SciMedCentral

JSM Sexual Medicine

Research Article

Expression and Differential Regulation of NR5A1/SF1 in Harderian Gland of Hamster

Luis Alberto García-Avila[1,2]*

[1]*National Polytechnic Institute, National School of Biological Sciences, Environmental Toxicology Laboratory, Mexico*

[2]*National Polytechnic Institute, National School of Biological Sciences, Immunochemistry Laboratory I, Mexico*

***Corresponding author**

Luis Alberto García-Avila, Av. Wilfrido Massieu s/n, Zacatenco Professional Unit, Mexico City, CP 07738, Mexico

Submitted: 27 February 2022

Accepted: 28 March 2023

Published: 29 February 2023

ISSN: 2578-3718

Copyright

© 2023 García-Avila LA, et al.

OPEN ACCESS

Keywords

- Steroidogenic factor 1
- NR5A1
- Harderian gland
- Steroidogenesis
- Sexual dimorphism

Abstract

The nuclear receptor of subfamily 5 group A member 1 (NR5A1), also called steroidogenic factor 1 (SF1), is critical to the steroidogenesis process, controlling the expression of cytochrome P450scc and other essential proteins for this process. There is evidence that the Harderian gland (HG) of the Syrian hamster (*Mesocricettus auratus*) expresses steroidogenic enzymes. Some present a dimorphic expression, suggesting that sex steroids play a role in said expression. However, the expression of NR5A1/SF1 in HG has not been identified, and whether this expression presents dimorphic profiles in HG and other steroidogenic tissues. In this study, part of the cDNA corresponding to NR5A1/SF1 was isolated, and its experimental sequence was obtained, which will serve to obtain the complete cDNA sequence of this factor. So far, the cDNA's central and 3' region has been sequenced and compared using the Multiple Sequence Alignment by CLUSTALW online tool, with the predicted sequence for the NR5A1/SF1 cDNA, obtaining an alignment score of 94.24%.

INTRODUCTION

A complex of multiple enzymes carries out the steroidogenic process; among them is the acute steroidogenesis regulatory protein (StAR) which controls the amount of cholesterol supply that enters the inner membrane of the mitochondria. This cholesterol is the precursor for the biosynthesis of steroid hormones [1]. The first reaction in this biosynthesis is the conversion of cholesterol to pregnenolone by the action of the cytochrome P450 side-chain cleavage enzyme (P450scc; CYP11A1). This enzyme resides on the matrix side of the inner mitochondrial membrane. Existing pregnenolone in mitochondria is converted into progesterone and other steroids in microsomal compartments of the cell [2].

Nuclear receptor subfamily 5 group A member 1 (NR5A1), also called steroidogenic factor 1 (SF1), has been identified as a critical factor in the expression of several steroidogenic enzymes, including cytochromes P450 such as CYP11A1, CYP17A1, CYP21A2, CYP11B1, CYP11B2, 3β-hydroxysteroid dehydrogenase, and the StAR protein [3]. It is an essential transcription factor in the regulation of various genes crucial for normal endocrine function, as well as for the reproductive physiology of the individual. The gene encoded by NR5A1 is highly expressed in steroidogenic tissues, where it controls several steps of gonadal and adrenal development [4]. Complete loss of function of NR5A1 in mice has been shown to result in dysgenesis of the gonads and adrenal primordia via apoptosis. However, the mechanism by which this apoptosis occurs is unknown [5]. In addition, steroidogenic organs, structures, and functions in non-steroidogenic tissues where NR5A1 is also expressed (such as the pituitary gland, ventromedial hypothalamic nucleus, and spleen) are also affected in mice, indicating that this factor is essential in the differentiation, proliferation, and survival of the cells where it is expressed [6,7].

The high level of expression of the NR5A1 gene in the male gonad after differentiation has been shown to play a vital role in gonadal differentiation, mainly regulating the TP53 pathway during development, which is essential for the cell survival of fetal Sertoli cells, and controlling the cell cycle of Sertoli cells, during differentiation [8]. In addition, mutations in NR5A1 are associated with premature ovarian failure in humans [9].

Johann Jacob Harder first described the Harderian gland (HG) in 1694, which is present in most terrestrial vertebrates and responsible for eyeball lubrication [10]. This gland is very well developed in rodents (rats, mice, hamsters, gerbils, and guinea pigs) and occupies a considerable part of the posterior area of the eyeball. In rodents, HG contains many lipids and porphyrins secreted via this pipeline [11].

In particular, HG from the Syrian hamster (*Mesocricettus auratus*) has been widely studied due to its marked sexual dimorphism; these dimorphic characteristics are observed in histology and lipid, porphyrin, and melatonin content. The

concentration of porphyrins is much higher in females than in males, in addition to the fact that females have high melatonin levels.

This HG secretory activity seems to be regulated by steroids and the sexual dimorphism shown in the Syrian hamster [12]. Although some studies have suggested that HG can be classified as steroidogenic [13], no studies confirm the expression of NR5A1/SF1 in hamster HG, the experimental cDNA sequence, or the deduced amino acid sequence of this factor of transcription have been reported. In this study, we focus on determining the experimental sequence of the cDNA corresponding to NR5A1/SF1, its expression in HG, and whether it presents dimorphic differences, being affected by sex steroids.

MATERIAL AND METHODS

Total RNA extraction

Total RNA was extracted from the hamster liver, testis, and ovary, using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The tissue was homogenized and incubated for 5 min at room temperature, then 200 µl of chloroform was added for each ml. of the initial solution. The samples were shaken vigorously and centrifuged at 12,000 xg for 15 min at 4 °C. The aqueous phase was extracted to add 500 µl of isopropanol for each ml of the initial solution. This mixture was incubated for 10 min at room temperature after time and was centrifuged at 12,000 xg for 10 min at 4 °C. The supernatant was discarded, while the precipitate was washed with 1 ml of 75% ethanol, and then centrifuged at 12,000 xg for 10 min and 4 °C, the supernatant was discarded, and the RNA was allowed to dry at room temperature. Total RNA was dissolved in nuclease-free water, and the purity and concentration were evaluated at 260/280 nm in a spectrophotometer (Beckman DU 650; Fullerton, CA, USA).

Complementary DNA synthesis

Complementary DNA (cDNA) was synthesized with SuperScrip First-Strand cDNA (Invitrogen, Carlsbad, California, USA). For the reaction, a mixture of RNA (3-5 µg), one µl of dNTP mix, and one µl of oligo dT (0.5 µg) were made and brought to a final volume of 10 µl with DEPC-treated water. The mixture was incubated for 5 min at 65 °C and then placed on ice for 1 min. In a separate tube, another mix was prepared to contain 2 µl of 10X RT solution, 4 µl of 25 mM MgCl2, 2 µl of 0.1 M DTT, and 1 µl of RNaseOUT (40 U/µl). The nine µl of this mixture were added to the first reaction and mixed carefully, to be later incubated for 2 min at 42°C, and at the end of this incubation, one µl of SuperScript II RT was added. It was incubated again for 50 min at 42 °C followed by 15 min at 70°C. At the end of the reaction, one µl of RNase H was added and incubated for 20 min at 37°C. The reaction was stored at −20°C until its use in PCR.

Synthesis of the 5' and 3' ends of the cDNA

The cDNA corresponding to the 5' and 3' ends was synthesized using the SMARTer RACE 5'/3' kit (Takara Inc, USA). For each of the reactions, a buffer mixture was prepared to contain four µl of 5X First-Strand, 0.5 µl of DTT (100mM), and one µl of dNTPs (20 mM) for a total volume of 5.5 µl each. On the other hand, in the case of the 5' reaction, 1 µg of RNA was mixed with one µl of 5'-CDS Primer A and brought to a total volume of 11 µl with sterile water. For the 3' reaction, 1 µg of RNA and one µl of 3'-CDS Primer A were mixed and brought to 12 µl with sterile water. These last two mixtures were incubated for 3 min at 72° C, followed by another at 42 °C for 2 min, to denature the RNA. A third mix was prepared using the 5.5 µl of the first prepared buffer, to which was added 0.5 µl of RNase Inhibitor (40 U/µl) and two µl of SMARTScribe Reverse Transcriptase (100 U), resulting in a total volume of 8 µl for each reaction, which was added to the denatured RNAs, leaving a 20 µl volume reaction, which was incubated for 90 min at 42 °C and 10 min at 70°C. The reaction products were diluted in 90 µl of Tris-EDTA pH 7.6.

Oligonucleotide design

The region corresponding to NR5A1/SF1 to amplify oligonucleotides was designed using the Integrated DNA Technologies, Inc. "PrimerQuest Tool" online tool (https://www.idtdna.com/Primerquest/Home/Index). The predicted sequence for the NR5A1/SF1 cDNA of *Mesocrisetus auratus* available in the GenBank of the National Center for Biotechnology Information (NCBI) was used. The oligonucleotides were designed to amplify an internal region of the NR5A1/SF1 sequence. In contrast, for the extreme regions, oligonucleotides were designed to amplify regions within 300 base pairs (bp) of both the 5' end and the end. 3', amplified using the rapid amplification of cDNA ends (RACE) technique.

Polymerase Chain Reaction (PCR)

Amplification of the central region corresponding to NR5A1/SF1 was performed using two pairs of oligos SF1-1PF, SF1-1PR, and SF1-2PF, SF1-2PR for the amplification reaction were mixed in one tube, four µl of 5X buffer. , 0.5 µl of dNTPs (10 mM), 0.1 µl of GoTaq polymerase, eight µl of MgCl2 (25mM), four µl of DMSO and eight µl of cDNA and with the conditions of 94 °C for 3 min of initial denaturation, 30 cycles with denaturation temperature at 94° for 30 sec, alignment 60°C for 30 sec, an extension of 72°C for 30 sec, followed by a final extension of 72°C for 3 min. The reaction products were visualized in a 1% agarose gel stained with ethidium bromide to purify the observed bands.

Rapid amplification of cDNA ends (RACE)

The 5' and 3' ends were amplified using the SMARTer RACE 5'/3' kit (Takara Bio USA, Inc.). Different mixtures were made containing 12.5 µl of 2X SeqAmp buffer solution., 0.5 µl of SeqAmp Polymerase, 2.5 µl of 10X Universal Primer Mix (UPM), 0.5 µl of 20 mM gene-specific oligonucleotide (GSP), and three µl of 5' or 3' cDNA as appropriate and completed with sterile water to a final volume of 25 µL. The reaction was carried out with 35 cycles of 94 °C for 30 sec, 68 °C for 30 sec, and 72°C for 3 min. The reaction products were visualized on a 1% agarose gel to purify the bands.

DNA purification on agarose gel

DNA purification in agarose gel was also performed using the E.Z.N.A® gel extraction kit (Omega Bio-tek, Inc. USA). The fragment of the gel containing the DNA band was excised and placed inside a 1.5 ml conical tube, where 500 µl of XP2 Binding was added, to be incubated at 60°C until the gel was completely dissolved, never exceeding 7 min of incubation. The mini-column was placed inside a 2 ml collection tube, and the DNA/agarose solution was added. The column was centrifuged at 10,000 xg for one minute, and the filtrate was discarded. 300 µl of XP2 Binding buffer was added and centrifuged at maximum speed (≤ 13000 xg) for 1 min, and the filtrate was discarded. 700 µl of SPW buffer solution, previously diluted with 100 ml of 100% ethanol, was added and centrifuged at maximum speed for 1 min, and the filtrate was discarded. This last washing step was repeated with SPW buffer. The empty column was centrifuged at maximum speed to eliminate any traces of alcohol. Then the column was transferred to a new collection tube, and 30 µl of elution buffer was added to the center of the column, avoiding touching the membrane, and incubated for 1 min and centrifuged at maximum speed for 1 min. Five µl of the purified DNA was used to run it on a 1% agarose gel. It was observed in a photo documenter (Molecular Imager ChemiDoc XRS+ with Image Lab Software, BioRad, Hercules, CA, USA).

Sequencing

Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Austin, TX, USA), with which PCR amplification of the product to be sequenced was performed. For said reaction, a mixture was made which contained one µl of BigDye Solution 5X, two µl of BigDye Terminator reaction mix, one µl of specific oligonucleotide (20 mM), five µl of DNA (5-20 ng) and was completed with water to a total volume of 10 µL. The reaction was carried out under the following conditions: 1 cycle at 96 °C for 1 min, 35 cycles at 96 °C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min. At the end of the reaction, the samples were purified using the BigDye XTerminator kit (Applied Biosystems, Austin, TX, USA). 45 µl of SAM solution and ten µl of BigDye Xterminator solution were added to each sample and shaken for 30 min. After this time, the samples were centrifuged at 3000 xg for 2 min. The supernatant was removed and placed in another tube. These samples were analyzed by capillary electrophoresis in an ABI PRISM 310 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA).

RESULTS

RNA extraction

RNA extraction from the testicle and ovary was performed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), the integrity of the RNA was observed in a 1% agarose gel, in which the full bands could be observed, corresponding to the 28S and 18S ribosomal subunits. Once the integrity of the RNA was corroborated, 1µg of it was used to carry out the reverse transcription reaction (RT) to obtain the cDNA.

Oligonucleotide design

Two pairs of oligonucleotides were designed using the "PrimerQuest Tool" online tool (Integrated DNA Technologies, Inc.) to amplify a core region of NR5A1/SF1. The sequence predicted for *Mesocrisetus auratus* NR5A1/SF1 cDNA is available in the GenBank of the National Center for Biotechnology Information and was used for design. The first pair of oligonucleotides, SF1-1PF and SF1-1PR, comprised an expected amplicon of 1000 bp, and the second pair of oligonucleotides, SF1-2PF and SF1-2PR, was expected to have an amplicon of 1014 bp.

Polymerase chain reaction

The polymerase chain reaction (PCR) was performed using hamster ovary and testis cDNA. For both cDNAs, three reactions were performed with a pair of oligonucleotides. These three reactions were carried out at different hybridization temperature gradients: 58° C, 60° C, and 62°C.

Considering the obtained amplifications at different gradients and cDNAs, it was decided to use the ovary cDNA, the oligonucleotides SF1-2PF and SF1-2PR, with a hybridization temperature of 58°C, to perform four reactions, which were run in a 1% agarose gel. Once the bands that possibly correspond to the central fragment of SF1/NR5A1 were identified, these bands were excised from the gel and purified by electro-elution. The purified product was rerun on a 1% agarose gel to observe the amount and integrity of the recovered fragment. Once the quality of the purified fragment was checked, it was labeled and stored until it was sequenced.

Rapid amplification of cDNA ends (RACE)

To perform the 5' and 3' RACE reaction, cDNA from hamster testis and the SMARTer RACE 5'/3' kit (Takara Bio USA, Inc.) were used. For each end, an amplification reaction was made with each designed oligonucleotide. These reactions were run on an agarose gel to observe the reaction products. Of the reactions carried out, it was only possible to observe an appropriate amplification with the oligonucleotide 5R2 of the possible fragment belonging to the 5' region of SF1/NR5A1. In the case of oligonucleotides 3R1 and 3R2 (corresponding to the 3' region), a slight amplification was observed, so it was decided to carry out more reactions to obtain a more significant amount of product and to be able to purify it.

Subsequently, two reactions were performed with each oligonucleotide: 5R2, 3F1, and 3F2. These reactions were run on a 1% agarose gel to observe the amplification bands of the product.

Once the possible product bands were observed, they were excised from the gel, and the products were extracted by electro-elution for purification. The recovered products were run on a new 1% agarose gel to observe their quantity and integrity. It was observed that only the amplified product with 5R2, corresponding to the 5' region, could be recovered, while those amplified with 3F1 and 3F2 were lost during the recovery and purification process.

Two more 3' RACE reactions were performed for each oligonucleotide: 3F1 and 3F2. The reactions were run on a now 2% agarose gel to avoid curling of the loaded products on the gel. It was observed that there was no amplification in any of the reactions carried out. More reactions continued, changing the hybridization temperature and the synthesis of new cDNA to use in said reactions, but they could not get amplified.

Sequencing of the regions obtained

The amplified and purified fragments of the central and 5' regions were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Austin, TX, USA) and analyzed by capillary electrophoresis in a 310 Genetic sequencer. ABI PRISM Analyzer (Applied Biosystems, Foster City, CA, USA).

The sequences obtained (Figure 1) were analyzed to construct the sequence obtained. This construct consists of 1155 sequenced bp, of which 65 are in the possible UTR region, and 1090 bp are located ahead of the translation start site.

The complete sequence obtained was compared using the Multiple Sequence Alignment by CLUSTALW online tool (Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan), with the sequence predicted for the NR5A1/SF1 cDNA (Figure 2), obtaining an alignment score of 94.24%.

DISCUSSION

The complexity of HG is evident. Expression of CYP450scc and 5α-reductases has been reported in hamster HG [14]. Androgen and estrogen receptors are known to exist in rat and hamster HG, suggesting that both testosterone and 5α-dihydrotestosterone affect secretory activity, as in the dimorphism of HG. In addition to the already known correlation between estrogens and the content of porphyrins in HG [15], member 2 of the G superfamily of the ATP-binding cassette (ABCG2) has been identified as involved in the regulation of the protoporphyrin IX transporter and other heme biosynthesis intermediates, which exhibits high expression in HG, with a dimorphic pattern, where females show greater expression than males [16-18].

The SOX9 protein, which can promote lipogenesis, is also expressed in HG, presenting a dimorphic expression being more significant in males than in females, suggesting that sex steroids may regulate this differential expression.

DAX1 is essential for differentiation during embryogenesis and is also expressed in HG but without showing sexual dimorphic expression. However, in adrenal glands, it presents a greater expression with a dimorphic behavior, being higher in females than in males, suggesting that sex steroids may control its activity. In addition, previous studies propose that DAX1 may be a protagonist in the differentiation, maintenance, and metabolism of HG in both sexes, with the interaction of other members of the nuclear receptor superfamily, such as NR5A1/SF1 and NR5A2.

Only part of NR5A1/SF1 has been sequenced, but the segment shows considerable similarity with the available sequence prediction. Even so, with the data obtained at the moment, it is impossible to know if NR5A1/SF1 has a differential expression in the Harderian gland and peripheral, steroidogenic, and non-steroidogenic tissues. For this result, it is necessary to obtain and completely sequence the cDNA corresponding to NR5A1/SF1.

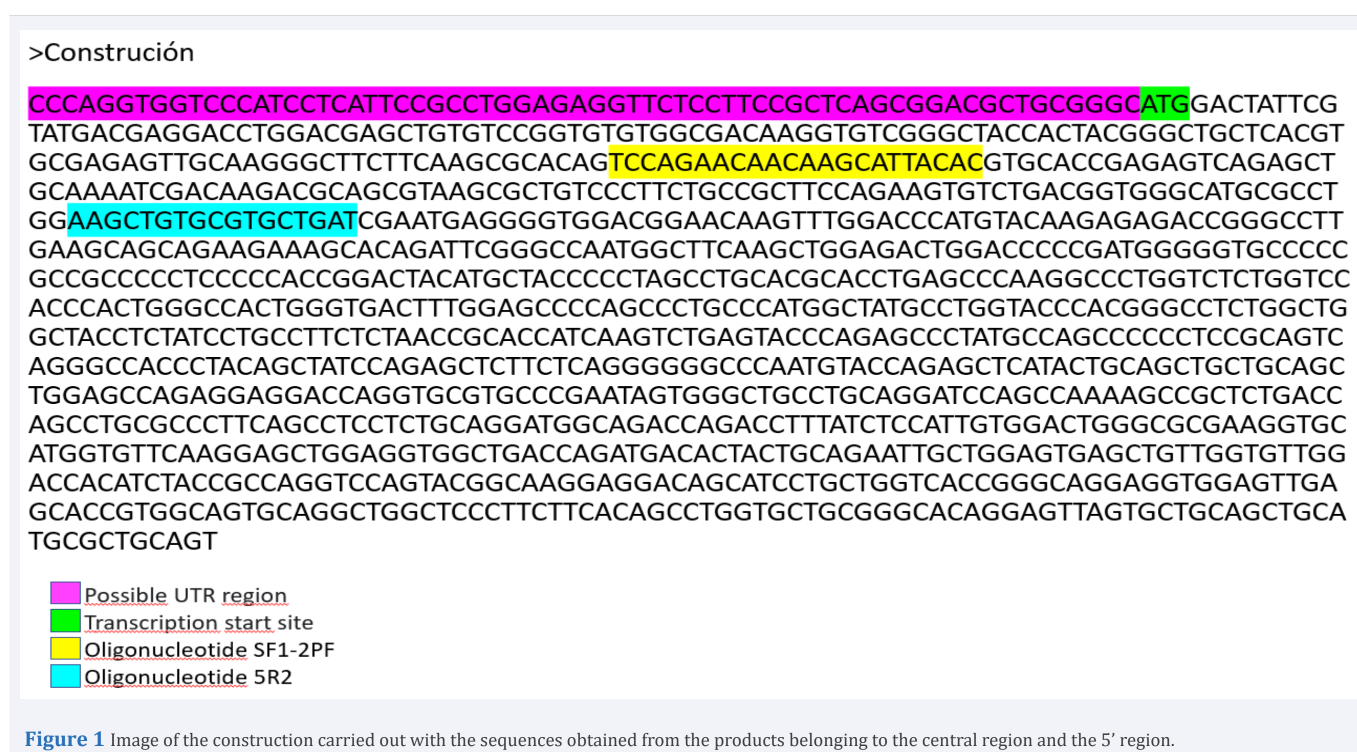

Figure 1 Image of the construction carried out with the sequences obtained from the products belonging to the central region and the 5' region.

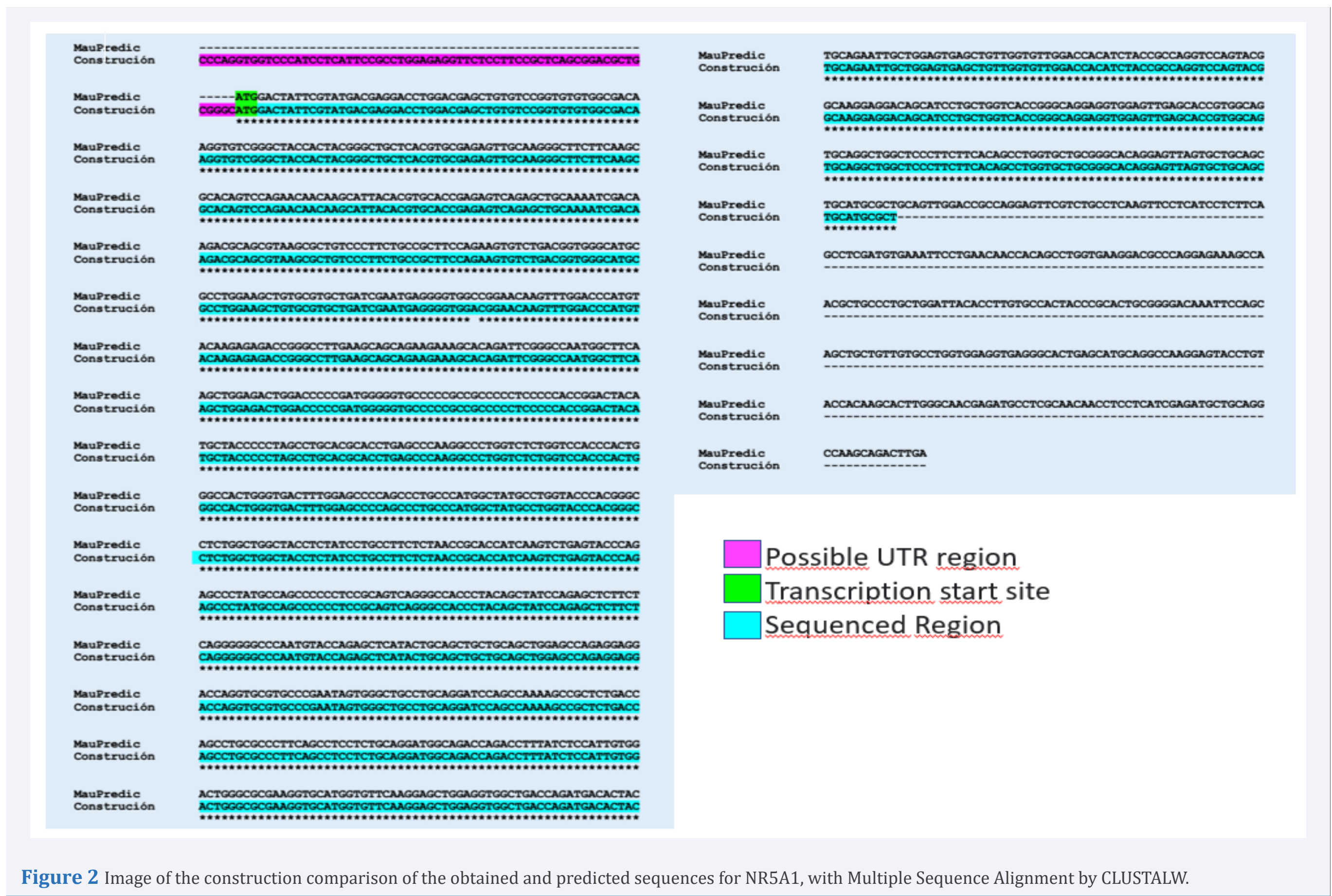

Figure 2 Image of the construction comparison of the obtained and predicted sequences for NR5A1, with Multiple Sequence Alignment by CLUSTALW.

REFERENCES

1. Kanimozhi V, Palanivel K, Akbarsha MA, Kadalmani B. Molecular mechanisms of tributyltin-induced alterations in cholesterol homeostasis and steroidogenesis in hamster testis: In vivo and in vitro studies. J Cell Biochem. 2018; 119: 4021-37.
2. Selvaraj V, Stocco DM, Clark BJ. Current knowledge on the acute regulation of steroidogenesis. Biol Reprod. 2018; 99: 13-26.
3. Ruggiero C, Doghman M, Lalli E. NR5A1 (nuclear receptor subfamily 5, group A, member 1). Atlas Genet Cytogenet Oncol Haematol. 2015; 19: 522-534.
4. Fabbri-Scallet H, de Sousa LM, Maciel-Guerra AT, Guerra-Junior G, de Mello MP. Mutation update for the NR5A1 gene involved in DSD and infertility. Hum Mutat. 2020; 41: 58-68.
5. Anamthathmakula P, Miryala CSJ, Moreci RS, Kyathanahalli C, Hassan SS, Condon JC, et al. Steroidogenic Factor 1 (Nr5a1) is Required for Sertoli Cell Survival Post Sex Determination. Sci Rep. 2019; 9: 4452.
6. Morohashi KI, Inoue M, Baba T. Coordination of Multiple Cellular Processes by NR5A1/Nr5a1. Endocrinol Metab (Seoul). 2020; 35: 756-64.
7. Lalli E, Doghman M, Latre de Late P, El Wakil A, Mus-Veteau I. Beyond steroidogenesis: novel target genes for SF-1 discovered by genomics. Mol Cell Endocrinol. 2013; 371: 154-9.
8. Lourenco D, Brauner R, Lin L, De Perdigo A, Weryha G, Muresan M, et al. Mutations in NR5A1 associated with ovarian insufficiency. N Engl J Med. 2009; 360: 1200-10.
9. Payne AP. The harderian gland: a tercentennial review. J Anat. 1994; 185: 1-49.
10. Chieffi G, Baccari GC, Di Matteo L, d'Istria M, Minucci S, Varriale B. Cell biology of the harderian gland. Int Rev Cytol. 1996; 168: 1-80.
11. Hussein OA, Elgamal DA, Elgayar SA. Structure of the secretory cells of male and female adult guinea pigs Harderian gland. Tissue Cell. 2015; 47: 323-35.
12. Santillo A, Chieffi Baccari G, Minucci S, Falvo S, Venditti M, Di Matteo L. The Harderian gland: Endocrine function and hormonal control. Gen Comp Endocrinol. 2020; 297: 113548.
13. Falvo S, Chieffi Baccaria G, Spaziano G, Rosati L, Venditti M, Di Fiore MM, et al. StAR protein and steroidogenic enzyme expressions in the rat Harderian gland. C R Biol. 2018; 341: 160-6.
14. Ramos L, Chavez B, Vilchis F. Cloning and differential expression of steroid 5 alpha-reductase type 1 (Srd5a1) and type 2 (Srd5a2) from the Harderian glands of hamsters. Gen Comp Endocrinol. 2010; 166: 388-95.
15. Falvo S, Chieffi Baccaria G, Spaziano G, Rosati L, Venditti M, Di Fiore MM, et al. StAR protein and steroidogenic enzyme expressions in the rat Harderian gland. C R Biol. 2018; 341: 160-6.
16. Mares L, Vilchis F, Chavez B, Ramos L. Expression and regulation of ABCG2/BCRP1 by sex steroids in the Harderian gland of the Syrian hamster (Mesocricetus auratus). C R Biol. 2019; 342: 279-89.
17. Mares L, Ramos L. Harderian SOX9: Molecular characterization and its dimorphic expression in hamster. Comp Biochem Physiol A Mol Integr Physiol. 2021; 258: 110981.
18. Ramos L, Mares L. Hamster DAX1: Molecular insights, specific expression, and its role in the Harderian gland. Comp Biochem Physiol A Mol Integr Physiol. 2021; 263: 111096.