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Evolution of steroid specificity in human, chicken, alligator, frog and zebrafish mineralocorticoid receptors: Allosteric interactions affect steroid specificity

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Abstract

We studied the response to aldosterone, 11-deoxycorticosterone, 11-deoxycortisol, cortisol, corticosterone, progesterone, 19-norprogesterone and spironolactone of human, chicken, alligator, frog and zebrafish full-length mineralocorticoid receptors (MRs) and truncated MRs, lacking the N-terminal domain (NTD) and DNA-binding domain (DBD), in which the hinge domain and ligand binding domain (LBD) were fused to a GAL4-DBD. Compared to full-length MRs, some vertebrate MRs required higher steroid concentrations to activate GAL4-DBD-MR-hinge/LBD constructs. For example, 11-deoxycortisol activated all full-length vertebrate MRs, but did not activate truncated terrestrial vertebrate MRs and was an agonist for truncated zebrafish MR. Progesterone, 19-norProgesterone and spironolactone did not activate full-length and truncated human, alligator and frog MRs. However, at 10 nM, these steroids activated full-length chicken and zebrafish MRs; at 100 nM, these steroids had little activity for truncated chicken MRs, while retaining activity for truncated zebrafish MRs, evidence that regulation of progestin activation of chicken MR resides in NTD/DBD and of zebrafish MR in hinge-LBD. Zebrafish and chicken MRs contain a serine corresponding to Ser810 in human MR, required for its antagonism by progesterone, suggesting novel regulation of progestin activation of chicken and zebrafish MRs. Progesterone may be a physiological activator of chicken and zebrafish MRs.

Short Title: Allosteric Regulation of steroid activation of for vertebrate MRs

Key Words: aldosterone, corticosteroids, progesterone, spironolactone, vertebrate MR evolution, allosteric regulation

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INTRODUCTION

The mineralocorticoid receptor (MR) belongs to the nuclear receptor family, a diverse group of transcription factors that also includes receptors for androgens (AR), estrogens (ER), glucocorticoids (GR) and progestins (PR), as well as other small lipophilic ligands, such as thyroid hormone and retinoids (*1-4*).

Aldosterone (Aldo) is the physiological activator for human MR in epithelial tissues, such as the kidney distal collecting tubules, and of the colon (*5-9*). The human MR has similar strong binding affinities for several corticosteroids: Aldo, cortisol (F), corticosterone (B) and 11-deoxycorticosterone (DOC), and for progesterone (Prog) (*10, 11*) (Figure 1). These steroids also have similar affinities for rat MR (*12-14*) and guinea pig MR (*13, 14*). Corticosteroids are transcriptional activators of human MR (*10, 15-18*), while, in contrast, Prog is an antagonist for human MR (*15, 17-19*) (Figure 1). Complicating Aldo activation of human, rat and mouse MRs is the substantially higher concentration in human serum of F and in rat and mouse serum of B compared to Aldo. For example, the concentration of F in human serum is from 500 to 1,000 times higher than that of Aldo, and under stress F increases further. In this case, human MR would be expected to be occupied by F, to the exclusion of Aldo (*5, 8, 20-22*). One contributor to selective occupation of the MR in epithelial cells by Aldo over F and B arises from 11β-hydroxysteroid dehydrogenase-type 2 (11β-HSD2), which selectively inactivates F and B (*5, 22-25*). Aldo is inert to 11β-HSD2, as is DOC, which lacks an 11β-hydroxyl, allowing both steroids to activate the MR in epithelial tissues. The MR also is found in brain, brain, heart, aorta, lung, adipose tissue, breast and ovary (*6, 7, 9, 26*), some of which lack 11β-HSD2. In those tissues, F and B would be expected to occupy the MR. Also important for selective occupation of the MR by Aldo is corticosteroid binding globulin, which preferentially sequesters F, B and DOC compared to Aldo (*12, 27*).

Despite the similar binding affinities of Aldo, F, B and DOC for the human MR, there is substantial variation in the half-maximal response (EC50) among these steroids for transcriptional activation of the MR. For example, Aldo has a substantially lower EC50 (higher activity) than F for human MR (*11, 15-18, 28, 29*). Also, fish MRs have a stronger response to Aldo than to F, B and S (*18, 29-33*). The basis for this difference among corticosteroids in transcriptional activation of these vertebrate MRs is still not well understood.

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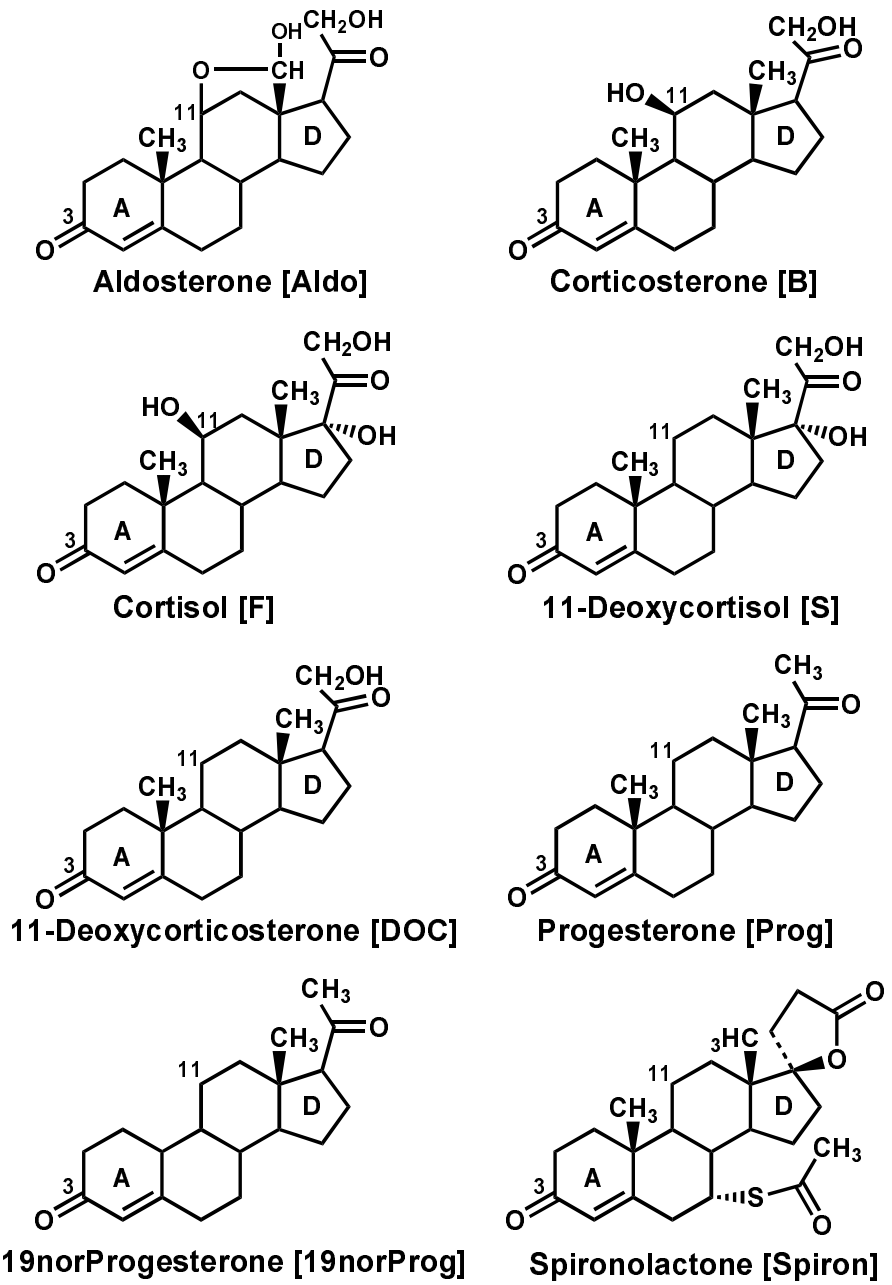

Figure 1. Corticosteroid and Progestin Structures.

Aldosterone (Aldo) the physiological ligand for terrestrial vertebrate MRs. Cortisol (F), corticosterone (B), 11-deoxycorticosterone (DOC) also are ligands for terrestrial vertebrate MRs. 11-deoxycorticosterone (DOC) and Cortisol (F) have been proposed to be mineralocorticoids in teleosts (*18*, *34-36*) because Aldo is not found in fish (*37*). 11-deoxycortisol (S) is a ligand for corticosteroid receptor (CR) in lamprey (*38*, *39*). Prog has high affinity for human MR (*15*, *17*, *18*), but is an antagonist at 10 nM. Spiron is an MR antagonist. However, Prog, 19norProg and Spiron are agonists for gar, sturgeon and zebrafish and trout MRs (*18*, *29*, *33*).

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Interestingly, Prog, 19nor-progesterone (19norProg) and spironolactone (Spiron) (Figure 1), which are antagonists for human MR (*15, 17, 18*), are agonists for several fish MRs (*18, 29, 33*). Data for progestin activation of frog, alligator and chicken MRs are absent. Thus, the timing of the evolution of antagonist activity of progestins and Spiron for the MR is not known.

An important structural property that influences transcriptional activation of the MR and other steroid receptors is their modular domain structure, which consists of an N-terminal domain (NTD) (domains A and B), a central DNA-binding domain (DBD) (domain C), a hinge domain (D) and a C-terminal ligand-binding domain (LBD) (domain E) (*11, 18, 40-42*) (Figure 2). Although the LBD alone on the MR is competent to bind steroids (*20, 40, 43-46*) allosteric interactions between the LBD and NTD are important in transcriptional activation of the human and zebrafish MR (*19, 29, 42, 47*)), as well as for the GR and other steroid receptors (*11, 48-57*).

Moreover, there are differences between the effects F and DOC on transcription due to interactions between the LBD and NTD in human MR (*19, 42*) and zebrafish MR (*29*). In human MR, DOC and F weakly promote the NTD/LBD interaction and gene transcription (*19*). In contrast, in zebrafish MR, F and DOC significantly induce the NTD/LBD interaction and increase transcription. The basis for these differences between human and zebrafish MR is not known, as well as the effect, if any, of inter-domain interactions on corticosteroid and progestin-mediated transcription in frog, alligator and chicken MRs.

To begin to fill in these gaps, we investigated activation of full-length MRs from human, chicken, alligator, frog (*Xenopus laevis*) and zebrafish and their truncated MRs, consisting of the GAL4 DBD fused to the D domain and E domain of the MR (MR-LBD), by a panel of corticosteroids (Aldo, F, B, DOC, 11deoxycortisol (S)) and progestins (Prog, 19norProg) and Spiron. We found significant differences between some full-length and truncated vertebrate MRs in their EC50s for DOC and S, which lack an 11β-hydroxyl, with truncated MRs having higher EC50s (weaker activation) than their corresponding full-length MRs. For example, although S is a transcriptional activator of full-length vertebrate MRs, only truncated chicken and zebrafish MRs are activated by S. Moreover, Prog, 19norProg and Spiron, which were transcriptional activators of full-length chicken and zebrafish MRs, were inactive for truncated chicken MR, but retained activity for truncated zebrafish MR. These results indicate that

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interactions between the A/B/C and D/E domains in vertebrate MRs are important in steroid specificity, with regulation of progestin activation of chicken MR residing in the NTD/DBD and of zebrafish MR in the hinge-LBD.

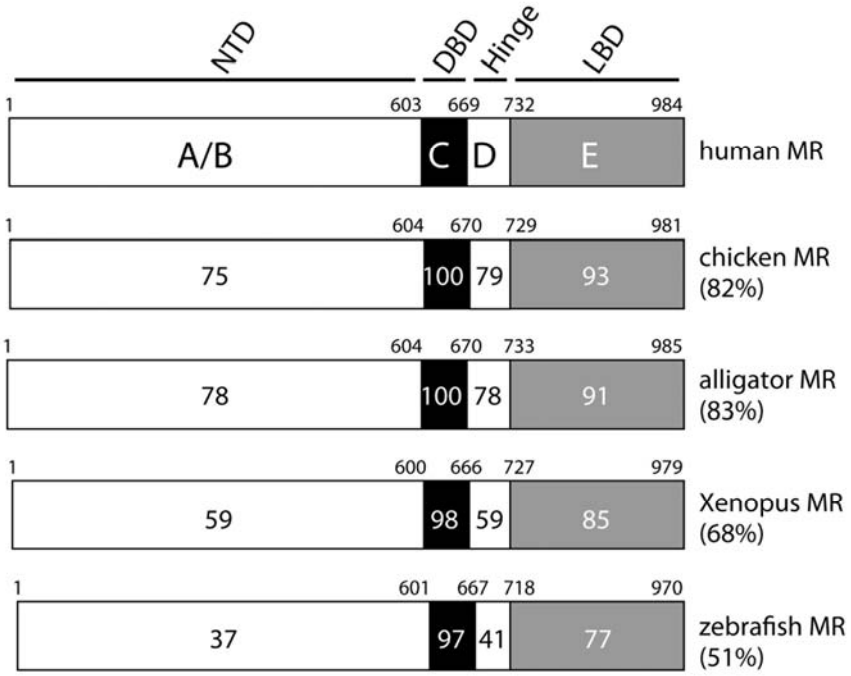

Figure 2. Comparison of domains in vertebrate MRs.
Domains A/B (NTD), C (DBD) D (hinge) and E (LBD) on MRs from human, chicken, alligator *Xenopus* and zebrafish are compared. Shown are the number of amino acids in each domain and the percent identical amino acids compared to human MR. GenBank accession numbers: human MR (NP_000892), chicken (ACO37437), alligator MR (NP_001274242), *Xenopus* MR (NP_001084074), zebrafish MR (NP_001093873).

Regarding transcriptional activation by progestins of chicken and zebrafish MRs, Geller et al. (*17*) found that at 1 nM, Prog, 19norProg and Spiron are transcriptional activators of a Ser810Leu mutant human MR. However, both chicken and zebrafish MRs contain a serine corresponding to Ser810 in wild-type human MR indicating that there are alternative mechanisms for progestin activation of chicken and zebrafish MRs. Our data suggests that Prog may be a physiological activator of chicken MR, as well as of zebrafish and other fish MRs (*18, 29, 33*). In this regard, Prog lacks an 11β-hydroxyl, and thus, like Aldo, Prog is inert

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to 11β-HSD2. Also caution is advised in assuming that studies of activation by some corticosteroids of constructs containing GAL4-DBD fused to MR-LBD are representative of full-length MR.

MATERIALS & METHODS

Chemical reagents

Cortisol (F), corticosterone (B), aldosterone (Aldo), 11-deoxycorticosterone (DOC), 11-deoxycortisol (S), progesterone (Prog), 19nor-progesterone (19norProg), and Spironolactone (Spiron) were purchased from Sigma-Aldrich. For reporter gene assays, all hormones were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO in the culture medium did not exceed 0.1%.

Construction of plasmid vectors

The full-coding regions and D/E domains of the MR from human, chicken, alligator, frog (*Xenopus*) and zebrafish were amplified by PCR with KOD DNA polymerase. The PCR products were gel-purified and ligated into pcDNA3.1 vector (KpnI-NotI site for human MR, and BamHI-NotI site for chicken, alligator, frog and zebrafish MRs) for the full-coding region or pBIND vector (MluI-NotI site for human, chicken, frog and zebrafish MR, and MluI-KpnI site for alligator MR) for D-E domains. As shown in Figure 2, the D domain begins at human MR (732), chicken MR (729), alligator MR (733), frog MR (727), and zebrafish MR (718).

Transactivation Assay and Statistical Methods

Human embryonic kidney 293 (Hek293) cells were used in the reporter gene assay, and transfection and reporter assays were carried out as described previously (*18, 57*). All transfections were performed at least three times, employing triplicate sample points in each experiment. The values shown are mean ± SEM from three separate experiments, and dose-response data and EC50 were analyzed using GraphPad Prism.

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RESULTS

Comparison of vertebrate MR domains

In Figure 2, we compare the A/B (NTD), C (DNA-binding domain, DBD), D (hinge region), and E (ligand-binding domain, LBD) domains on human MR with corresponding domains on chicken, alligator, *Xenopus*, and zebrafish MRs. These phylogenetically diverse MRs have strong conservation of the C domain (97-100%) and E domain (77-91%) with substantially less conservation in the A/B domain (36-78%) and D domain (42-78%). 100% identity in the amino acid sequence of the DBD in human, chicken and alligator MRs is important because it eliminates sequence differences in their DBDs as contributing to differences in transcriptional activation by corticosteroids and progestins of these MRs.

Transcriptional activation by corticosteroids of full-length and truncated human, chicken, alligator, *X. laevis*, and zebrafish MRs

First, we screened a panel of steroids at 1 nM and 10 nM for transcriptional activation of full-length and truncated human (mammalian), chicken (avian), alligator (reptilian), *Xenopus* (amphibian), and zebrafish (teleost fish) MRs. Aldo, F, B and DOC were strong activators of full-length human, chicken, alligator and zebrafish MR (Figure 3 A1-C1, E1). Aldo, F and B were strong activators of full length Xenopus MR, while DOC was a weaker activator (Figure 3D1). S was a good activator of chicken and zebrafish MRs, and a weaker activator of human, alligator and Xenopus MRs.

In contrast, in parallel experiments with truncated MRs, lacking the A/B domain and containing a GAL4-DBD instead of the MR DBD, S and DOC, which lack an 11β-hydroxyl had substantially lower or little transcriptional activity for truncated human, chicken and Xenopus MRs (Figure 3A2, B2, D2). For example, at 10 nM, S had little transcriptional activity for truncated human, chicken and Xenopus MRs, and DOC lost most of its activity for truncated Xenopus MR and substantial activity for truncated human MR and chicken MR. F, which contains an 11β-hydroxyl, had little activity for truncated Xenopus MR and diminished activity for truncated human and

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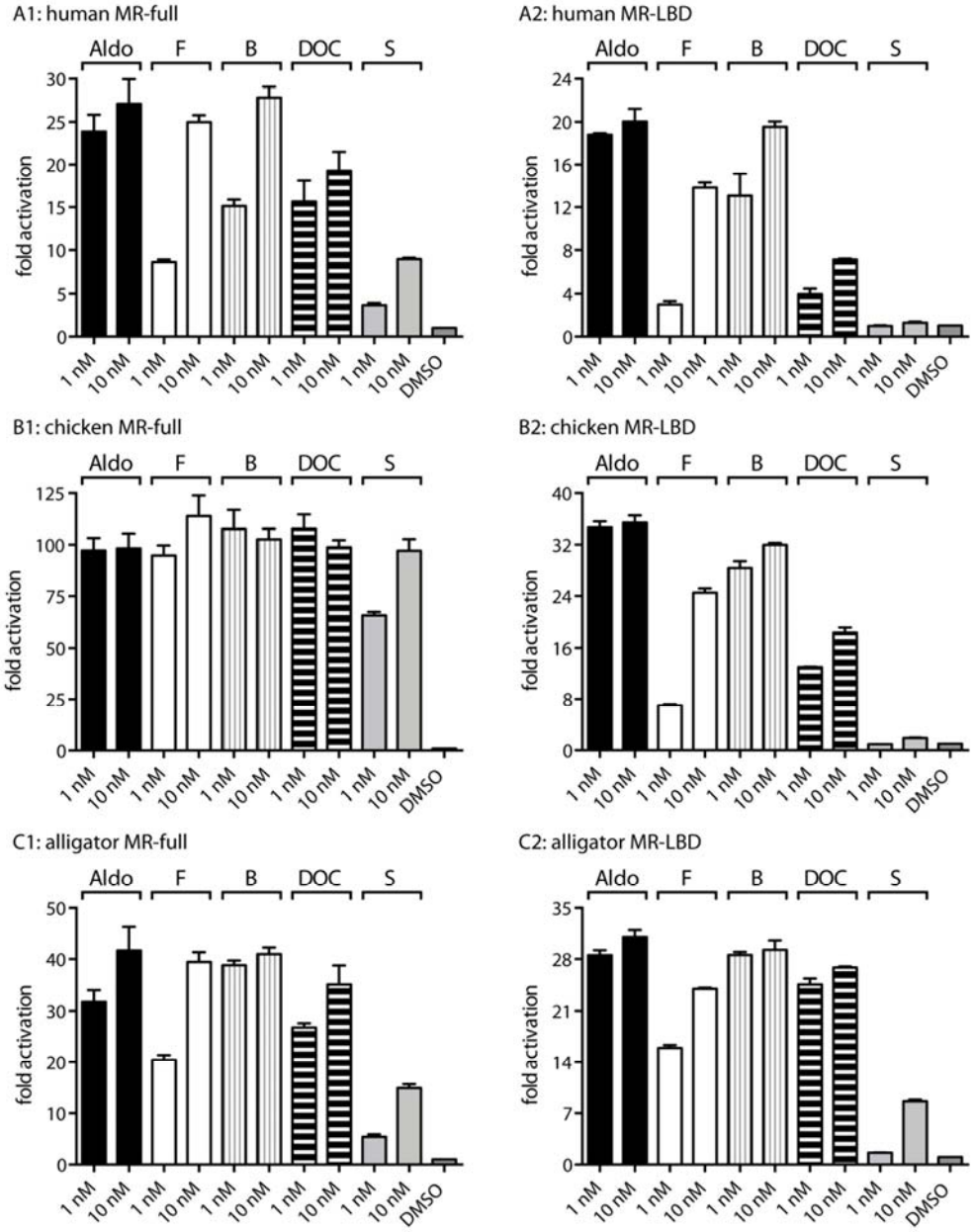
chicken MR. The response to corticosteroids by truncated zebrafish MR was different from truncated terrestrial vertebrate MRs (Figure 3E2). Truncated zebrafish MR retained activity for all corticosteroids.



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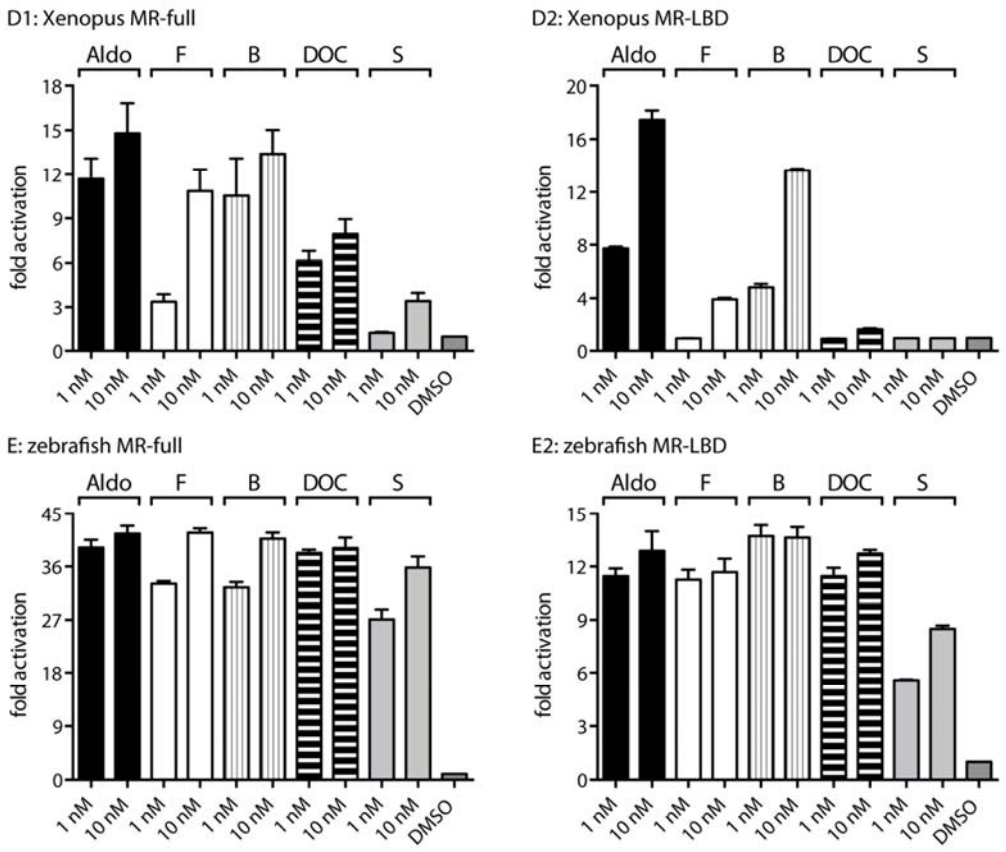

Figure 3. Corticosteroid activation of human, alligator, *Xenopus* and zebrafish full-length MRs and LBD MRs.

Full-length human MR (A1), chicken MR (B1), alligator MR (C1), *Xenopus* MR (D1), and zebrafish MR (E1) were expressed in HEK293 cells with an MMTV-luciferase reporter. Plasmids for corresponding truncated MRs, human (A2),chicken (B2), alligator (C2), *X. laevis* (D2) and zebrafish (E2) containing the D domain and LBD (E domain) fused to a GAL4-DBD were expressed in HEK293 cells with a luciferase reporter containing GAL4 binding site. Cells were treated with 1 nM or 10 nM Aldo, F, B, DOC, S or vehicle alone (DMSO). Results are expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

Transcriptional activation by Prog, 19norProg and Spiron of full-length and truncated human, chicken, alligator, *X. laevis*, and zebrafish MRs

Based on previous studies showing the Prog, 19norProg and Spiron were transcriptional activators of fish MRs (*18, 29, 33*), we screened these steroids at concentrations of 10 nM, 100 nM and 1 μM for transcriptional activation of full length

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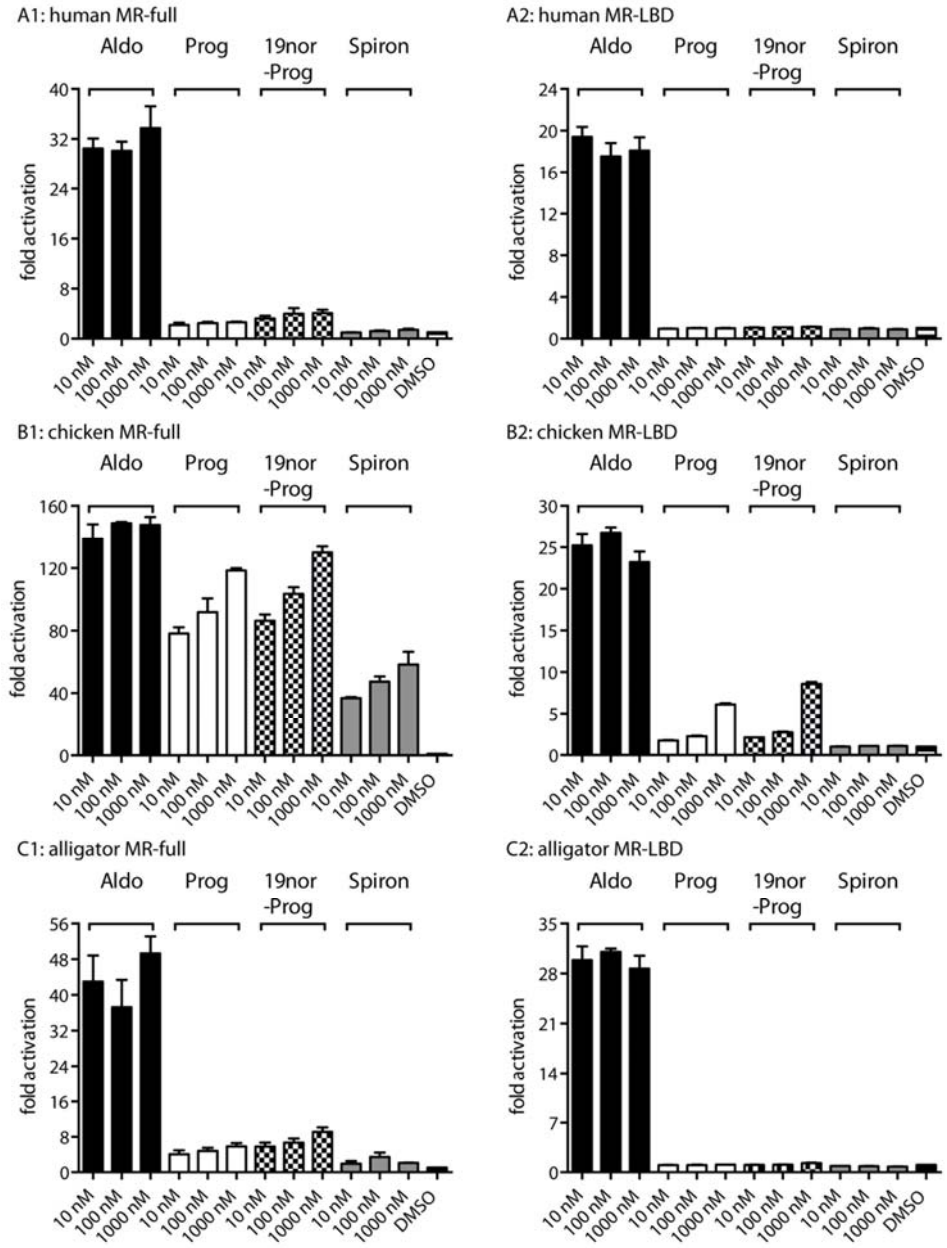
and truncated terrestrial vertebrate and zebrafish MRs (Figure 4). At 1 μM, neither Prog, 19norP nor Spiron were transcriptional activators of full-length human, Xenopus and alligator MRs. As previously reported, Prog, 19norP and Spiron activated transcription by full-length zebrafish MR (*18*). Unexpectedly, Prog, 19norP and Spiron activated full-length chicken MR (Figure 4B1).

Prog, 19norProg and Spiron had no activity for truncated human, chicken, alligator and Xenopus MRs. However, Prog, 19norProg and spiron activated truncated zebrafish MRs (Figure 4E2).



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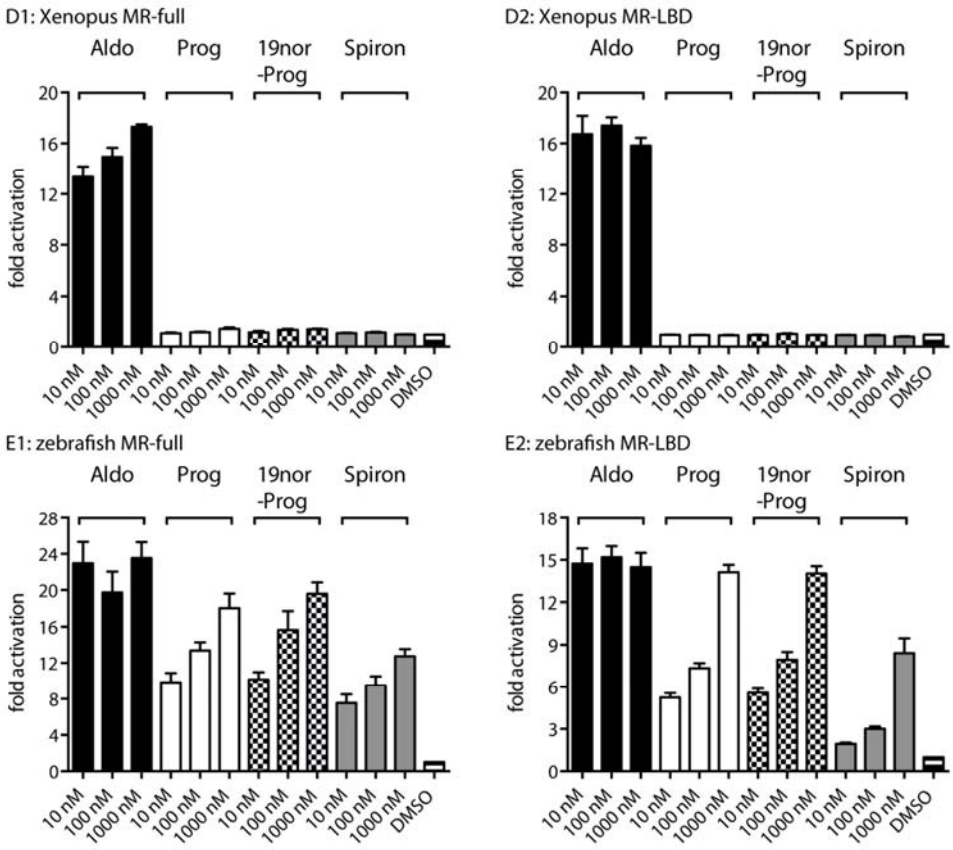

Figure 4. Prog, 19norProg or Spiron activation of human, alligator, *Xenopus* and zebrafish full-length and truncated MRs.

Full-length human MR (A1), chicken MR (B1), alligator MR (C1), *Xenopus* MR (D1), and zebrafish MR (E1) were expressed in HEK293 cells with an MMTV-luciferase reporter. Plasmids for corresponding truncated MRs, human (A2),chicken (B2), alligator (C2), *X. laevis* (D2) and zebrafish (E2) containing the D domain and LBD (E domain) fused to a GAL4-DBD were expressed in HEK293 cells with a luciferase reporter containing GAL4 binding site. Cells were treated with 10 nM, 100 nM or 1 µM Prog, 19norProg or Spiron or vehicle alone (DMSO). Results are expressed as means ± SEM, n=3. Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

EC50 values for corticosteroid activation of full-length and truncated human, chicken, alligator, *X. laevis* and zebrafish MRs

Full-length vertebrate MRs

To gain a quantitative measure of corticosteroid activation of vertebrate MRs,

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we determined the concentration-dependent activation of full-length vertebrate MRs by Aldo, F, B, DOC and S (Figure 5, Table 1). Full-length chicken and zebrafish MRs have EC50s that are below 1 nM for Aldo, F, B, DOC and S. Human, alligator and Xenopus MRs have strongest responses to Aldo, B and F and weaker responses to DOC and S.

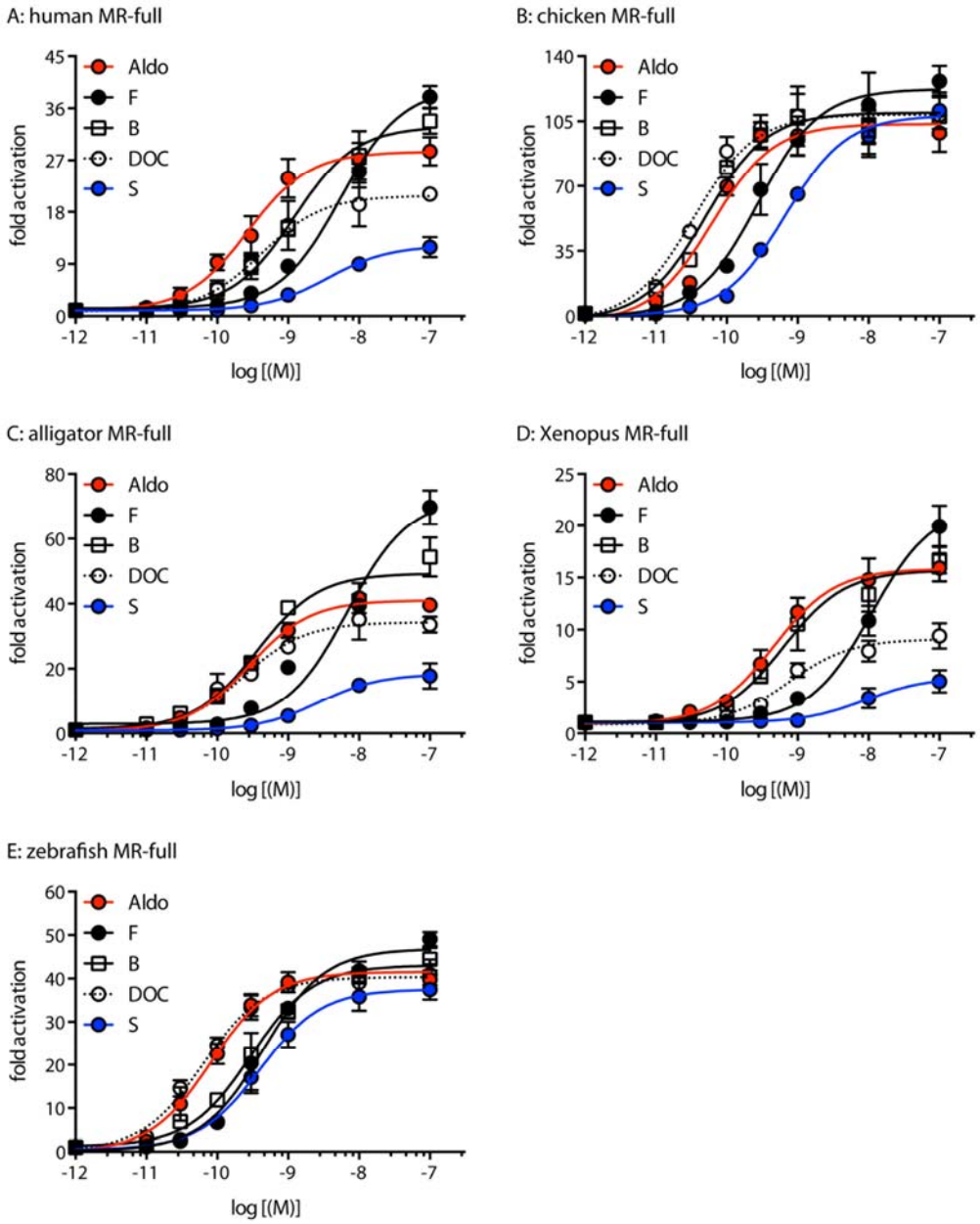

Figure 5. Concentration-dependent transcriptional activation by corticosteroids of full-length human, chicken, alligator, *Xenopus* and zebrafish MRs.

Plasmids encoding full-length MRs A: human MR, B: chicken MR, C: alligator MR, D: *Xenopus* MR and E: zebrafish MR were expressed in HEK293 cells treated with

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increasing concentrations of steroid or vehicle alone (DMSO). Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

Table 1. EC50 activities for 3-ketosteroid transcriptional activation of full-length vertebrate MRs

MR	Aldo	B	F	DOC	S
	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)
Human	2.7×10^{-10}	1.2×10^{-9}	5.5×10^{-9}	4.2×10^{-10}	3.6×10^{-9}
	100%	119%	133%	74%	42%
Chicken	6.2×10^{-11}	5.1×10^{-11}	2.8×10^{-10}	3.4×10^{-11}	6.7×10^{-10}
	100%	109%	128%	110%	112%
Alligator	2.8×10^{-10}	3.6×10^{-10}	6.9×10^{-9}	2.3×10^{-10}	2.7×10^{-9}
	100%	138%	176%	85%	45%
Xenopus	4.6×10^{-10}	6.2×10^{-10}	1.1×10^{-8}	7.6×10^{-10}	9.1×10^{-9}
	100%	105%	126%	59%	31%
Zebrafish	8.2×10^{-11}	3.0×10^{-10}	4.4×10^{-10}	6.3×10^{-11}	4.0×10^{-10}
	100%	112%	123%	103%	94%

(%) Relative induction compared to Aldosterone induced activation.

Truncated vertebrate MRs

To investigate the role of the NTD and DBD we determined the concentration-dependent transcriptional activation of truncated terrestrial vertebrate MRs by Aldo, F, B, Aldo, DOC and S. Transcriptional activation by S, DOC and F was dramatically lowered for some terrestrial vertebrate MRs that lacked MR NTD-DBD (Figure 6 and Table 2). For example, S had little activity for truncated human, alligator and Xenopus MRs. DOC and F had less activity for truncated Xenopus and human MRs. Interestingly, truncated zebrafish MR retained a good response to corticosteroids.

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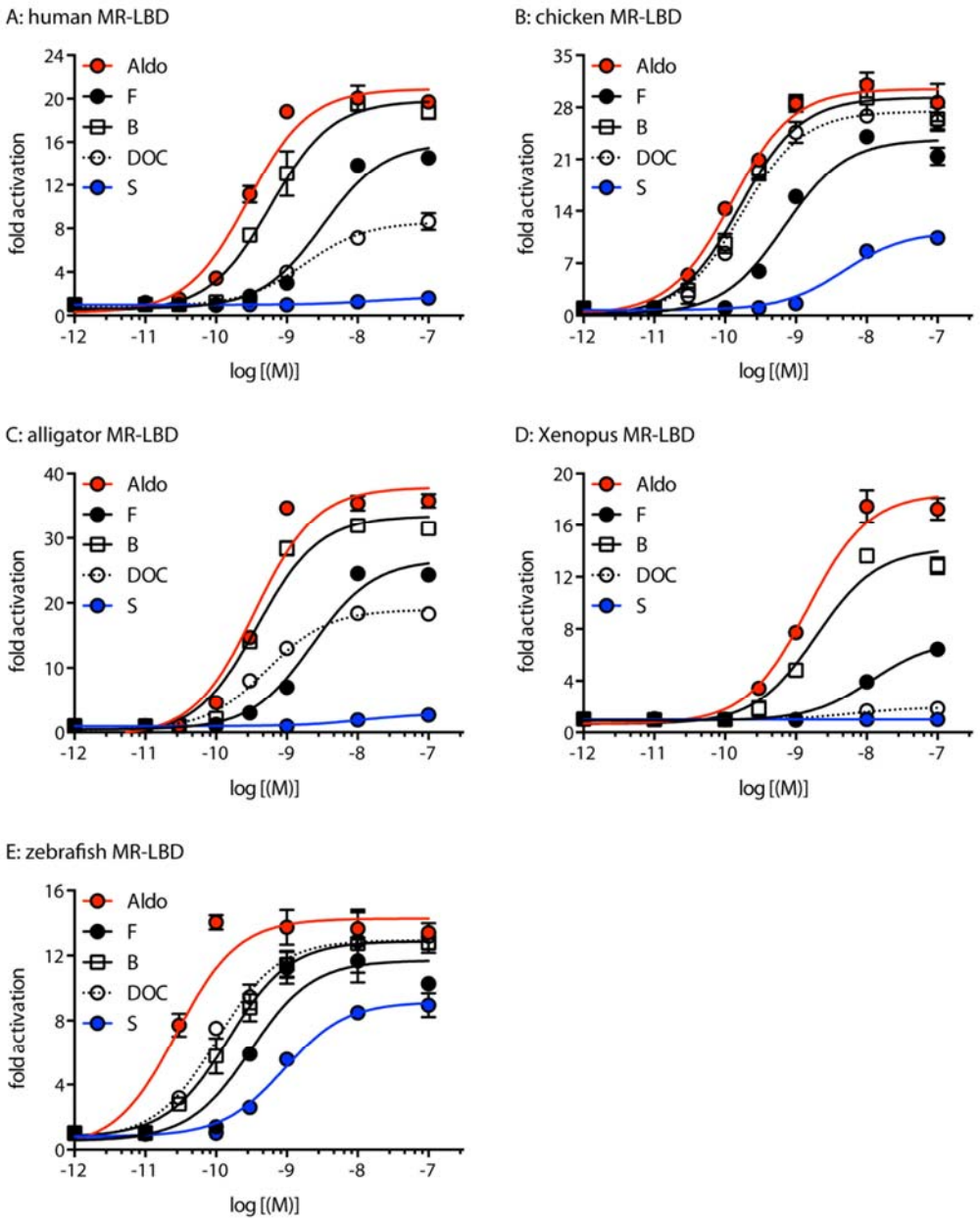

Figure 6. Concentration-dependent transcriptional activation of truncated human, chicken, alligator, *Xenopus* and zebrafish MRs.

Plasmids encoding the GAL4-DBD fused to the D domain and LBD of MRs (A: human, B: chicken, C: alligator, D: *Xenopus*, E: zebrafish)) were expressed in HEK293 cells treated with increasing concentrations of Aldo, F, and DOC or vehicle alone (DMSO). Y-axis indicates fold-activation compared to the activity of control vector with vehicle (DMSO) alone as 1.

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Table 2. EC50 activities for 3-ketosteroid transcriptional activation of GAL4-DBD-MR-LBD of vertebrate MRs

MR	Aldo	B	F	DOC	S
	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)	EC50 (M)
Human	2.8×10^{-10}	5.9×10^{-10}	3.2×10^{-9}	1.8×10^{-9}	-
	100%	95%	74%	44%	8%
Chicken	1.3×10^{-10}	1.6×10^{-10}	6.9×10^{-10}	1.7×10^{-10}	4.7×10^{-9}
	100%	92%	75%	92%	36%
Alligator	3.5×10^{-10}	3.8×10^{-10}	2.3×10^{-9}	5.2×10^{-10}	-
	100%	88%	68%	51%	8%
Xenopus	1.5×10^{-9}	1.9×10^{-9}	1.2×10^{-8}	-	-
	100%	74%	37%	10%	6%
Zebrafish	2.7×10^{-11}	1.5×10^{-10}	3.1×10^{-10}	1.0×10^{-10}	9.1×10^{-10}
	100%	96%	77%	99%	67%

(%) Relative induction compared to Aldosterone induced activation.

DISCUSSION

Although it is thirty years since the human MR was cloned (*10*), data on transcriptional activation of terrestrial vertebrate MRs by corticosteroids and progestins is modest. For the most part, the focus has been on activation of full-length human MR by Aldo and F, and in some studies by B, DOC and S (*11, 18, 19, 42, 44, 58, 59*). Transcriptional activation by Aldo and B of full-length chicken MR (*60*) and by Aldo, B, F and DOC full-length alligator MR (*61*) also have been studied. Data for Prog, 19norProg and Spiron in terrestrial vertebrates is limited to human MR for which Prog and 19norProg have low activity, while Spiron is an MR antagonist (*15, 17, 18, 44*). In contrast, Prog, 19norProg and Spiron activate the MR in zebrafish, trout, gar and sturgeon (*18, 29, 33*). Data on the influence of the NTD on transcriptional activation by corticosteroids and progestins is limited to human, rat and zebrafish MRs (*19, 29, 42, 62*), with no data on chicken, alligator and Xenopus MRs.

Here we fill in some gaps in our knowledge of transcriptional activation by corticosteroids and progestins of full-length and truncated MRs in chicken, alligator and Xenopus and truncated zebrafish MR. The DBDs in human, chicken and alligator

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MRs are identical and the DBDs in other vertebrate MRs are strongly conserved (Figure 2) suggesting that differences in transcriptional activation by corticosteroids and progestins of full-length and truncated MRs are mainly are due to interactions between the NTD and hinge-LBD.

The higher EC50s for corticosteroid activation of truncated terrestrial vertebrate MRs compared to full-length MRs (Tables 1 and 2) indicate that the NTD and LBD are important in transcriptional activation of the MR. The loss of activity for truncated MRs varies with the steroid and the vertebrate. Aldo and B have the smallest change in EC50 for full-length and truncated terrestrial vertebrate MRs. Unexpectedly S does not activate any truncated terrestrial vertebrate MR, while DOC loses activity for truncated Xenopus MR and has diminished activity for truncated human MR. In contrast to terrestrial vertebrate MRs, truncated zebrafish MR retains activity for all corticosteroids. Our results are in agreement with Rogerson and Fuller (*42*) and Pippal et al (*19, 29*), who found that truncated (GAL4-DBD-MR-LBD) human and zebrafish MRs had lower responses to Aldo than truncated MRs incubated with the NTD domain. They also reported that Aldo could not activate transcription by truncated human Glu962Ala MR, but Aldo could activate NTD+truncated-Glu962Ala MR, demonstrating the importance of human MR NTD in transcriptional activation of human MR. DOC and F promoted an increase in transcriptional activation for NTD+GAL4-DBD-zebrafishMR-LBD, but only had a weak effect for NTD+GAL4-DBD-human LBD. Our results extend this role of the NTD to transcriptional activation of chicken, alligator and Xenopus MRs by Aldo, F, B, DOC and S and transcriptional activation of human MR by B and S (Tables 1,2, Figures 3,5).

Prog, 19norProg and Spiron are enigmatic ligands for vertebrate MRs. These steroids are antagonists for human MR (*15, 17-19*), and as reported here for alligator and Xenopus MRs (Figure 4), and agonists for zebrafish MR (*18, 29*), trout MR (*33*), sturgeon MR and gar MR (*18*) and as reported here for chicken MR, which was unexpected (Figure 4). Indeed, Prog may be a transcriptional activator of chicken MR as well as of fish MRs. Due to the absence of an 11β-hydroxyl on Prog, it is inert to 11β-HSD2.

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For terrestrial vertebrate MRs, the only previous example of a Prog-activated MR was the Ser810Leu mutant human MR, which was studied by Geller et al. (*17*). Ser810Leu MR was activated by 1 nM Prog, 19norProg and Spiron. However, both chicken and zebrafish MRs, as well as other Prog-activated fish MRs, contain a serine corresponding to Ser810 in wild-type human MR. This indicates that alternative mechanisms are involved in progestin activation of chicken, zebrafish and other fish MRs. Our experiments with truncated chicken and zebrafish MRs provide clues to regulation of Prog activation of chicken and zebrafish MRs. The lack of Prog, 19norProg and Spiron activation of truncated chicken MR indicates that allosteric interactions between the NTD and hinge-LBD domains of full-length chicken MR are important in transcriptional activation by these steroids, while activation of truncated zebrafish MR indicates that the hinge-LBD domains are important in transcriptional activation of full-length zebrafish MR.

The evolution of Prog as an agonist and antagonist of the MR is not fully understood. Evidence that Prog, 19norProg and Spiron are transcriptional activators of sturgeon and gar MRs (*18*), two basal ray-finned fish that evolved before teleosts (zebrafish and trout), suggests that Prog was an ancestral transcriptional activator for ray-finned fish MR. Moreover, at 100 nM, Prog is a transcriptional activator of sea lamprey (*Petromyzon marinus*) and hagfish *Myxine glutinosa* CRs (*63*). The CR is the common ancestor of the MR and GR (*8, 20, 64*). Elucidating the response to Prog of full-length MRs from cartilaginous fishes (Chondrichthyes), such as sharks, rays and skates, and from lobe-finned fish (Sarcopterygii), such as coelacanths and lungfish, is needed to determine when Prog antagonist activity arose in vertebrates.

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