I
1123 Bgl II
1257 Bal I
2150 EcoR V
2583 Afl II
2863 BstE II
2986 PflM I
3007 PpuM I
3521 Not I
3522 Eag I
3763 Dra III
3869 Nae I
Sfi I 4420
Nco I 4381
Bsu36 I 4131
AlwN I 5026
Sca I 5986
Aat II 6426
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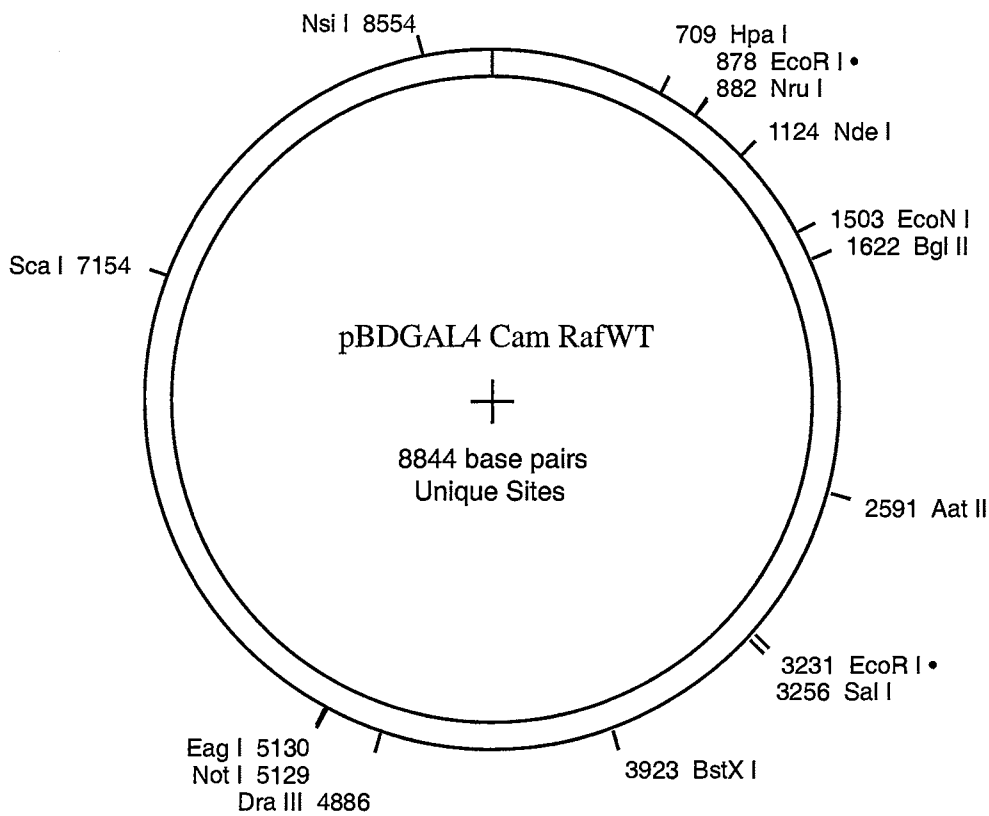
7846 base pairs
Unique Sites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882 Nru I
1124 Nde I
1503 EcoN I
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Sca I 7154
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Unique Sites
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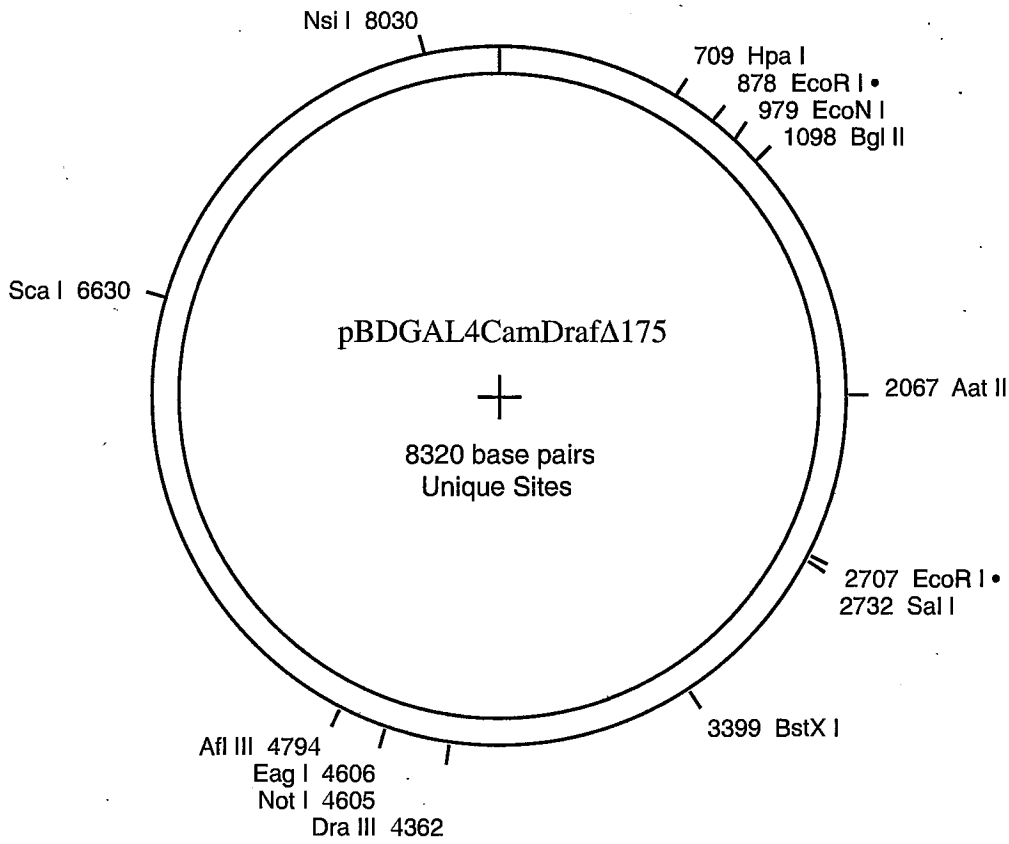
Eag I 5130
Not I 5129
Dra III 4886
3923 BstX I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Sca I 6630
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8320 base pairs
Unique Sites
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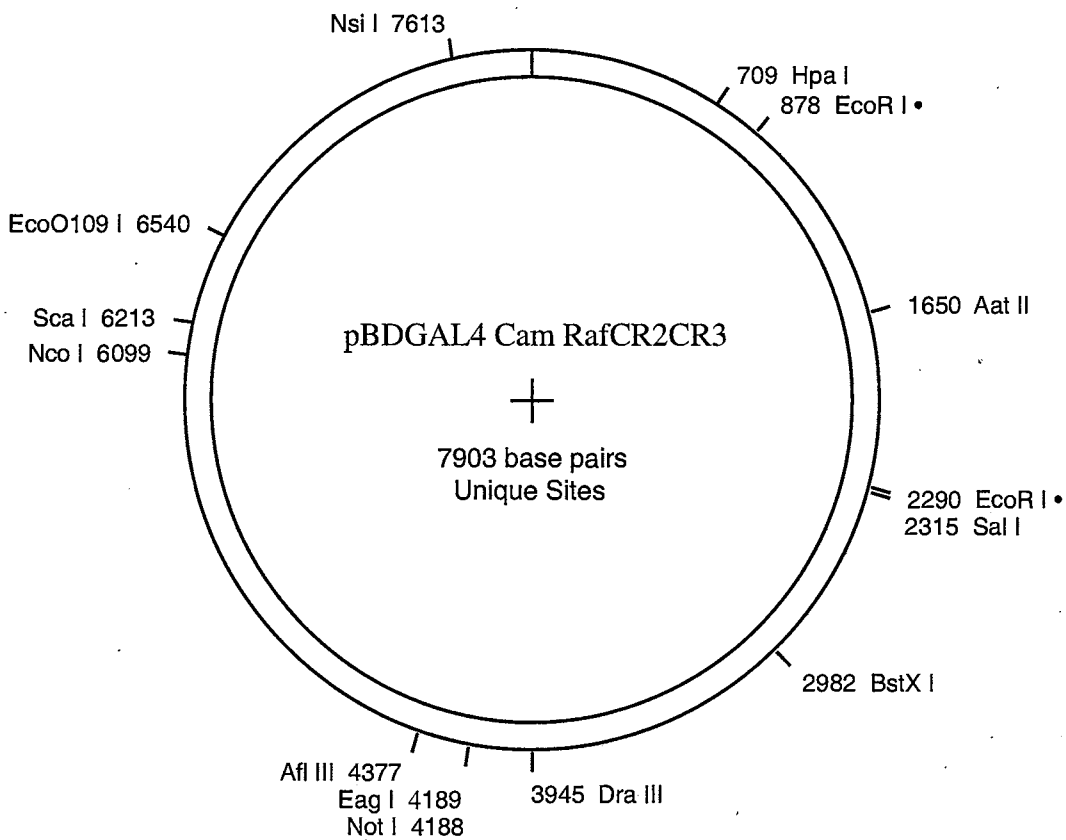
2707 EcoR I •
2732 Sal I
3399 BstX I
Afl III 4794
Eag I 4606
Not I 4605
Dra III 4362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878 EcoR I •
EcoO109 I 6540
Sca I 6213
Nco I 6099
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7903 base pairs
Unique Sites
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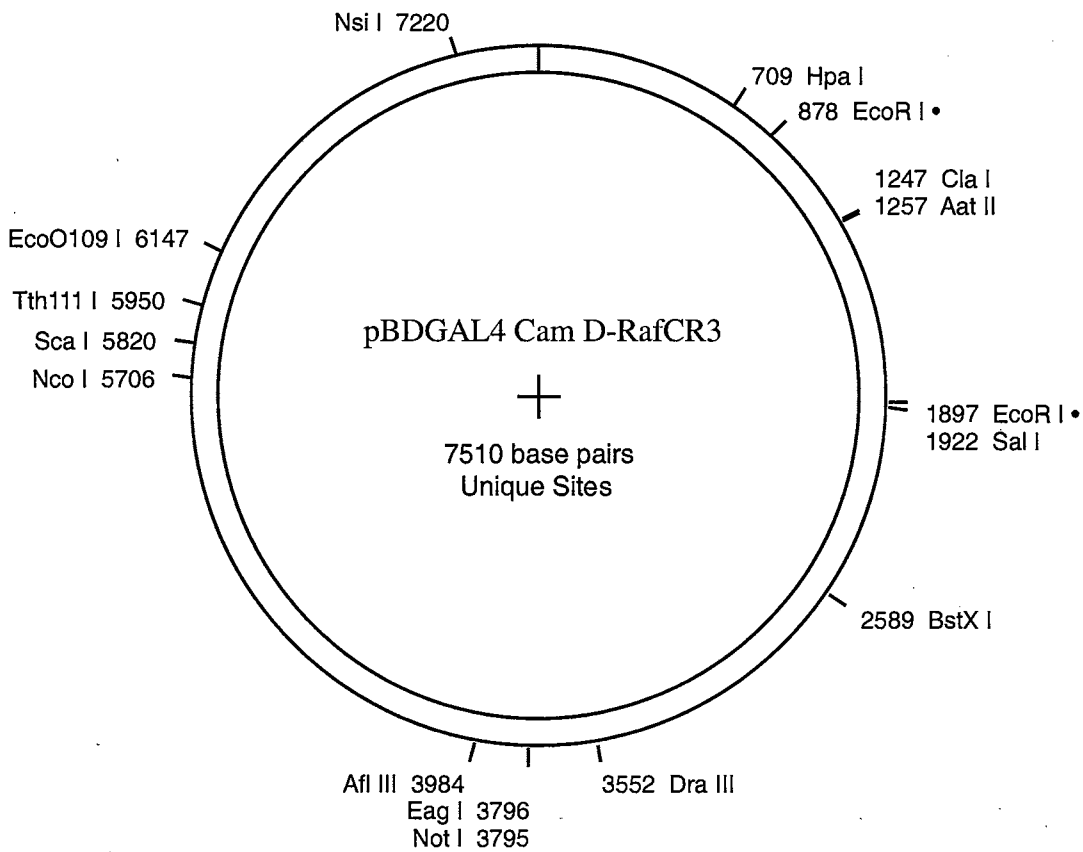
Eag I 4189
Not I 4188
3945 Dra III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878 EcoR I •
1247 Cla I
1257 Aat II
EcoO109 I 6147
Tth111 I 5950
Sca I 5820
Nco I 5706
pBDGAL4 Cam D-RafCR3
7510 base pairs
Unique Sites
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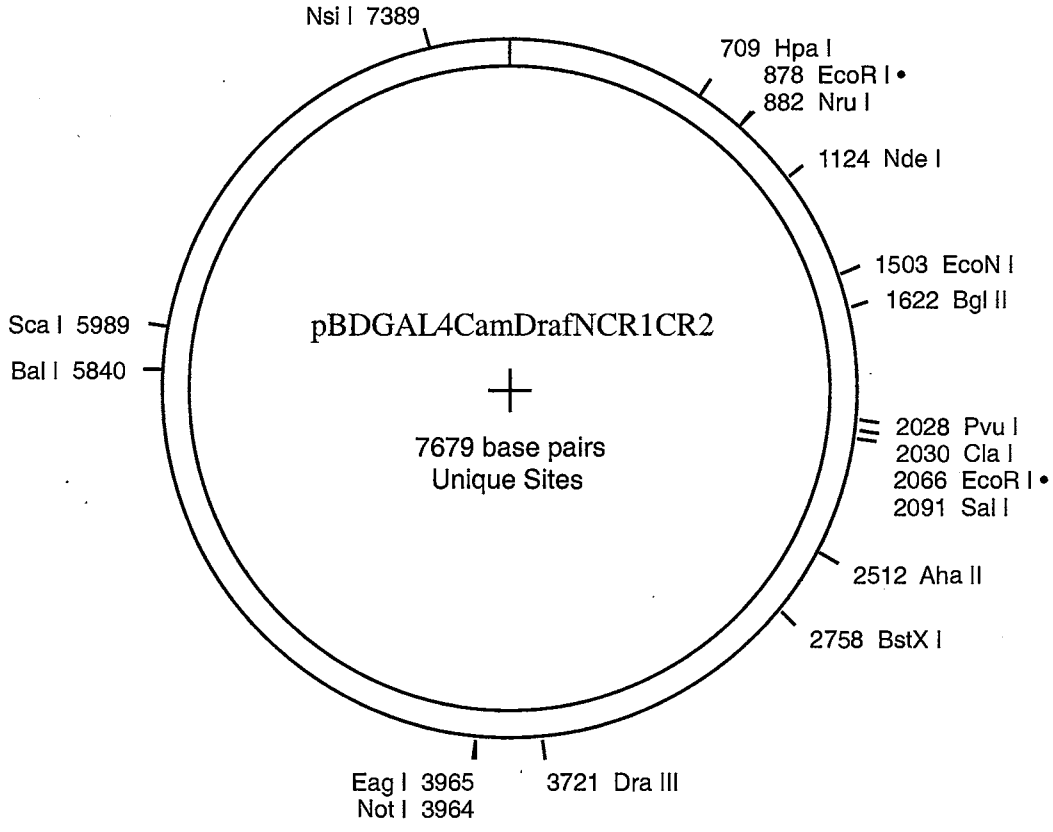
2589 BstX I
Afl III 3984
Eag I 3796
Not I 3795
3552 Dra III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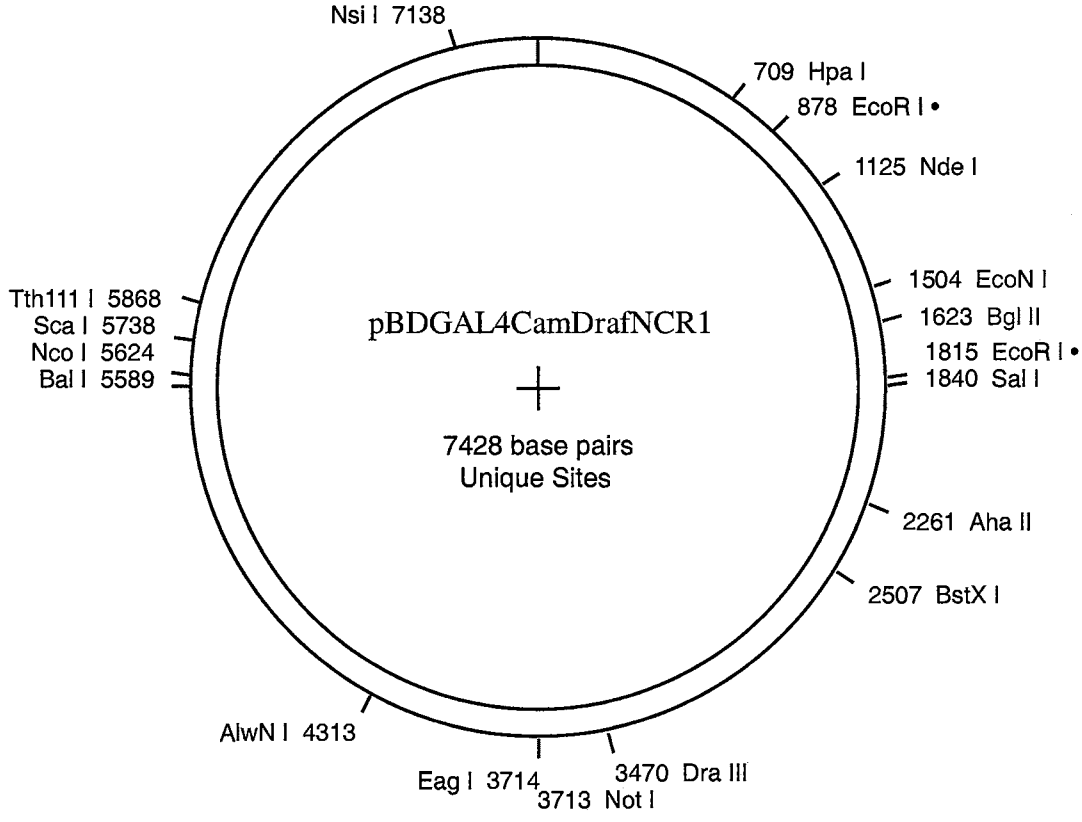
2028 Pvu I
2030 Cla I
2066 EcoR I •
2091 Sal I
2512 Aha II
2758 BstX I
3721 Dra III
Eag I 3965
Not I 3964
Bal I 5840
Sca I 5989
pBDGAL4CamDrafNCR1CR2
7679 base pairs
Unique Sites


Nsi I 7138
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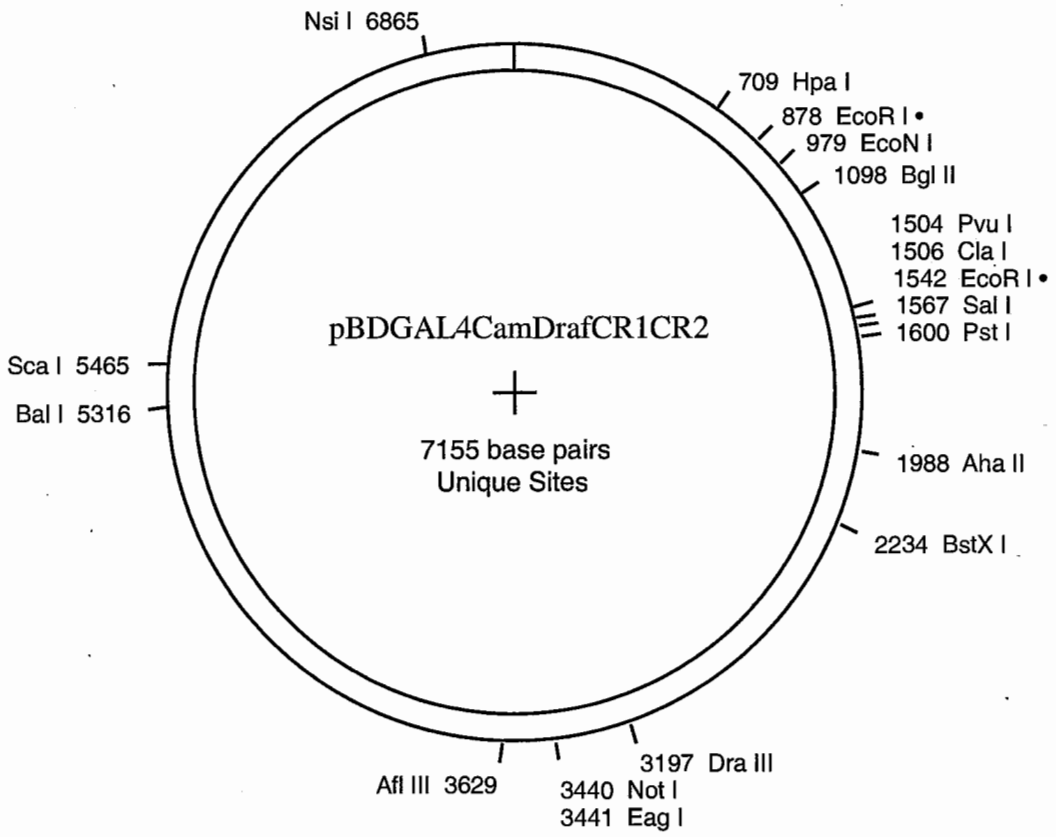
1840 Sal I
2261 Aha II
2507 BstX I
3470 Dra III
3713 Not I
Eag I 3714
AlwN I 4313
Tth111 I 5868
Sca I 5738
Nco I 5624
Bal I 5589
pBDGAL4CamDrafNCR1
7428 base pairs
Unique Sites


Nsi I 6865
709 Hpa I
878 EcoR I •
979 EcoN I
1098 Bgl II
1504 Pvu I
1506 Cla I
1542 EcoR I •
1567 Sal I
1600 Pst I
pBDGAL4CamDrafCR1CR2
7155 base pairs
Unique Sites
1988 Aha II
2234 BstX I
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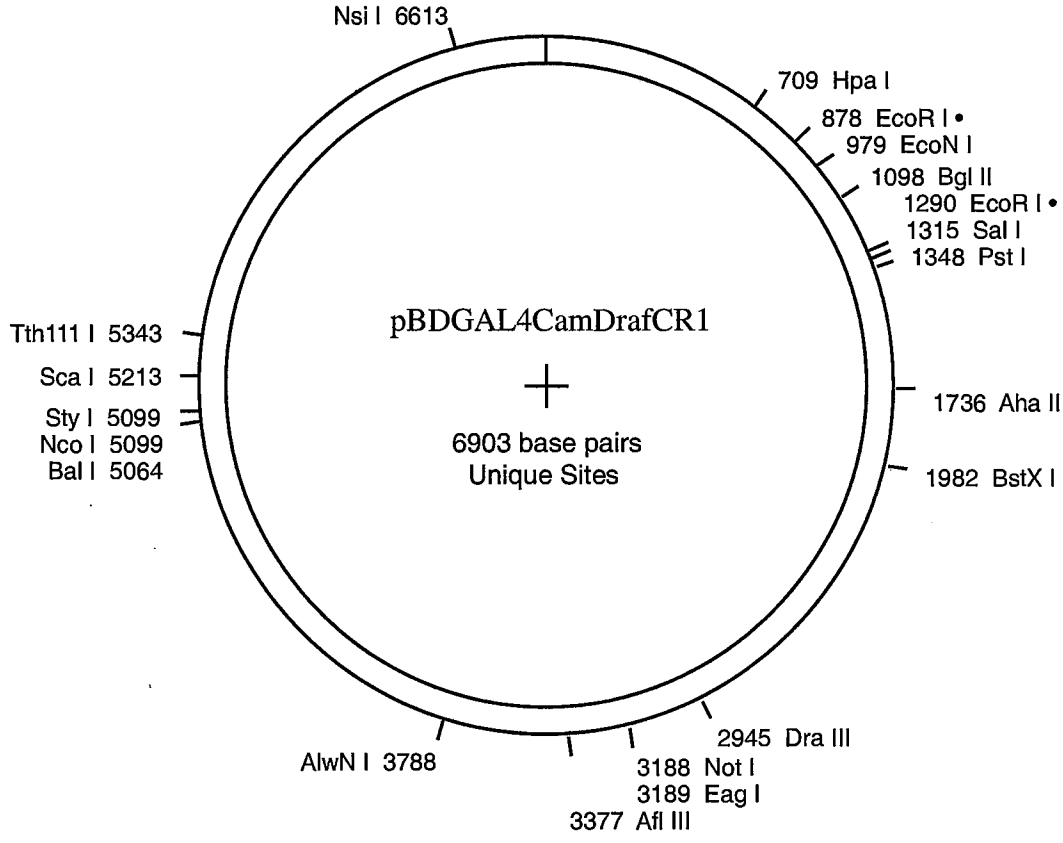
3441 Eag I
Afl III 3629
Bal I 5316
Sca I 5465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6903 base pairs
Unique Sites
1736 Aha II
1982 BstX I
2945 Dra III
3188 Not I
3189 Eag I
3377 Afl III
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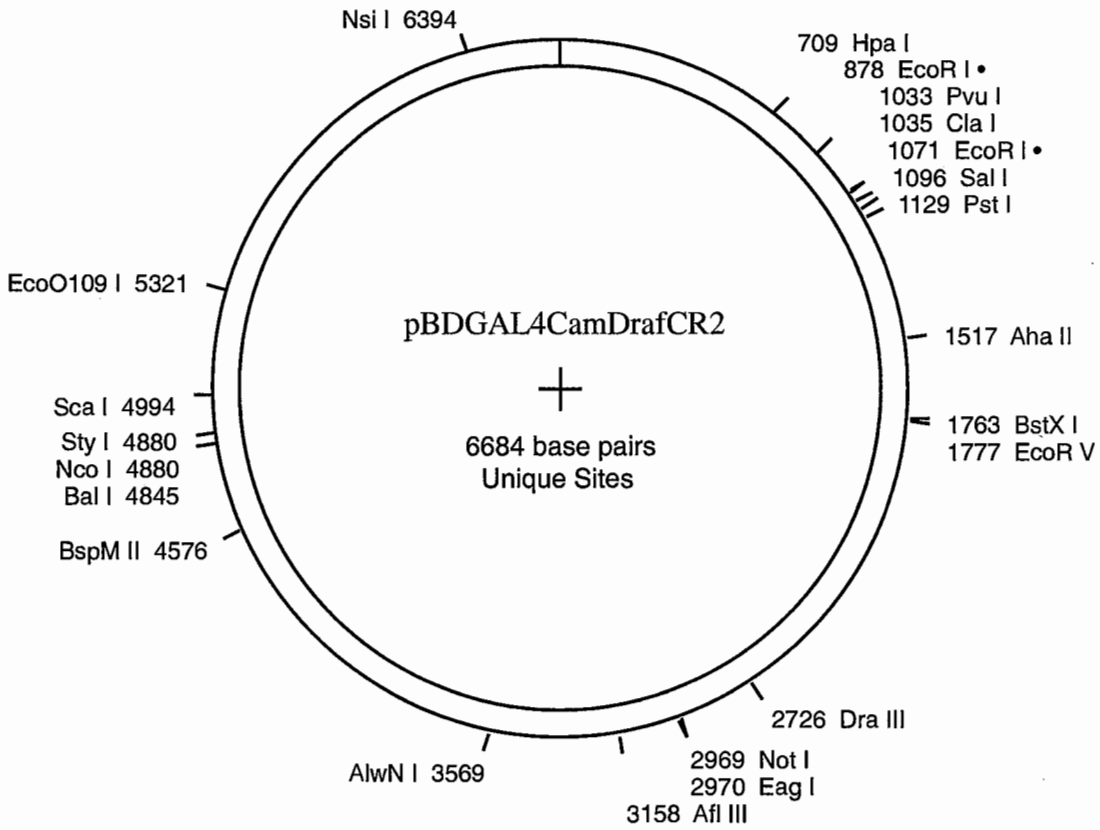
Tth111 I 5343
Sca I 5213
Sty I 5099
Nco I 5099
Bal I 5064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1096 Sal I
1129 Pst I
EcoO109 I 5321
pBDGAL4CamDrafCR2
1517 Aha II
Sca I 4994
Sty I 4880
Nco I 4880
Bal I 4845
6684 base pairs
Unique Sites
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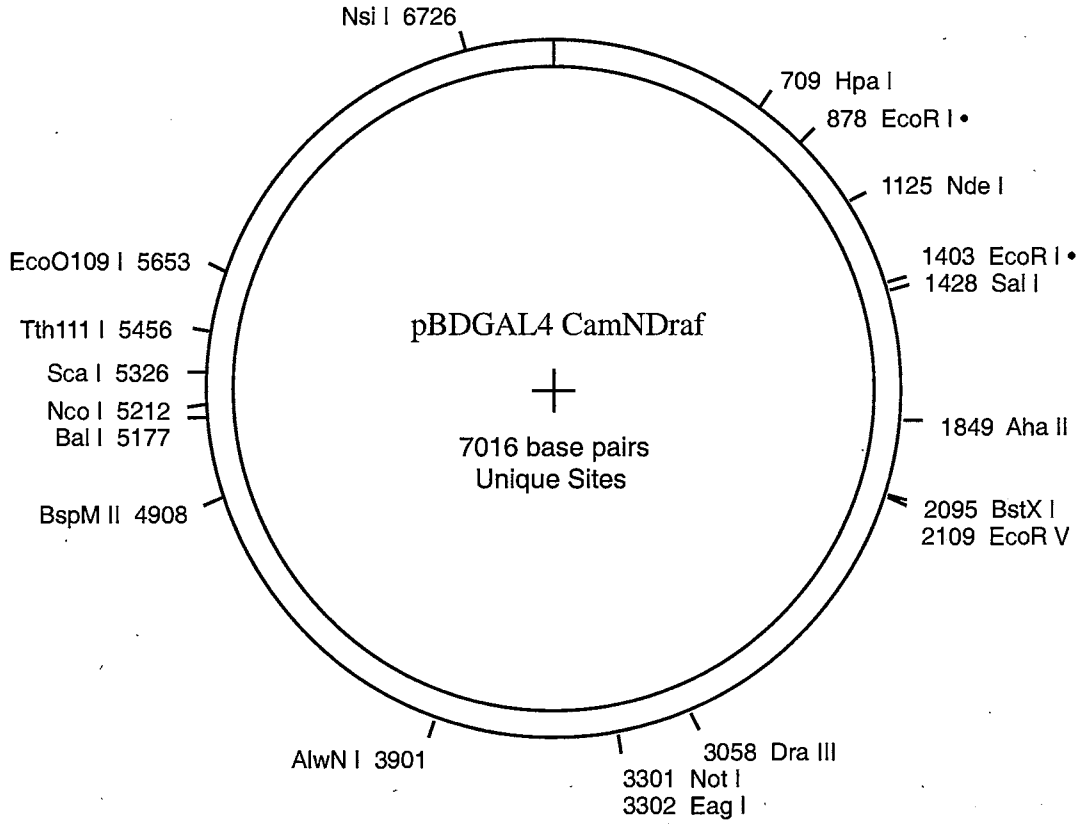
1777 EcoR V
BspM II 4576
2726 Dra III
AlwN I 3569
2969 Not I
2970 Eag I
3158 Afl III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709 Hpa I
878 EcoR I •
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3058 Dra III
3301 Not I
3302 Eag I
AlwN I 3901
BspM II 4908
Bal I 5177
Nco I 5212
Sca I 5326
Tth111 I 5456
EcoO109 I 5653
pBDGAL4 CamNDraf
7016 base pairs
Unique Sites

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