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N-terminal Domain Influences Steroid Activation of the Atlantic Sea Lamprey Corticoid Receptor

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Abstract

Lampreys are jawless fish that evolved about 550 million years ago at the base of the vertebrate line. Modern lampreys contain a corticoid receptor (CR), the common ancestor of the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), which first appear in cartilaginous fish, such as sharks. Until recently, 344 amino acids at the amino terminus of adult lamprey CR were not present in the lamprey CR sequence in GenBank. A search of the recently sequenced lamprey germline genome identified two CR sequences, CR1 and CR2, containing the 344 previously un-identified amino acids. CR1 also contains a novel four amino acid insertion in the DNA-binding domain (DBD). We studied corticosteroid and progesterone activation of CR1 and CR2 and found their strongest response was to 11-deoxycorticosterone and 11-deoxycortisol, the two circulating corticosteroids in lamprey. Based on steroid specificity, both CRs are close to elephant shark MR and distant from elephant shark GR. HEK293 cells that were transfected with full-length CR1 or CR2 and the MMTV promoter have about 3-fold higher steroid-mediated activation compared to HEK293 cells transfected with these CRs and the TAT3 promoter. Deletion of the amino-terminal domain (NTD) of lamprey CR1 and CR2 to form truncated CRs decreased transcriptional activation by about 70% in HEK293 cells that were transfected with MMTV, but increased transcription by about 6-fold in cells transfected with TAT3. This indicated that the promoter has an important effect on NTD regulation of transcriptional activation of the CR by steroids. Our results also indicate that the entire lamprey CR sequence is needed for an accurate determination of steroid-mediated transcription.

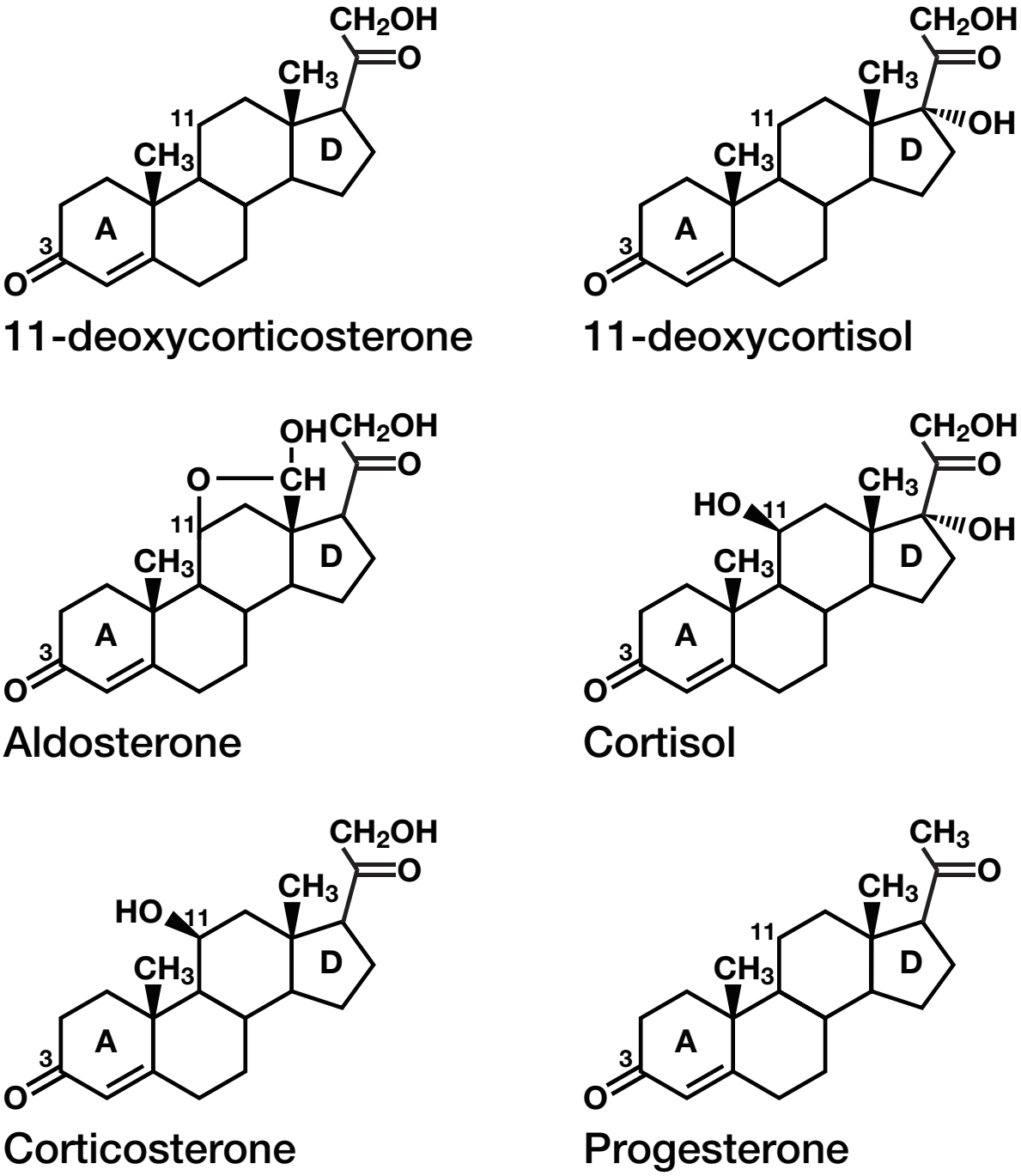
Key words: Atlantic sea lamprey, corticoid receptor, mineralocorticoid receptor, glucocorticoid receptor, evolution.

Running title: Regulation of Corticoid Receptor Transcription

1. Introduction.

The sea lamprey (Petromyzon marinus), belongs to an ancient group of jawless vertebrates known as cyclostomes, which last shared a common ancestor with vertebrates about 550 million years ago [1–3]. As an outgroup to the jawed vertebrates, lampreys are important for studying early events in the evolution of vertebrates [1–8]. Lampreys and hagfish, the other extant cyclostome lineage, contain a corticoid receptor (CR), which is the common ancestor to both the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) [4,9]. The MR and GR first appear as separate steroid receptors in sharks and chimeras [4,9–13]. The CR, MR and GR belong to the nuclear receptor family of transcription factors, which also contains the progesterone receptor, estrogen receptor and androgen receptor [14–18].

Early studies from Thornton's laboratory provided important insights into corticosteroid activation of lamprey CR [4]. These studies reported that several corticosteroids (Figure 1), including aldosterone, cortisol, corticosterone,11-deoxycorticosterone and 11-deoxycortisol activated lamprey CR. Although aldosterone, the physiological mineralocorticoid in humans and other terrestrial vertebrates [13,19–25], is the strongest activator of lamprey CR [4], neither lampreys nor hagfish synthesize aldosterone [4]. Later studies with lamprey revealed that 11-deoxycortisol and 11-deoxycorticosterone (Figure 1) are the circulating corticosteroids in sea lamprey [26–28].



68

69 **Figure 1. Structures of Corticosteroids and Progesterone.** 11-deoxycorticosterone and 11-
70 deoxycortisol are circulating corticosteroids in lamprey [26–28]. Aldosterone is the
71 physiological mineralocorticoid in terrestrial vertebrates [13,19–24]. Aldosterone is not
72 synthesized by lampreys [4]. Cortisol and corticosterone are glucocorticoids in terrestrial

73 vertebrates and fish [29–32]. Progesterone is an antagonist for human MR [16,33–35], but an
74 agonist for fish MR [34,36,37].

75

76 Until recently, due to complexities in sequencing and assembly of the lamprey's highly
77 repetitive and GC rich genome [3,38,39], DNA encoding 344 amino acids at the amino terminus
78 of lamprey CR was present on a separate contiguous sequence that was not joined with the rest
79 of the lamprey CR sequence and therefore not retrieved with BLAST searches of GenBank. The
80 recent sequencing of the sea lamprey germline genome [40] provided contiguous DNA for two
81 CR isoforms, CR1 and CR2, that encode the previously unassembled 344 amino acids at the
82 amino terminus. The sequences of lamprey CR1 and CR2 reveal that like other nuclear
83 receptors, lamprey CR is a multi-domain protein consisting of an N-terminal domain (NTD), a
84 central DNA-binding domain (DBD), a hinge domain and a C-terminal ligand-binding domain
85 (LBD) [4,14,41,42] (Figure 2). The DBD and LBD in lamprey CR are conserved in vertebrate
86 MRs and GRs (Figure 2) [9,43], while their sequences in the NTD and hinge domains have
87 diverged (Figure 2). The only sequence difference between lamprey CR1 and CR2 is a four
88 amino acid insertion in the DBD in CR1 is not present in the DBD in CR2 or in other GRs and
89 MRs. Except for the twelve nucleic acid insertion in the DBD of CR1, the nucleic acid
90 sequences are conserved throughout between CR1 and CR2 (Supplement Figure 1).

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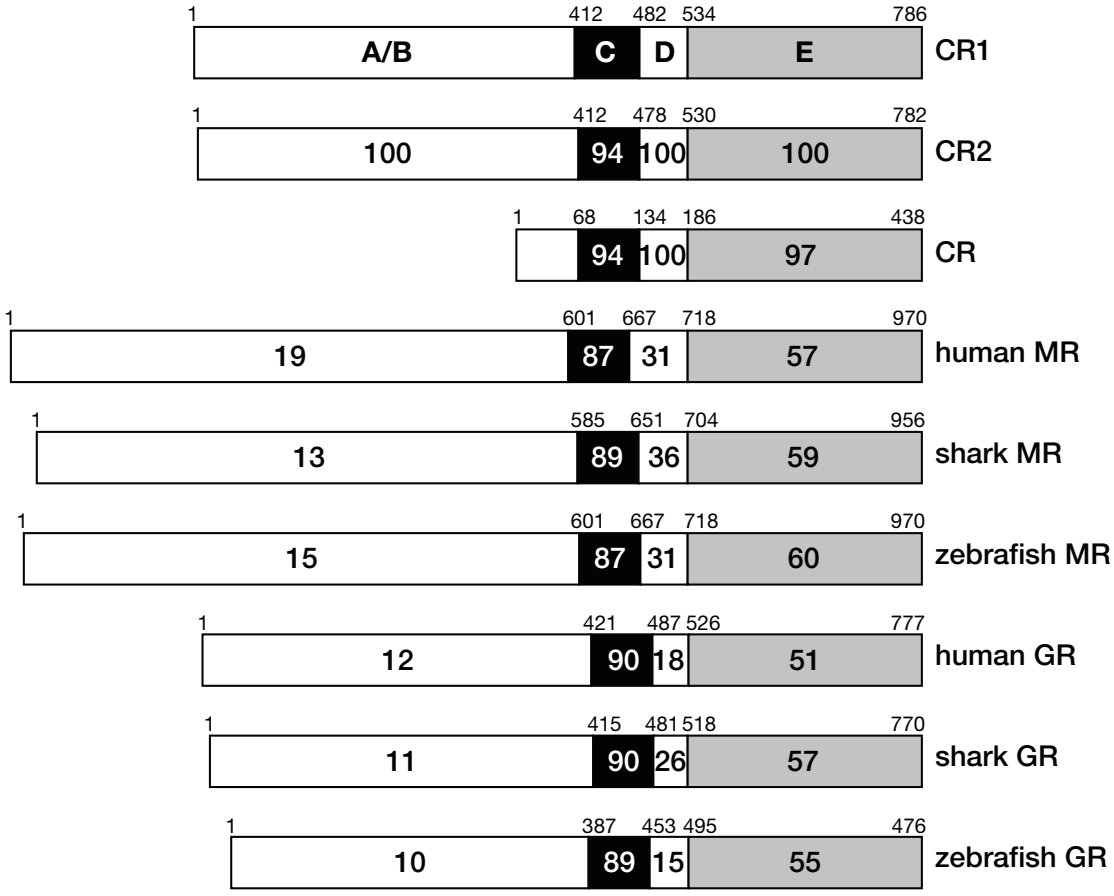


Figure 2. Comparison of the functional domains of lamprey CR1 to CR2, previously cloned lamprey CR and selected vertebrate MRs (human, elephant shark, zebrafish) and GRs (human, elephant shark, zebrafish). Lamprey CR1 and human MR and GR have 87% and 90% identity in the DBD and 57% and 51% identity in the LBD, respectively. Lamprey CR1 and elephant shark MR and GR have 89% and 90% identity in the DBD and 59% and 57% identity in the LBD, respectively. Lamprey CR1 and zebrafish MR and GR have 87% and 89% identity in the DBD and 60% and 55% identity in the LBD, respectively. This strong conservation of the DBD and LBD contrasts with the low sequence identity of 10-19%% and 15-36% between their NTD and hinge domains, respectively. Lamprey CR [4,43] is a partial CR sequence that was cloned from adult lamprey.

105 With the full sequences of the two lamprey CR isoforms in hand, we investigated four
106 questions about this receptor that shares common ancestry with gnathostome GRs and MRs
107 [9,13,43]. First, what is the response of lamprey CR to a panel of physiological corticosteroids
108 (aldosterone, cortisol, corticosterone, 11-deoxycorticosterone and 11-deoxycortisol) (Figure 1)
109 and progesterone for vertebrate GRs and MRs, and, second, how does this response compare to
110 the response to these steroids by elephant shark GR and MR [12,44]? Comparison of
111 corticosteroid activation of lamprey CR with elephant shark GR and MR can provide insights
112 into the evolution of corticosteroid specificity in the GR and MR.

113 Third, what is the role, if any, of the NTD in transcriptional activation of lamprey CR?
114 The NTD on human GR contains an activation function 1 (AF1) domain that is very important in
115 GR activation by steroids [45–53]. The NTD on human MR also contains an AF1 domain,
116 although it is a much weaker activator of the MR [46,54–56] compared to the AF1 on human
117 GR. The NTD on elephant shark GR also contains a strong AF1 [12]. The low sequence
118 identity (less than 20%) between the NTD in lamprey CR and in elephant shark MR and GR
119 raises the question: Is there AF1 activity in lamprey CR or did a strong AF1 evolve after CR
120 duplication and divergence to form vertebrate GR and MR?

121 Fourth, what is the role of the MMTV [57,58] and TAT3 [59] promoters in steroid-
122 mediated transcriptional activation of lamprey CR? That is, does lamprey CR have different
123 responses to corticosteroids in cells co-transfected with MMTV or TAT3 promoters, as we found
124 for cells co-transfected with either MMTV or TAT3 and either elephant shark MR [60] or GR
125 [12]. Comparison of activation of lamprey CR in cells with either MMTV or TAT3 with that of
126 elephant shark GR and MR could indicate whether lamprey CR was closer to the GR or to the
127 MR, and thus shed light on the evolution of the GR and MR from their common ancestor.

In this report, we used two metrics for evaluating activation by steroids of lamprey CR and other receptors. The first metric was the half maximal response (EC50) to various steroids, and the second metric was the strength (fold-activation) of transcription. Combined, these two metrics provide insights into the relevance of a steroid as a physiological ligand for the CR.

Our initial experiments focused on lamprey CR1 because RNA-Seq analysis indicates that CR1 is more highly expressed than CR2 (greater than 99%) in lamprey tissue. However, CR1 and CR2 have similar EC50s for corticosteroids. We find that the EC50s for activation by 11-deoxycorticosterone and 11-deoxycortisol, the two circulating corticosteroids in lampreys [26–28], of full-length lamprey CR1 in HEK293 cells with MMTV were 0.16 nM and 1.5 nM, respectively. These are the lowest EC50s for CR1 among the corticosteroids that we studied. Aldosterone, cortisol and corticosterone had EC50s from 2 nM to 9.9 nM for activation of CR1 in cells with MMTV. For truncated CR1, which lacks the NTD, the EC50 of 11-deoxycorticosterone for lamprey CR1 in HEK293 cells with MMTV was 0.4 nM, while EC50s of the other corticosteroids for lamprey CR1 increased from 3 to 6-fold.

Comparison of corticosteroid activation of CR with that of elephant shark MR and GR reveals that full-length and truncated elephant shark MR and the CR have similar EC50s for 11-deoxycorticosterone, 11-deoxycortisol and other corticosteroids, in contrast to full-length and truncated elephant shark GR, which has a negligible response to 11-deoxycortisol and weak responses to other corticosteroids [12], indicating that, based on steroid specificity, elephant shark MR is a closer to CR1 and CR2 than is elephant shark GR, which has diverged more from its common ancestor with the MR.

Interestingly, we found differences between the effect of the MMTV and TAT3 promoters on fold-activation of transcription of full-length CR and of truncated CR.

Unexpectedly, fold-activation of full-length CR1 to corticosteroids was about 3 to 4-fold higher in cells with the MMTV promoter than in cells transfected with the TAT3 promoter. Removal of the NTD on CR1 decreased fold-activation by corticosteroids of truncated CR1 in cells with MMTV by about 70% indicating that there is an activation function in the NTD. In contrast, compared to full-length CR1, transcriptional activation by corticosteroids of truncated CR1 in cells with TAT3 increased by about 6-fold, indicating that the CR1 NTD represses steroid activation in the presence of TAT3. These data indicate that regulation by the NTD evolved before the evolution of the GR and MR from their common ancestor in cartilaginous fishes, with divergence of specificity for various corticosteroids evolving in elephant shark GR and MR [12].

2. RESULTS

2.1 Comparison of functional domains on lamprey CR to domains on selected vertebrate MRs and GRs.

To begin to understand the evolution of the MR and GR from the CR, we compared the sequences of functional domains on lamprey CRs with corresponding domains in human, elephant shark and zebrafish MRs and GRs (Figure 2). The sequences of LBD and hinge domain of lamprey CR1 and CR2 have more similarity to the LBD and hinge domain on vertebrate MRs than to the GRs as has been reported for lamprey CR [9,13,43,61]. The strong conservation of the DBD and LBD contrasts with the low sequence identity of 10-19% between the NTDs and 15-36% between the hinge domains. The low similarity of the NTD on the CR to the NTD on elephant shark MR and GR of 13% and 12%, respectively, indicates that there was rapid evolution of the NTD during the divergence of a distinct MR and GR from their CR ancestor.

Moreover, the NTD of elephant shark GR has only 21% sequence identity with the NTD on elephant shark MR, additional evidence for rapid evolution of the NTD early in the evolution of the MR and GR [12].

Although most of the sequence divergence among these receptors occurred in the NTD and hinge domain, there is an insertion of four amino acids in the DBD of CR1 that is not found in the DBD of either CR2 or the MR and GR (Figure 3). Otherwise, the DBD in both CRs is highly conserved in the MR and GR, with CR2 being closest to the DBD in the other MRs and GRs. Based on this analysis of the DBD, CR2 appears to be related to the common ancestor of the MR and GR.

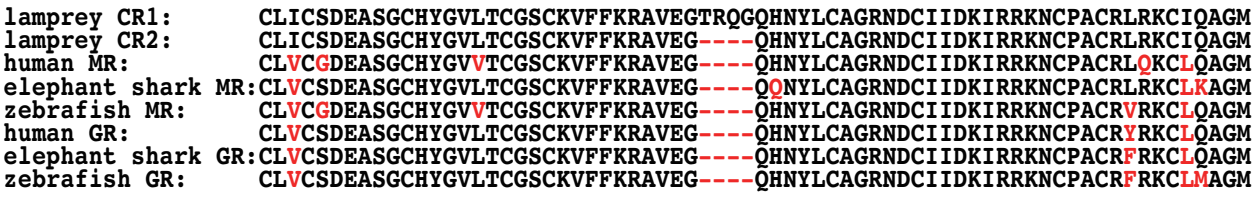


Figure 3. Comparison of the DNA-binding domains on lamprey CR1, CR2, elephant shark MR and GR and human MR and GR. The DNA-binding domain of lamprey CR1 has a unique insertion of four amino acids. Otherwise the DNA-binding domain on CR1 and CR2 are identical. Differences between the amino acid sequence of lamprey CR and selected vertebrate MRs and GRs are shown in red. Comparison of the nucleic acid sequences of CR1 and CR2 (Supplement Figure 1) reveal that except for the twelve nucleic acid insertion in CR1, the nucleic acid sequences are conserved between CR1 and CR2.

2.2 RNA-Seq analysis indicates that lamprey CR1 is the predominate CR expressed in lamprey.

To gain an insight into the relative biological importance of CR1 and CR2 in lamprey, we used RNA-Seq analysis to investigate the relative expression of lamprey CR1 and CR2 in lamprey tissues using databases in GenBank [3,40]. As shown in Table 1, RNA-Seq analysis reveals that expression of lamprey CR1 is substantially higher than CR2. Indeed, CR1 expression is over 100-fold higher than CR2 in the intestine and kidney in the larval stage, in the parasitic and adult stages, as well as in the parasitic liver and in the adult intestine, kidney and brain.

Table 1. RNA-Seq Analysis of Expression of Lamprey CR1 and CR2.

	Larval Stage		Parasitic Stage				Adult Stage		
	Intestine	Kidney	Proximal intestine	Distal intestine	Kidney	Liver	Brain	Intestine	Kidney
CR1	21.78	10.36	23.23	19.44	21.49	24.96	2471	35.48	30.32
CR2	0.1	0.13	0.22	0.32	0.09	0	0	0.2	0.35

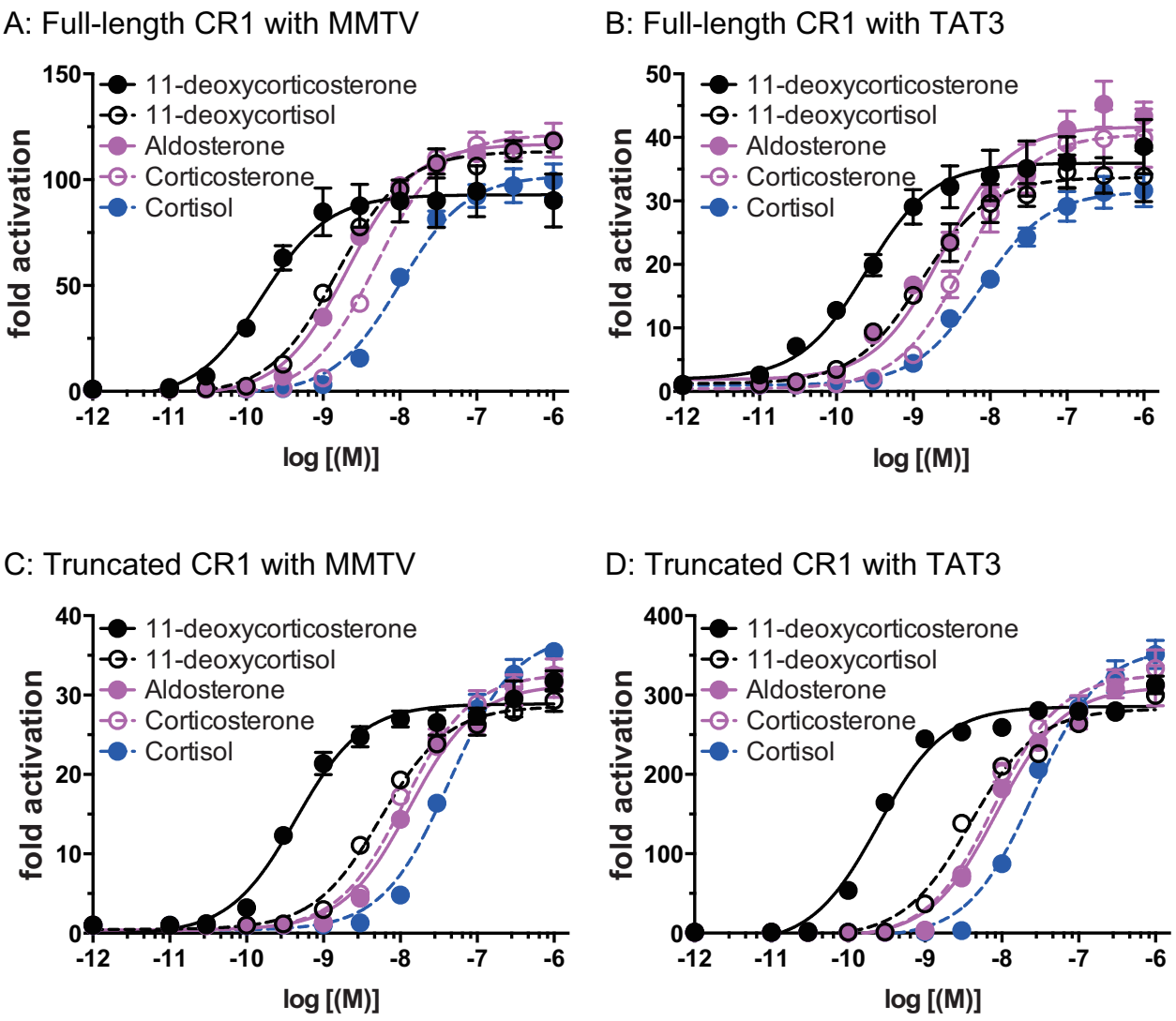
Single-end RNA-Seq reads of sea lamprey, *Petromyzon marinus* for intestine and kidney from larval stage, intestine, kidney and liver from parasitic stage, brain, intestine and kidney from adult stage, were downloaded from database of National Center for Biotechnology Information (accession number: PRJNA50489). The relative measure of transcript abundance is FPKM (fragments per kilobase of transcript per million mapped reads) [62]. FPKM values were estimated by normalizing gene length, followed by normalizing for sequencing depth.

2.3 Corticosteroid-dependent and promoter-dependent activation of full-length and truncated lamprey CR1 and CR2.

To gain a quantitative measure of corticosteroid activation of full-length and truncated lamprey CR1 and CR2, we determined the concentration dependence of transcriptional activation by corticosteroids of full-length lamprey CR1 transfected into HEK293 cells with either an MMTV-luciferase promoter (Figure 4A) or a TAT3 luciferase promoter (Figure 4B). A parallel

study was done with truncated lamprey CR1 (Figure 4 C, D). Luciferase levels were used to calculate an EC50 value and fold-activation for each steroid for lamprey CR1 in HEK293 cells with either MMTV (Table 2) or TAT3 (Table 2).

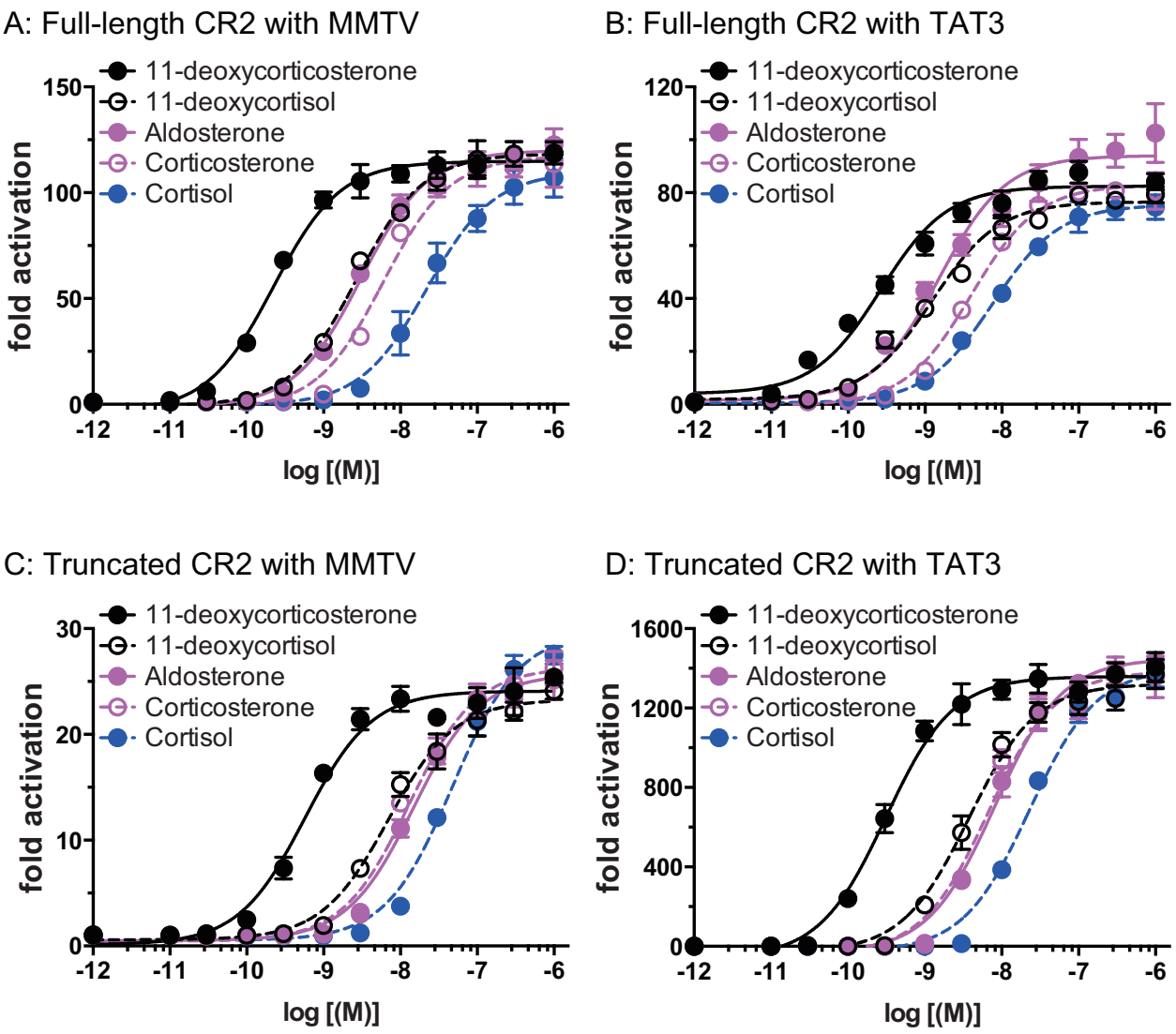
Similar experiments were performed for corticosteroid activation of full-length and truncated lamprey CR2 in HEK293 cells, containing either the MMTV or TAT3 promoters (Figure 5). EC50 values and fold-activation for each steroid for lamprey CR2 in the presence of either MMTV or TAT3 are shown in Table 2.



224

225 **Fig. 4. Concentration-dependent transcriptional activation by corticosteroids of full length**
226 **and truncated lamprey CR1.** Plasmids for full-length or truncated lamprey CR1 were
227 expressed in HEK293 cells with either an MMTV-luciferase promoter or a TAT3-luciferase
228 promoter. Cells were treated with increasing concentrations of either aldosterone, cortisol,
229 corticosterone, 11-deoxycortisol, 11-deoxycorticosterone or vehicle alone (DMSO). Results are
230 expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the activity of
231 vector with vehicle (DMSO) alone as 1. A. Full-length CR1 with MMTV-luc. B. Full-length
232 CR1 with TAT3-luc. C. Truncated lamprey CR1 with MMTV-luc. D. Truncated lamprey CR1
233 with TAT3-luc.

234



235

236 **Fig. 5. Concentration-dependent transcriptional activation by corticosteroids of full length**
237 **and truncated lamprey CR2.** Plasmids for full-length or truncated lamprey CR2 were
238 expressed in HEK293 cells with either an MMTV-luciferase promoter or a TAT3-luciferase
239 promoter. Cells were treated with increasing concentrations of either aldosterone, cortisol,
240 corticosterone, 11-deoxycortisol, 11-deoxycorticosterone or vehicle alone (DMSO). Results are
241 expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the activity of
242 vector with vehicle (DMSO) alone as 1. A. Full-length CR2 with MMTV-luc. B. Full-length
243 CR2 with TAT3-luc. C. Truncated lamprey CR2 with MMTV-luc. D. Truncated lamprey CR2
244 with TAT3-luc.

245

The results in Figures 4, 5 and 6 and Table 2 show that steroid-mediated transcriptional activation of full-length and truncated lamprey CR1 and CR2 in HEK293 cells is different in the presence of the MMTV and TAT3 promoters. In cells containing the MMTV promoter and full-length CR1, 11-deoxycortisol and 11-deoxycorticosterone, the two circulating corticosteroids in lamprey [26–28] have EC50s of 1.5 nM and 0.16 nM respectively (Table 2). These are the lowest EC50s of the tested corticosteroids, although aldosterone, corticosterone and cortisol also have low EC50s, which vary from 2.1 nM to 9.9 nM. In cells with MMTV, loss of the NTD raises the EC50 for corticosteroids, although the values for 11-deoxycorticosterone (0.4 nM) and 11-deoxycortisol (5.6 nM) are low. Cortisol has EC50 of 43 nM. Loss of the NTD results in a decline of fold-activation of CR1 by about 75%.

Analysis of Figure 4A and 4B and Table 2 shows that activation of full-length CR1 by corticosteroids is about 3-fold higher in HEK293 cells with the MMTV promoter than in cells with the TAT3 promoter. Removal of the NTD from CR1 leads to a decrease in fold-activation of about 75% by corticosteroids in cells with MMTV promoter (Figure 4C vs 4A). In contrast, in HEK293 cells with truncated CR1 and TAT3, corticosteroid stimulated transcription is about 7-fold higher than in cells with full-length CR1 and TAT3 (Table 2). Moreover, in cells with truncated CR1 and TAT3 corticosteroid-mediated transcriptional activation is about 50-fold higher than in cells with truncated CR1 and MMTV (Table 2).

Table 2. Corticosteroid and Progesterone Activation of Lamprey CR1 and CR2 in HEK293 cells with an MMTV promoter or a TAT3 promoter.

MMTV-luc		**DOC**	**S**	**ALDO**	**CORT**	**Cortisol**	**Progesterone**
Lamprey CR1 Full Seq.	EC50 (nM)	0.16	1.5	2.1	4.8	9.9	16.2
	Fold-Activation (±SEM)	95 (± 12)	106 (± 2.5)	112 (± 2.5)	116 (± 6.0)	92 (± 5.3)	81 (± 6.1)

Lamprey CR1 Truncated Seq.	EC50 (nM)	0.4	5.6	13.2	11.2	43.1	53.8
	Fold-Activation (±SEM)	27 (±1.0)	26 (± 1.3)	26 (± 0.9)	29 (± 1.7)	28 (± 1.8)	14 (± 0.4)
Lamprey CR2 Full Seq.	EC50 (nM)	0.2	2.7	3.1	5.9	14.2	26.4
	Fold-Activation (±SEM)	113 (± 4.8)	116 (± 9)	113 (± 6.4)	110 (± 7.0)	888 (± 6.2)	70 (± 5.2)
Lamprey CR2 Truncated Seq.	EC50 (nM)	0.6	7.0	14.9	13.0	47.8	63.5
	Fold-Activation (±SEM)	23.0 (±0.8)	21.3 (±0.9)	23.3 (± 0.8)	23.3 (± 0.8)	21.2 (± 0.8)	9.7 (± 1.0)
TAT3-luc		**DOC**	**S**	**ALDO**	**CORT**	**Cortisol**	**Progesterone**
Lamprey CR1 Full Seq.	EC50 (nM)	0.24	1.3	2.4	4.7	7.5	35.2
	Fold-Activation (±SEM)	36 (± 4)	35 (± 2.6)	41 (± 2.9)	39 (± 2.4)	29 (± 2.3)	14 (± 0.3)
Lamprey CR1 Truncated Seq.	EC50 (nM)	0.24	2.3	8.6	7.9	26	45.3
	Fold-Activation (±SEM)	280 (± 9.7)	264 (± 2.3)	267 (± 10.1)	280 (± 19.4)	284 (± 13.7)	89(± 1.1)
Lamprey CR2 Full Seq.	EC50 (nM)	0.26	1.2	1.5	4	7.4	16.7
	Fold-Activation (±SEM)	88 (±4.1)	79 (± 2.8)	93.4 (± 6.8)	80.4 (± 1.7)	71 (± 5.8)	41 (± 1.6)
Lamprey CR2 Truncated Seq.	EC50 (nM)	0.31	3.7	8.2	6.6	23.0	49.7
	Fold-Activation (±SEM)	1281 (± 51)	1232 (± 66)	1325 (± 16)	1215.5 (± 69)	1183 (± 55.6)	381 (± 22.7)

Full Seq. = full receptor sequence, Truncated Seq. = Receptor with NTD deleted

DOC = 11-deoxycorticosterone, ALDO = aldosterone, S = 11-deoxycortisol, CORT = Corticosterone

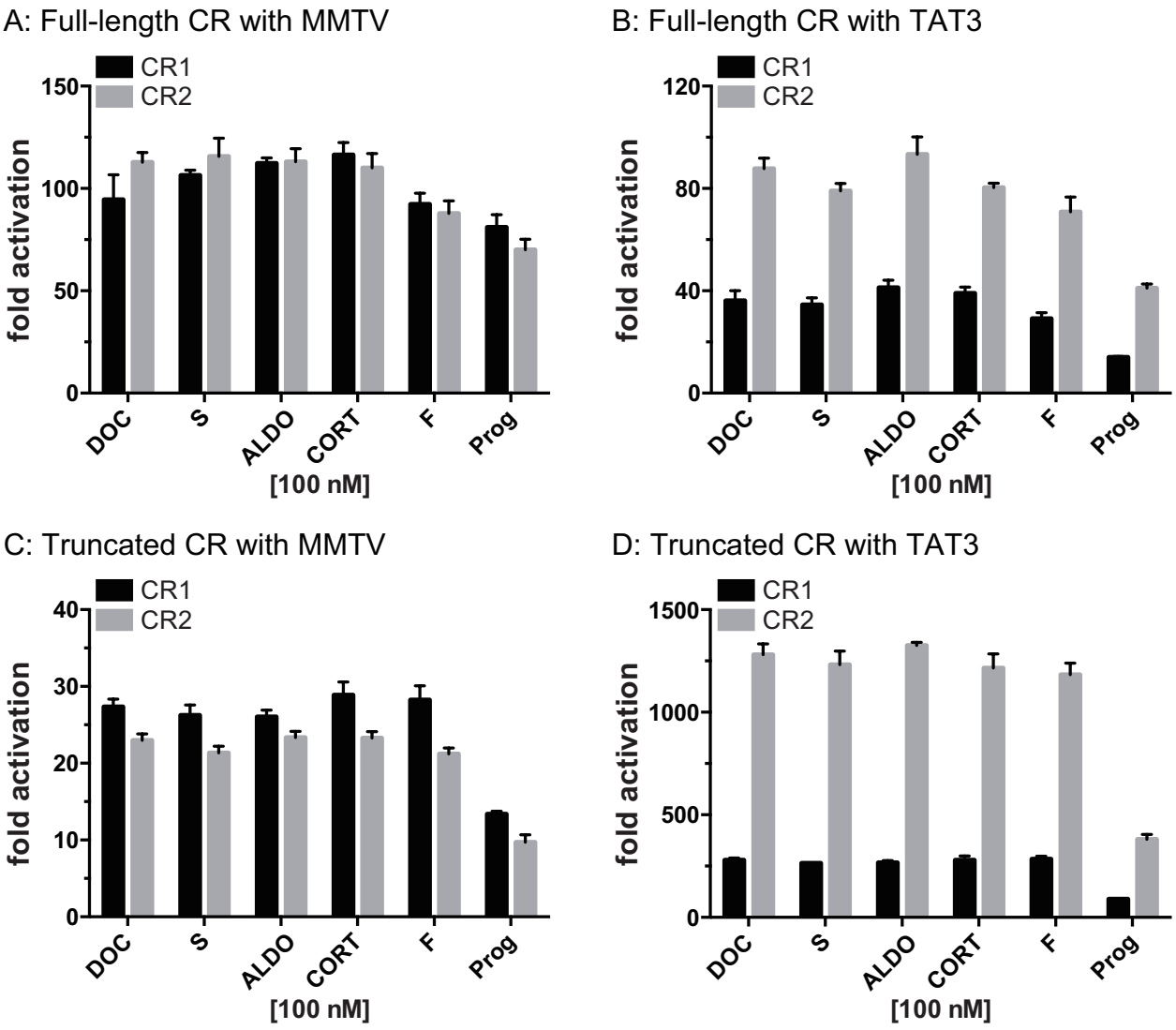


Figure 6. Graphical analysis of activation by steroids at 100 nM of transcription of full-length and truncated CR1 and CR2.

Plasmids for full-length or truncated lamprey CR2 were expressed in HEK293 cells with either an MMTV-luciferase promoter or a TAT3-luciferase promoter. Cells were treated with 100 nM concentrations of either aldosterone, cortisol, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, progesterone or vehicle alone (DMSO). Results are expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the activity of vector with vehicle (DMSO) alone as 1. A. Full-length CR1 and CR2 with MMTV-luc. B. Full-length CR1 and CR2 with TAT3-luc. C. Truncated lamprey CR1 and CR2 with MMTV-luc. D. Truncated lamprey CR1 and CR2 with TAT3-luc.

DOC = 11-deoxycorticosterone, ALDO = aldosterone, S = 11-deoxycortisol, CORT = Corticosterone, F = cortisol, Prog = progesterone.

2.4 Corticosteroid-dependent and promoter-dependent activation by corticosteroids of full-length and truncated lamprey CR2.

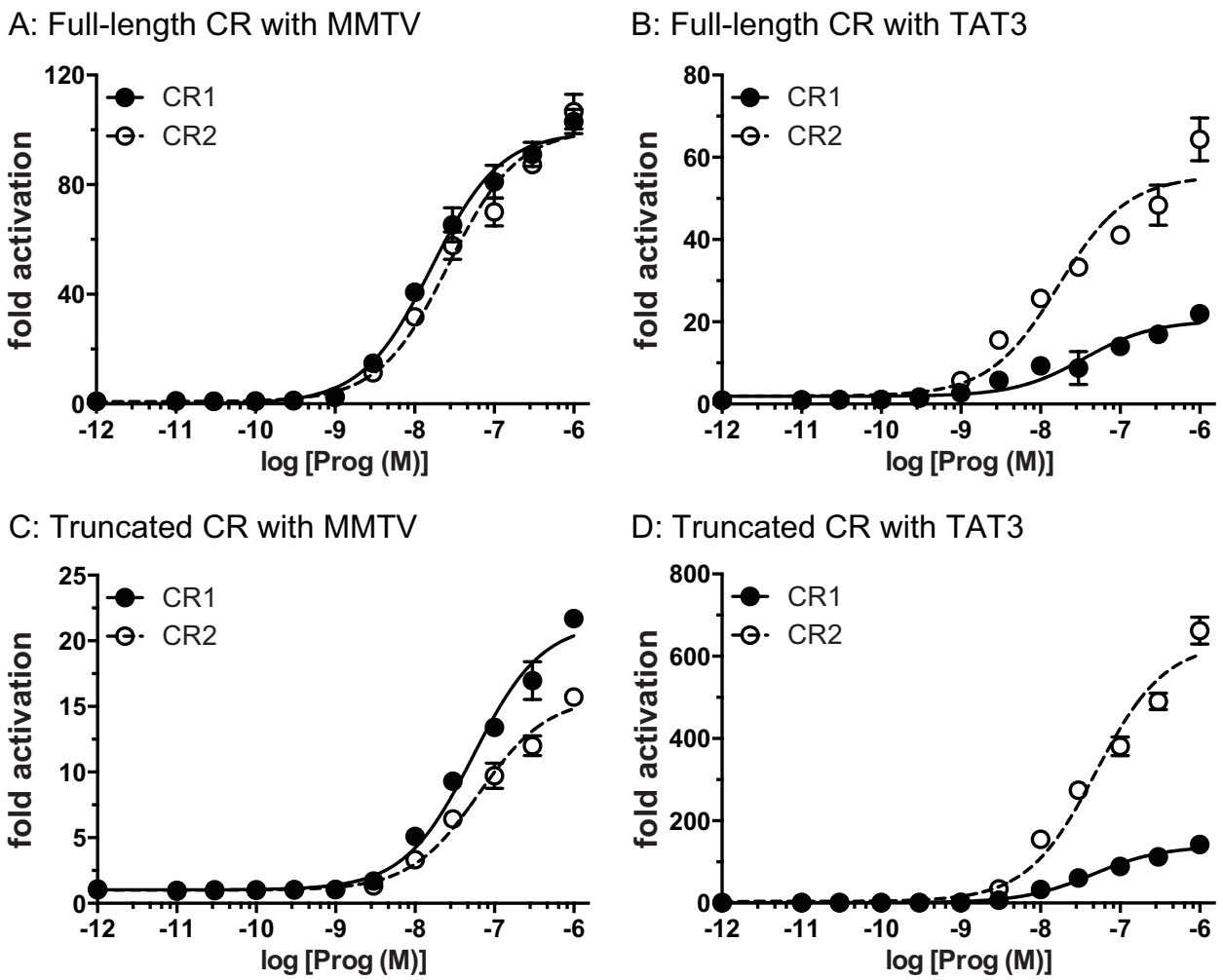
Corticosteroids in HEK293 cells with full-length CR2 and the MMTV promoter have slightly higher EC50s and a similar fold-activation compared to corticosteroids in HEK293 cells with full-length CR1 with the MMTV promoter (Table 2, Figure 6). However, steroid-mediated transcriptional activation of full-length and truncated lamprey CR2 in HEK293 cells is different in the presence of the MMTV and TAT3 promoters (Figure 5, Table 2). In HEK293 cells with TAT3, there is about 30% lower fold-activation for full-length CR2 compared to fold-activation for full-length CR2 in cells with MMTV. In contrast, fold-activation for truncated CR2 is about 15-fold higher in the presence of TAT3 than full-length CR2 and 55 to 60 higher activation than for truncated CR2 with MMTV.

2.6 Progesterone activates transcription of full-length and truncated CR1 and CR2.

Although progesterone has a strong affinity for human MR [33,34,63], progesterone is an antagonist of the mineralocorticoid receptor [33–35]. However, progesterone is an agonist for elephant shark MR [44]. We find that progesterone is an agonist for lamprey CR1 and CR2. Progesterone stimulates luciferase activity in HEK293 cells transfected with either full-length CR1 and CR2 and either an MMTV-luciferase promoter (Figure 7A) or a TAT3 luciferase promoter (Figure 7B). For full-length CR1, progesterone has an EC50 of 16.2 nM with MMTV and 35.2 nM with TAT3. For full-length CR2, progesterone has an EC50 of 26.4 nM with

303 MMTV (Table 2) and 16.7 nM with TAT3 (Table 2). These EC50s are higher than that for 11-
304 deoxycortisol and 11-deoxycorticosterone. Fold activation of CR1 and CR2 differs for the
305 MMTV and TAT3 promoters (Figure 6).

306 We also studied progesterone activation of truncated CR1 and CR2 in HEK293 cells that
307 were transfected with either the MMTV or TAT3 promoters. Truncated CR1 and CR2 lost
308 substantial activity for progesterone. In this assay, progesterone had an EC50 of 53.8 nM for
309 truncated CR1 in HEK293 cells with MMTV (Table 2) and an EC50 of 45.3 nM for truncated
310 CR1 in HEK293 cells with TAT3 (Table 2). Progesterone had an EC50 of 63.5 nM for truncated
311 CR2 in HEK293 cells with MMTV (Table 2) and an EC50 of 49.7 nM for truncated CR2 in
312 HEK293 cells with TAT3 (Table 2).



313

314 **Fig. 7. Concentration-dependent transcriptional activation by progesterone of full length**
315 **and truncated lamprey CR.** Plasmids for full-length or truncated lamprey CRs were expressed
316 in HEK293 cells with either an MMTV-luciferase promoter or a TAT3-luciferase promoter.
317 Cells were treated with increasing concentrations of progesterone or vehicle alone (DMSO).
318 Results are expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the
319 activity of vector with vehicle (DMSO) alone as 1. A. Full-length CR with MMTV-luc. B. Full-
320 length CR with TAT3-luc. C. Truncated lamprey CR with MMTV-luc. D. Truncated lamprey
321 CR with TAT3-luc.

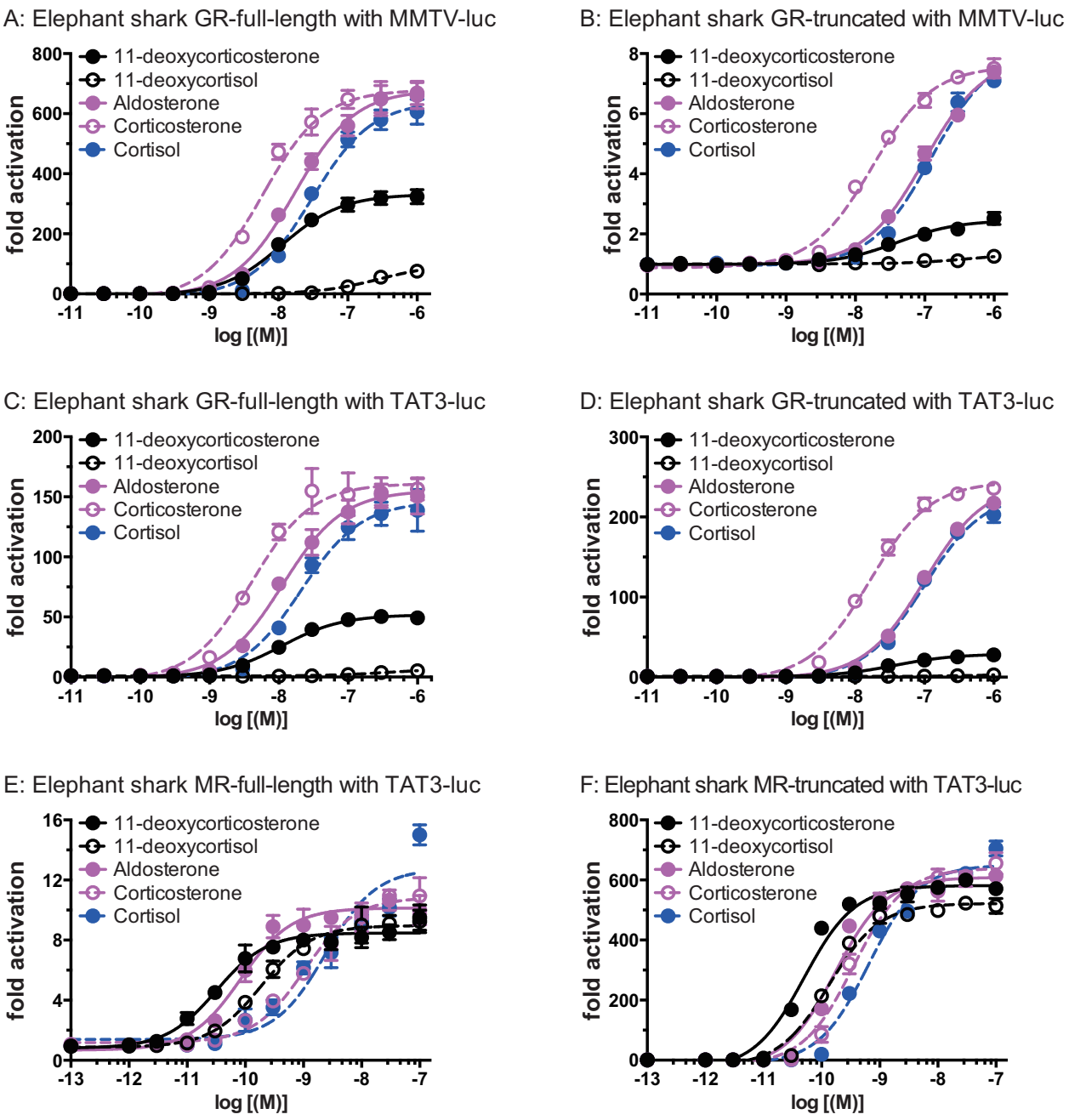
322

323 **2.7 Corticosteroid-dependent and promoter-dependent activation by corticosteroids of full-**
324 **length and truncated elephant shark MR and GR.**

325 To gain an insight into corticosteroid activation of the MR and GR early in their
326 evolution, we compared corticosteroid activation of lamprey CR with activation of the MR and
327 GR in elephant shark (*Callorhinchus milii*), a cartilaginous fish belonging to the oldest group of
328 jawed vertebrates. Elephant shark occupy a key position spanning an ancestral node from which
329 ray-finned fish and terrestrial vertebrates diverged about 450 million years ago from bony
330 vertebrates [64,65].

331 We previously studied corticosteroid activation of elephant shark GR [12,44] and MR
332 [12] in HEK293 cells containing the MMTV promoter. To complete our dataset for an
333 evolutionary analysis of the CR with elephant shark MR and GR, we investigated corticosteroid
334 activation of elephant shark GR and MR in HEK293 cells containing TAT3. Figure 8 and Table
335 3 show our results. For full-length elephant shark GR, only corticosterone retains an EC50 value
336 close to the EC50 of lamprey CR1. The other corticosteroids have substantially higher EC50s,
337 and are unlikely to be physiological ligands for this GR. Notably, 11-deoxycortisol has little
338 activity for elephant shark GR (EC50=289 nM), which compares to an EC50 of 1.5 nM for
339 lamprey CR1 and 2.7 nM for lamprey CR2. However, fold-activation by corticosteroids of full-
340 length elephant shark GR is higher than for full-length lamprey CR1 with over 600-fold
341 activation by aldosterone, corticosterone and cortisol. Truncated elephant shark GR loses
342 substantial activity for all corticosteroids indicating that the NTD is important in the EC50 and
343 fold-activation of elephant GR.

344



345

346 **Fig. 8. Concentration-dependent transcriptional activation by corticosteroids of full length**
347 **and truncated elephant shark GR and elephant shark MR.** Plasmids for full-length or
348 truncated elephant shark GR were expressed in HEK293 cells with either an MMTV-luciferase
349 promoter or a TAT3-luciferase promoter. Plasmids for full-length or truncated elephant shark
350 MR were expressed in HEK293 cells with a TAT3-luciferase promoter. Cells were treated with
351 increasing concentrations of either aldosterone, cortisol, corticosterone, 11-deoxycortisol, 11-
352 deoxycorticosterone or vehicle alone (DMSO). Results are expressed as means ± SEM, n=3. Y-

353 axis indicates fold-activation compared to the activity of vector treated with vehicle (DMSO)
354 alone as 1. A. Full-length elephant shark GR with MMTV-luc. B. Truncated elephant shark GR
355 with MMTV. C. Full-length elephant shark GR with TAT3. D. Truncated elephant shark GR
356 with TAT3. E. Full-length elephant shark MR with TAT3-luc. F. Truncated elephant shark MR
357 with TAT3.

358

359 **Table 3. Corticosteroid Activation of elephant shark GR and MR in HEK293 cells with an**
360 **MMTV promoter or a TAT3 promoter.**

MMTV-luc		**DOC**	**S**	**ALDO**	**Corticosterone**	**Cortisol**
Elephant Shark GR Full Seq.	EC50 (nM)	11.2	289	17.1	5.9	30
	Fold-Activation (±SEM)	297 (± 22)	23.4 (± 0.5)	561 (± 34)	648 (± 30)	514 (± 25)
Elephant Shark GR Truncated Seq.	EC50 (nM)	40.5	-	98.4	17.5	124
	Fold-Activation (±SEM)	2.0 (± 0.1)	1.1 (± 0.03)	4.7 (± 0.2)	6.4 (± 0.23)	4.2 (± 0.06)
Elephant Shark MR Full Seq. *	EC50 (nM)	0.1	0.22	0.14	0.61	1.6
	Fold-Activation (±SEM)	8.6 (± 0.4)	8.0 (± 0.5)	9.6 (± 1.0)	9.1 (± 0.7)	10.1 (± 0.9)
Elephant Shark MR Truncated Seq. *	EC50 (nM)	0.09	0.28	0.26	0.58	1.06
	Fold-Activation (±SEM)	6.9 (± 0.15)	6.6 (± 0.5)	7.3 (± 0.8)	8.2 (± 0.4)	8.1 (± 0.95)
TAT3-luc		**DOC**	**S**	**ALDO**	**Corticosterone**	**Cortisol**
Elephant Shark GR Full Seq.	EC50 (nM)	11.0	-	11.4	4.0	21
	Fold-Activation (±SEM)	48 (± 3.5)	2.1 (± 0.2)	137.3 (± 10)	152 (± 18)	124 (± 10.2)
Elephant Shark GR Truncated Seq.	EC50 (nM)	37.4	-	100.3	16.3	99.6
	Fold-Activation (±SEM)	21.4 (± 0.8)	1.3 (± 0.04)	125 (± 5)	216 (± 8)	122 (± 5.7)
Elephant Shark MR Full Seq.	EC50 (nM)	0.03	0.2	0.08	1.0	2.4
	Fold-Activation (±SEM)	9.5 (± 0.84)	9.2 (± 0.74)	10 (± 1)	11 (± 1.2)	15 (± 0.7)

Elephant Shark MR Truncated Seq.	EC50 (nM)	0.05	0.13	0.17	0.35	0.64
	Fold-Activation (±SEM)	571 (± 11)	514 (± 24.6)	613 (± 17.5)	656 (± 35.3)	705 (± 24.6)

Full Seq. = full receptor sequence, Truncated Seq. = Receptor with NTD deleted

DOC = 11-deoxycorticosterone, ALDO = aldosterone, S = 11-deoxycortisol

*: Katsu et al. N-terminal Domain Regulates Steroid Activation of Elephant Shark Glucocorticoid and Mineralocorticoid Receptors. J. Steroid Biochem. Mol .Biol. 210, 105845 (2021).

Unlike full-length elephant shark GR, full-length elephant shark MR in cells with the MMTV promoter has EC50s varying from 0.1 nM (11-deoxycorticosterone) to 1.6 nM (cortisol), which are close to their EC50s for lamprey CR1 and CR2. Truncated elephant shark MR in cells with MMTV retains these low EC50s (Table 3). As found for lamprey CR in cells with TAT3, removal of the NTD in elephant shark MR leads to a substantial increase of 50-fold in transcriptional activation by corticosteroids (Figure 8, Table 3). These data also support lamprey CR and elephant shark MR as closer to each other than to elephant shark GR.

3. Discussion

Sea lamprey and hagfish, the two extant cyclostomes, occupy a critical position in the evolution of vertebrates [1–3,6], and the sea lamprey CR occupies a critical position in the evolution of the MR and GR in vertebrates [4,8,9,13,43,61], two steroid receptors that are important regulators of vertebrate physiology [10,19,20,22,31,32,41]. The assembly of the lamprey germline genome [40] provided an opportunity to determine the response to corticosteroids of full-length CR for comparison to elephant shark GR and MR and provide insights into the evolution of glucocorticoid and mineralocorticoid signaling. Analysis of the CR

in the lamprey genome identified two CRs, CR1 and CR2, which differ only in an insertion of four amino acids in the DBD of CR1. The DBD in elephant shark MR and GR [12], as well as in other vertebrate MRs and GRs lack these extra four amino acids found in CR1, suggesting that vertebrate MRs and GRs are descended from an ancestral CR2-like gene. Expression of CR1 and CR2, as determined by RNA-Seq revealed that CR1 comprises over 99% of expressed CR in lamprey kidney, intestine and brain (Table 1).

Our analysis of steroid activation of lamprey CR1 and CR2 found that 11-deoxycorticosterone and 11-deoxycortisol (Figure 1) have the lowest EC50s and a high fold-activation of transcription, consistent with the evidence that these are the circulating steroids in lamprey [26–28]. Overall, full-length lamprey CR1 and CR2 have similar EC50s and fold-activation for corticosteroids in cells transfected with MMTV promoter. Truncated lamprey CR1 and CR2 also have similar EC50s and fold-activation for corticosteroids in cells transfected with MMTV promoter. However, in HEK293 cells transfected with TAT3, compared to full-length CR1, full-length CR2 has about 2.25-fold higher activation in the presence of corticosteroids and truncated CR2 has about 4.5-fold higher fold activation than truncated CR1. It appears that a complex mechanism regulating CR-mediated transcription involving the NTD and the MMTV and TAT3 promoters evolved before the evolution of distinct GR and MR genes in an ancestral cartilaginous fish. NTD regulation of corticosteroid activation of the CR also indicates that the entire sequence of lamprey CR regulates transcription by corticosteroids.

Progesterone activates transcription by full-length lamprey CR1 and CR2, in contrast to the lack of progesterone-mediated transcriptional activation of human MR [12,33–35,44]. Progesterone also activates transcription by full-length elephant shark MR (Table 3), but not elephant shark GR [12,44].

Analysis of the effect of deleting the NTD in the CR and in elephant shark MR and GR on corticosteroid activation of these receptors in HEK293 cells containing TAT3 support the hypothesis that lamprey CR and elephant shark MR as functionally more similar to one another than to elephant shark GR. A similar conclusion comes from comparison of the responses of lamprey CRs and elephant shark MR and GR to corticosteroids.

Although 11-deoxycortisol and 11-deoxycorticosterone are the circulating corticosteroids in lamprey, studies of these two steroids in live lamprey find that only 11-deoxycortisol is biologically active [26,27,66]. This contrasts with our results in cell culture in which both steroids, as well as other corticosteroids, stimulate transcription of CR1 and CR2 (Figures 4 and 5). There may be additional regulatory mechanisms that lead to preferential activity of 11-deoxycortisol in lamprey and inactivity of 11-deoxycorticosterone. It appears that the Atlantic sea lamprey has more secrets to share.

4. Materials and Methods

4.1 Chemical reagents

Cortisol, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, and aldosterone were purchased from Sigma-Aldrich. For reporter gene assays, all hormones were dissolved in dimethyl-sulfoxide (DMSO); the final DMSO concentration in the culture medium did not exceed 0.1%.

4.2 Gene expression analysis

To determine the expression level of CR gene, single-end RNA-seq reads of sea lamprey for seven stages of embryos, intestine and kidney from larval stage, intestine, kidney and liver from

parasitic stage, brain, intestine and kidney from adult stage, were downloaded from database of National Center for Biotechnology Information (accession number: PRJNA50489). Reference genome assembly and gene annotation were also downloaded from NCBI database (accession ID: GCF_010993605.1). RNA-seq reads of various tissues were independently aligned to the reference sequences using RSEM (v1.3.3) [57]. The relative measure of transcript abundance is FPKM (fragments per kilobase of transcript per million mapped reads).

4.3 Construction of plasmid vectors

Full-length mineralocorticoid receptor sequence of sea lamprey, *Petromyzon marinus*, was registered in Genbank (accession number: XM_032955475). Based on the registered sea lamprey MR sequence we synthesized DNA containing the full-length sequence. Full-length sea lamprey CR1 was ligated into pcDNA3.1 vector (Invitrogen). CR2 construction was performed using KOD-Plus-mutagenesis kit (TOYOBO). All cloned DNA sequences were verified by sequencing.

4.4 Transactivation assay and statistical methods

Transfection and reporter assays were carried out in HEK293 cells, as described previously [12]. The cells were transfected with 100 ng of receptor gene, reporter gene containing the Photinus pyralis luciferase gene and pRL-tk, as an internal control to normalize for variation in transfection efficiency; pRL-tk contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter. Each assay had a similar number of cells, and assays were done with the same batch of cells in each experiment, which is consistent with similar fold-activation in each assay at 1 □M and 100 nM (Figure 6). All experiments were performed in triplicate. Promoter activity was calculated as firefly (P. pyralis)-lucifease activity/sea pansy (R.

reniformis)-lucifease activity. The values shown are mean ± SEM from three separate experiments, and dose-response data, which were used to calculate the half maximal response (EC50) for each steroid, were analyzed using GraphPad Prism. Comparisons between two groups were performed using paired *t*-test. $P < 0.05$ was considered statistically significant.

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5.2 Author contributions: Y.K. and M.E.B. designed the research. Y.K. and X.L. carried out the research and analyzed data. R.J., Z.C., Y.K., K.B. performed the cell culture and DNA construction. Y.K. and M.E.B. wrote the paper. All authors gave final approval for publication.

5.3 Competing Interests: We have no competing interests.

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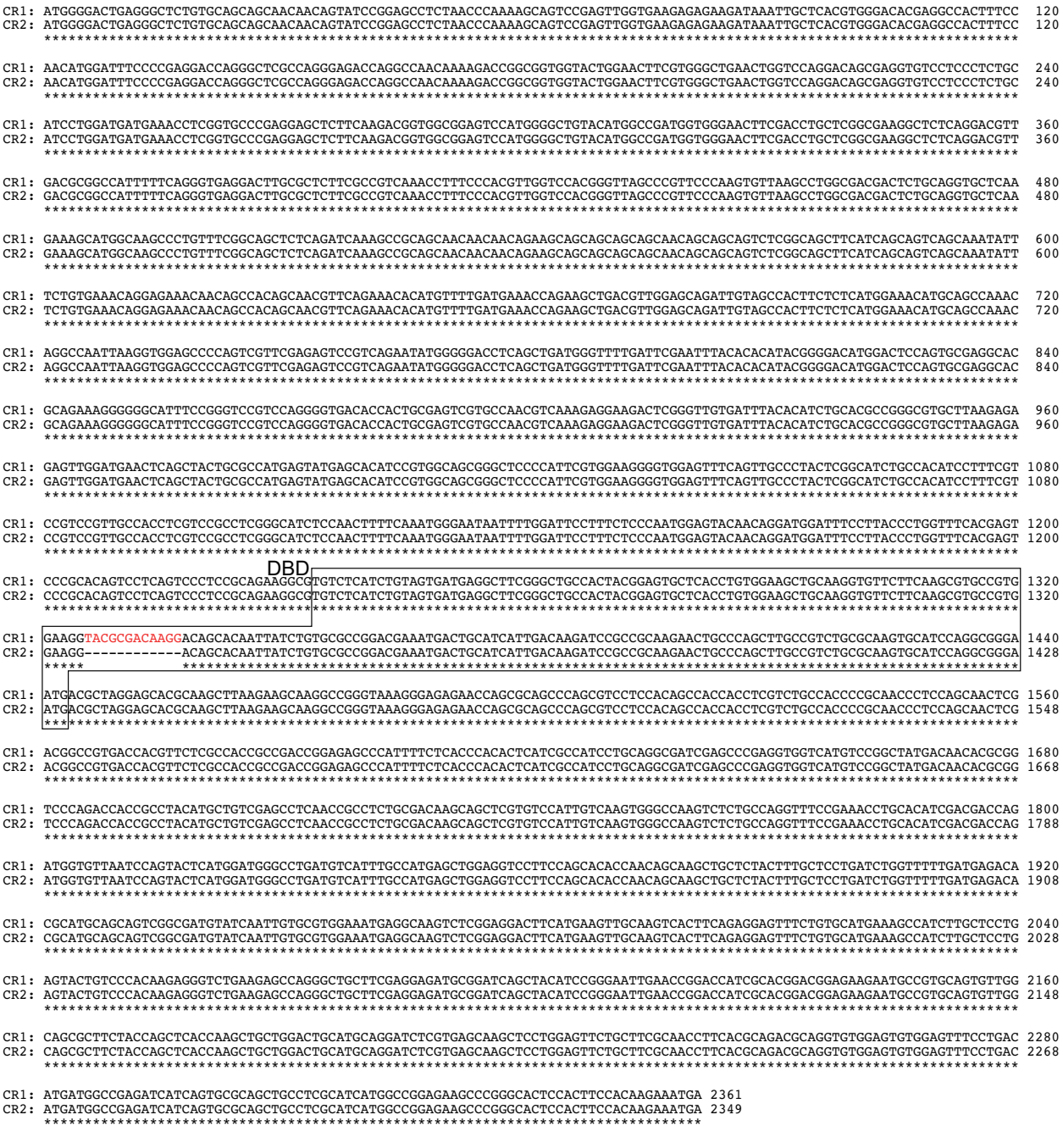

Supplement Figure 1. Alignment of the nucleic acid sequences of lamprey CR1 and CR2.

With exception of the 12 nucleic acid, TACGCGACAAGG, insert in lamprey CR1, the sequences of CR1 and CR2 are identical.

Accession for CR1 nucleotide sequence: XM_032955475.

Accession for CR2 nucleotide sequence: XM_032955480