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ASW: a gene with conserved avian W-linkage and female specific expression in chick embryonic gonad

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Abstract Vertebrates exhibit a variety of sex determining mechanisms which fall broadly into two classes: environmental or genetic. In birds and mammals sex is determined by a genetic mechanism. In mammals males are the heterogametic sex (XY) with the Y chromosome acting as a dominant determiner of sex due to the action of the testis-determining factor, SRY. In birds females are the heterogametic sex (ZW); however, it is not known whether the W chromosome carries a dominant ovary-determining gene, or whether Z chromosome dosage determines sex. Using an experimental approach, which assumes only that the sex-determining event in birds is accompanied by sex-specific changes in gene expression, we have identified a novel gene, *ASW* (Avian Sex-specific W-linked). The putative protein for ASW is related to the HIT (histidine triad) family of proteins. *ASW* shows female-specific expression in genital ridges and maps to the chicken W chromosome. In addition, we show that, with the exception of ratites, *ASW* is linked to the W chromosome in each of 17 bird species from nine different families of the class Aves.

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The sequence reported in this paper has been deposited in the GenBank database (accession no. AF148455, chicken *ASW*; accession no. AF148456, zebra finch *ASW*).

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Introduction

Commitment of cell or tissue types to specific developmental fates is achieved in most instances by the action of highly conserved key regulatory genes. A conspicuous exception to this rule is the large variety of mechanisms, both environmental and genetic, that have evolved to commit sexually indifferent genital primordia to ovarian or testicular developmental pathways. Given the repercussions of improper or diminished functioning of reproductive organs, it is remarkable that there has been such lability in the primary signalling event even among closely related taxa (Wolf 1999).

In vertebrates gonadogenesis is a highly conserved morphogenetic process with the rudiments of the gonads first appearing as epithelial folds along the ventromedial surface of the embryonic kidney, the mesonephros. As would be expected, many genes such as *SOX9*, and *AMH* (Anti-Müllerian Hormone), have conserved temporal, spatial and sex-specific patterns of expression implying a conserved role in sexual differentiation (Capel 1998; Kent et al. 1996). As yet the genetic switch initiating development of either testis or ovary is known only in mammals. The *SRY* gene, encoding an HMG box DNA-binding protein, has been shown to be both necessary and sufficient to initiate testicular development in mouse and humans (Gubbay et al. 1990; Koopman et al. 1991; Sinclair et al. 1990). The conservation of its DNA binding domain and its conserved linkage to the mammalian Y chromosome implies that it plays the same role of initiating testis development in all mammals.

Birds differ from mammals in that the female is the heterogametic sex (ZW) and the male is homogametic (ZZ). In male birds the development of the testis is almost identical to that of mammals. However, in female birds gonadogenesis is often asymmetric such that the left ovary develops normally while the right regresses to a vestige.

The mammalian Y and avian W chromosomes, while not syntenic, are similar in that both are sex limited, small in size, heterochromatic and apparently gene poor. During female meiosis an homologous pairing segment is observed between the short arm of the Z and W chromosomes, as is the case with the mammalian X and Y chromosomes (Solari 1988). Although the data are scant, dosage compensation as observed in mammals appears to be absent in birds such that neither avian Z chromosome is inactivated in the male (reviewed in Thorne 1996).

While the Z and W chromosomes must carry genes that in some way regulate avian sex determination, the underlying genetic mechanism is not clear. The mammalian testis-determining gene, *SRY*, has not been detected in birds and does not appear to play a role in avian sex determination (Griffiths 1991). Nevertheless, sex determination in birds may be similar to that in mammals in that the W chromosome may harbour a dominant sex-determining gene that initiates ovarian development in ZW female embryos. Alternatively, avian sex determination may more resemble the genic balance mechanism seen in *Drosophila* such that the ratio of Z chromosomes to autosomes determines sex rather than the presence or absence of the W (Thorne 1996). Clues to the mechanism of sex determination can be gleaned from individuals with sex chromosome aneuploidy. If the W dominant hypothesis were correct, diploid individuals with sex chromosome aneuploidy such as ZO males or ZZW females should be observed. Conversely, the observation of a ZO female or a ZZW male would support the Z chromosome genic balance model. Unfortunately, the critical evidence from these genotypes is lacking in birds (Thorne 1996). No ZO individuals have ever been found, suggesting they may be embryo lethal (Kagami et al. 1995), and no definitive data exist on either ZZW females or males. In triploid ZZW chickens the left gonad initially develops as an ovary (supporting W dominance) but later forms an ovotestis, suggesting a testis can form in the presence of a W chromosome (supporting Z dosage; Thorne and Sheldon 1993).

In order to identify the key regulatory genes involved in avian sex determination it was necessary to take an experimental approach which does not assume one switch mechanism in favour of the other; a dominant W effect versus Z chromosome dose. For this reason we chose to assay for sex-specific transcripts expressed around the time that sexual dimorphism first becomes apparent in the developing chick gonad. Due to the extremely limited amounts of RNA from dissected chick embryonic gonads available for differential screening we employed two PCR-based screening methods; differential display (DDPCR; Liang and Pardee 1992) and representational difference analysis (RDA) of cDNA (Hubank and Schatz 1994; Lisitsyn and Wigler 1993).

Materials and methods

Animals

All adult and embryonic fowl tissues were obtained from White Leghorn/Austrolorp (*Gallus gallus*) stock (Research Poultry Farms). Various bird DNAs were obtained from blood extracted from wild-caught and captive populations.

Dissection and sexing of chicken embryos

Chicken eggs were incubated in a humidified incubator at 37.8°C. Embryo age was estimated by days (d) of incubation and staged according to Hamburger and Hamilton (1951). Mesonephros and/or genital ridges were dissected from developing chicken embryos at the appropriate stages. DNA was extracted from a small amount of tail tissue for sexing by PCR. Embryos were sexed using primers *Xho*I (5′-AACTACCACTTTTCTCACGG-3′) and *Xho*II (5′-TTCAGAGTGATAACGCATGG-3′) specific for a *Xho*I repeat on the chicken W chromosome (Kodama et al. 1987). RNA was extracted using the single step method (Chomczynski and Sacchi 1987).

Differential display

DDPCR was performed essentially according to (Liang and Pardee 1992) with the following alterations. Anchor primers (T_{11}MN; Operon) were combined in reactions according to the last base (e.g. T_{11} MG consists of T_{11}AG, T_{11}CG and T11GG). Total RNA was reverse-transcribed using Superscript reverse transcriptase (BRL) at 42°C for 1 h. PCR was performed on a Corbett microcapillary thermal cycler under the following conditions: 94°C for 15 s; 42°C for 2 min; 72°C for 30 s, for 35 cycles using [α-^{33}P]dATP. To eliminate false positives, display reactions were performed in duplicate using independent RNA samples. Display products were run on 6% polyacrylamide/7M urea sequencing gels. Candidate bands were excised; the DNA eluted, reamplified and cloned into pCRscript (Stratagene) for further analysis.

Representational difference analysis

RDA of cDNA was performed essentially according to Hubank and Schatz (1994) with modifications as reported by O'Neill and Sinclair (1997).

Rapid amplification of cDNA ends

Rapid amplification of cDNA ends (RACE) PCR was performed using the Marathon kit (Clontech) according to the manufacturer's instructions.

Cloning of ASW

A primer was prepared from the sequence of the differential display clone for use in RACE PCR. The 5′ extension products were cloned into pBluescript (Stratagene). The ASW full-length transcript was derived from the RACE clones and the DDPCR product. We produced a female chicken genomic library and screened it with the differential display product to isolate an ASW genomic clone. The ASW genomic clone was sequenced and compared to the ASW cDNA clone to determine the intron/exon boundaries.

Southern analysis

Southern blots were prepared using 10 µg of male and female DNA digested with restriction enzymes as indicated. DNA was electrophoresed on a 1% agarose gel and transferred to Hybond N+ (Amersham) according to the manufacturer's instructions. Either the original 371-bp DDPCR clone or the 657-bp full-length *ASW* cDNA probe (100 ng) was labelled with [α-^{32}P]dCTP using random priming. Probes were hybridized with the membrane at 65°C overnight. Membranes were washed to either high stringen-

cy (0.1×SSC/0.1% SDS, 65°C) or low stringency (2×SSC/0.1% SDS, 65°C). Blots were exposed to Kodak Biomax film.

Northern analysis

Twenty micrograms of total RNA was electrophoresed on a 1% agarose/formaldehyde gel and transferred to Hybond N (Amersham) according to the manufacturer's instructions. The *ASW* DDPCR product was radiolabelled as above and used as a probe in a blot hybridization carried out at 50°C overnight in 5×SSC/50% Denhart's/0.5% SDS. Blots were washed to 0.1% SSC/0.1%SDS and exposed to Kodak Biomax film. Blots were reprobed with β-actin to confirm equal loading and transfer of RNA sample.

In situ hybridization

Chicken embryos were dissected to remove the genital ridges and mesonephros or left intact. Antisense and sense *ASW* probes were prepared and hybridized for whole mount as described by Andrews et al. (1997) and for tissue sections according to Western et al. (1999).

Zebra finch cDNA library

Zebra finch ovary cDNA library was kindly provided by Dr. A. Arnold (Department of Physiological Sciences, UCLA).

Results

Identification of a female specific transcript in genital ridge

DDPCR and RDA were used to compare male and female transcripts from chicken embryonic genital ridges. In an attempt to find genes expressed differentially in developing gonads, transcripts were compared at three different stages of development; E4.5, E5.5 and E6.5 (Hamburger and Hamilton stages 24–29). These stages comprise a window around the time sexual dimorphism in the gonad is first apparent (Thorne 1996). In order to constitute comprehensive coverage of transcripts expressed in genital ridge by DDPCR, the complement of 12 dinucleotide anchored primers were used in conjunction with 40 different random 10mers. One primer set used in DDPCR generated a band of 371 bp seen only in female samples in all three stages (data not shown). DNA was eluted from this band, re-amplified using the same primer set, cloned and sequenced (Fig. 1A). The transcript represented by this clone was named the *ASW* (Avian Sex-specific W-linked) gene.

Both 3′ and 5′ RACE PCR were performed to generate a full-length cDNA from the DDPCR fragment. The failure of any extension in the 3′ RACE suggested that the DDPCR fragment represented the extreme 3′ end of the transcript. The presence of a consensus cytoplasmic poly-adenylation signal 50 bp from the T11-MN binding site supported the conclusion that the display clone is the 3′ end of a genital ridge mRNA. The 5′ RACE extended the *ASW* DDPCR fragment an additional 236 bp upstream of the DDPCR 10mer binding site (Fig. 1A).

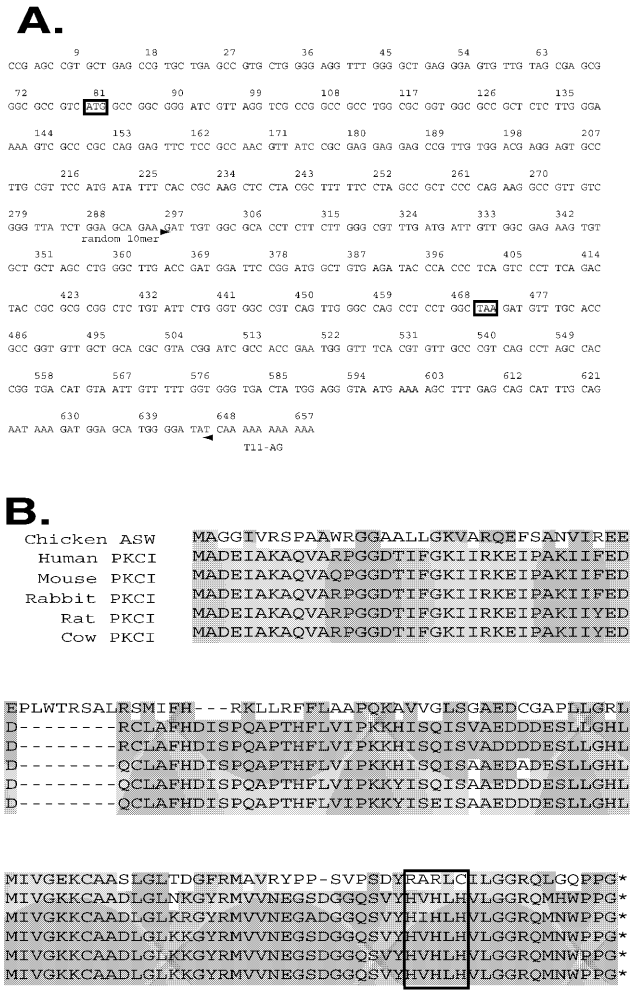

Fig. 1 **A** Chicken ASW cDNA sequence (GenBank accession no. AF148455) and deduced amino acid sequences; *arrows* DDPCR primer binding sites; *boxes* start and stop codons; intron/exon boundaries occur at 203 bp and 309 bp. **B** Alignment of *ASW* deduced amino add sequence with *PKCI* genes from human (GenBank accession no. U51004), mouse (GenBank accession no. U6001), rabbit (EMBL accession no. Y11175), rat (GenBank accession no. U09407) and cow (GenBank accession no. U09405)

Analysis of cDNA fragments generated by RDA also revealed several female-specific fragments (data not shown). Once these cDNAs were cloned and sequenced, they were found to be fragments of the *ASW* transcript identified by DDPCR and RACE. Two independent differential screening methods therefore identified *ASW* as a transcript expressed in female genital ridge and absent in male genital ridge. Several RACE and RDA clones showed slight sequence degeneracy, indicating that *ASW* may be an amplified gene (see below).

The full-length *ASW* cDNA contained an uninterrupted open reading frame (ORF) encoding a putative polypeptide of 130 amino acids. An *ASW* genomic clone was isolated, sequenced and compared to the *ASW* cDNA. This revealed that ASW has two introns at 203 bp and 309 bp (Fig. 1A). Comparison of the *ASW* sequence to GenBank revealed highest similarity (41% at the protein

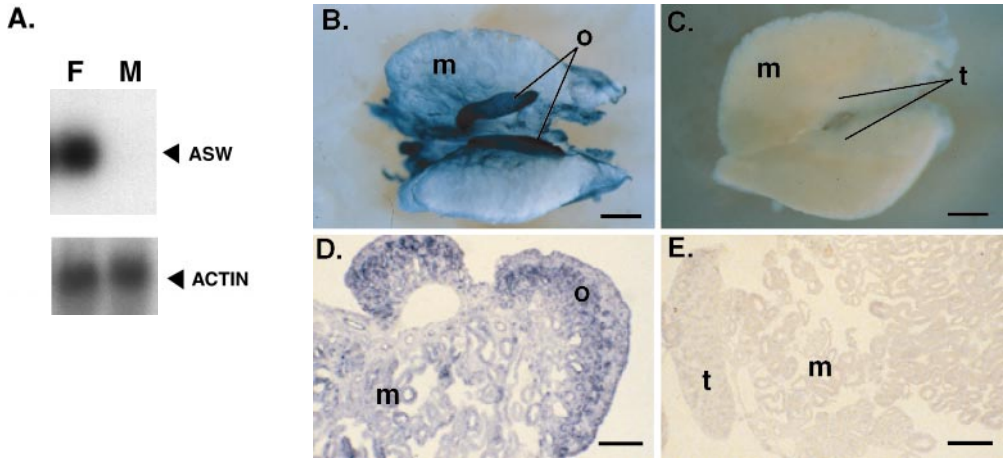

Fig. 2A–E Expression analysis of *ASW*. **A** Northern blot of total RNA from 5.5-day chick embryos probed with *ASW* cDNA, stripped and re-probed with β-actin. *F* Female; *M* male. **B** Whole-mount *in situ* hybridization of *ASW* antisense RNA probe on dissected urogenital system of 6.5-day female chick embryo. *m* Mesonephros; *o* ovary. **C** Same as **B**, 6.5-day male sample. *m* Mesonephros; *t* testis. **D** In situ hybridization of ASW antisense RNA probe on sectioned 6.5-day female urogenital system. *m* Mesonephros; *o* ovarian medulla. **E** Same as **D**, 6.5-day male sample

level) to a putative protein kinase C inhibitor (*PKCI*) identified in cattle. *PKCI* is a member of the highly conserved, ubiquitous HIT (histidine triad) family of proteins, designated as such because of an invariant His-X-His-X-His motif near the carboxy-terminus of all the proteins in this family (Seraphin 1992). Figure 1B shows sequence alignment of *ASW* to several *PKCI*. It should be noted that the histidine triad motif is missing in the *ASW* sequence.

Spatial and temporal expression profile of *ASW*

Female-specific expression of *ASW* in chick genital ridge was initially confirmed on a northern blot of total RNA from E5.5 male and female embryos (Fig. 2A). Additionally, northern analysis indicated the size of the *ASW* transcript to be approximately 650 bp. To determine the exact location of *ASW* expression RNA in situ hybridization was performed on both whole-mounted and sectioned urogenital tissues from males and females also taken at E5.5. Whole-mount in situ hybridization shows high levels of ASW transcripts in female genital ridge relative to the underlying mesonephros (Fig. 2B), while *ASW* expression is totally absent in the male urogenital system (Fig. 2C). Figure 2D shows expression to be more concentrated in the medulla of the developing ovary relative to the overlying cortex, but again completely absent in developing testis (Fig. 2E). More extensive northern and in situ analyses show that *ASW* is expressed in the presumptive ovary as early as E4.5, and that expression persists throughout embryonic development and in adult ovary (data not shown).

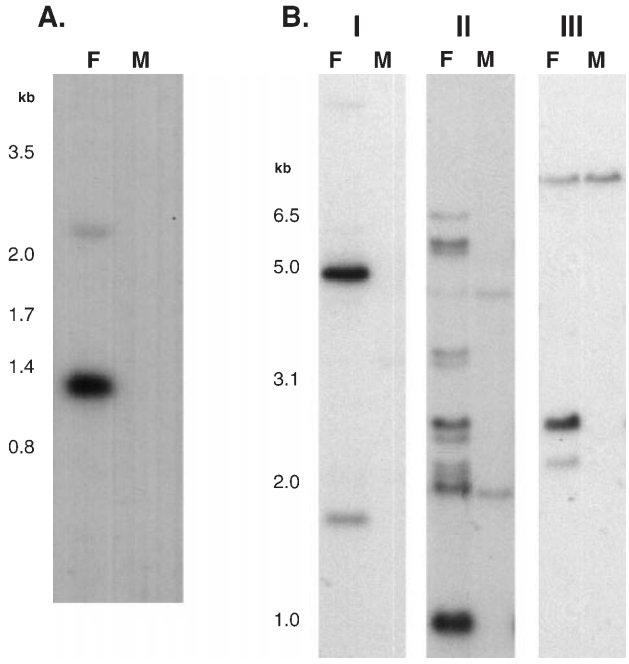

Fig. 3A, B Southern blots of avian genomic DNA hybridized with *ASW* cDNA. **A** *Hind*III digest of chicken (*Gallus gallus*). **B** *Taq*I digest of: *I* New Zealand pigeon (*Hemiphaga novaseelandiae*); *II* Moorhen (*Gallinula tenebrosa*); *III* Kakapo (*Strigops habroptilus*). Molecular weight marker sizes are given in kilobases (kb)

Conserved W-linkage of *ASW*

Since *ASW* expression was detected in females but not at all in males, the possibility existed that the gene is linked to the female-limited W chromosome. To test this, a Southern blot of whole genomic DNA from male and female fowl was probed with the full-length *ASW* cDNA. The presence of an intense band on the Southern blot only in lanes with female DNA indicated W-linkage of the *ASW* gene (Fig. 3A). The intensity of the *ASW* signal in female lanes (exposure time 4 h) again suggested that *ASW* is amplified on the W chromosome.

Since a candidate sex-determining gene might be expected to be W-linked in all birds, we tested whether

Table 1 Bird species which show W-linkage for *ASW*

Common name	Scientific name	Order	*ASW* female specific
Chicken	*Gallus domesticus*	Galliformes (pheasants, guineafowl)	Yes
Fiordland crested penguin	*Eudyptes pachyrhynchus*	Sphenisciformes (penguins)	Yes
Adelie penguin	*Pygoscelis adeliae*	Sphenisciformes (penguins)	Yes
Kakapo	*Strigops habroptilus*	Psittaciformes (cockatoos, parrots)	Yes
Crimson rosella	*Platycercus elegans*	Psittaciformes (cockatoos, parrots)	Yes
Fairy wren	*Malurus* sp.	Passeriformes (perching, song birds)	Yes
Scrubwren	*Sericomus* sp.	Passeriformes; (perching, song birds)	Yes
Zebra finch	*Poephila guttata*	Passeriformes (perching, song birds)	Yes
New Zealand pigeon	*Hemiphaga novaseelandiae*	Columbiformes (pigeons, doves)	Yes
Moorhen	*Gallinula tenebrosa*	Gruiformes (rails, cranes, allies)	Yes
Tasmanian hen	*Gallinula mortierii*	Gruiformes (rails, cranes, allies)	Yes
Swamp hen, pukeko	*Porphyrio porphyrio*	Gruiformes (rails, cranes, allies)	Yes
Takahe	*Notornis mantelli*	Gruiformes (rails, cranes, allies)	Yes
Black stilt	*Himantopus novazealandiae*	Charadrifformes (waders, gulls)	Yes
Brown skua	*Catharacta lonnbergi*	Charadriiformes (waders, gulls)	Yes
Grey-faced petrel	*Pterodroma macroptera*	Procellarfiformes (tube-nosed seabirds)	Yes
Kookaburra	*Dacelo novaguineae*	Coracifformes (kingfishers)	Yes
Emu	*Dromaius novaehollandiae*	Struthioniformes (ratites)	No
Ostrich	*Struthio camelus*	Struthioniformes (ratites)	No

linkage of *ASW* to the W chromosome is conserved among birds or is unique to the domestic fowl. Southern blots of genomic DNA from a broad sampling of birds were probed with the *ASW* cDNA, and in all cases, with the exception of ratites (emus and ostriches), *ASW* hybridized to a band(s) present only in females. Figure 3B shows examples of *ASW* cDNA hybridized to Southern blots of male and female DNA of various bird species, and a summary of birds tested for *ASW* W-linkage is shown in Table 1. To date, *ASW* has shown W-linkage in all neognathae tested. Longer exposure times showed *ASW* hybridizing bands shared by females and males, indicating a similar gene on the Z chromosome or autosomes and suggesting cross-hybridization of *ASW* to other HIT family members (see "Discussion").

ASW sequences from zebra finch

The conserved W-linkage of ASW made it a potentially useful tool for sex-genotyping birds of various species. To determine the sequence conservation of *ASW* in birds a cDNA library from zebra finch ovary was screened with the chicken *ASW* cDNA. The alignment of a zebra finch cDNA sequence with chicken *ASW* is shown in Fig. 4. It should be noted that none of six independent *ASW* cDNA clones isolated from the zebra finch library has an ORF corresponding to the one in domestic fowl.

Discussion

ASW is one of only two genes reported which have conserved linkage to the avian W chromosome (Ellegren 1996; Griffiths and Tiwari 1995). However, *ASW* is the only one shown to have female-specific expression in the developing gonad. The chicken homologue of the mouse chromo-helicase DNA binding protein (CHD) is the only other gene reported, which codes for a polypeptide and has a W-linked copy in all non-ratite birds (Ellegren 1996). The expression profile of CHD in chickens has not been reported, but it does have a Z-linked homologue. Sequence conservation between the Z- and W-linked copies of CHD suggest that protein function has been maintained in both copies (Griffiths and Korn 1997).

By contrast, *ASW* is exclusively W-linked and consequently is expressed only in females. Weak hybridization of *ASW* to male chicken DNA indicates the existence of a similar gene(s) which may be Z linked and/or autosomal. These related sequences in chicken have not yet been characterized; however, we have made attempts to identify *ASW* homologues in human and alligator (our unpublished results). Initially *ASW* was used as a probe on zoo and Noah's ark blots, and hybridizing bands were seen in both reptiles and mammals. Screening of genomic libraries from alligator and human with *ASW* resulted in the isolation of the *PKCI* gene from which *ASW* may be derived. It is possible that the weakly hybridizing bands in male birds correspond to other members of the HIT gene family in birds.

It is clear from the intense hybridization signal of *ASW* to female bird DNA and from the variation in sequence among *ASW* cDNAs that the gene has undergone amplification and mutation on the W chromosome. Amplification and degeneration of genes on the mammalian Y chromosome has been widely reported and is exemplary of the accumulation of mutation by Muller's ratchet on the nonrecombining segments of sex chromosomes (e.g. mammalian Y and the avian W). Most of the chicken *ASW* cDNAs under study have an ORF corresponding to the ORF in HIT family homologues while the zebra finch cDNAs do not. It is possible that there are copies with the proper ORF in zebra finch which were not detected in our screen.

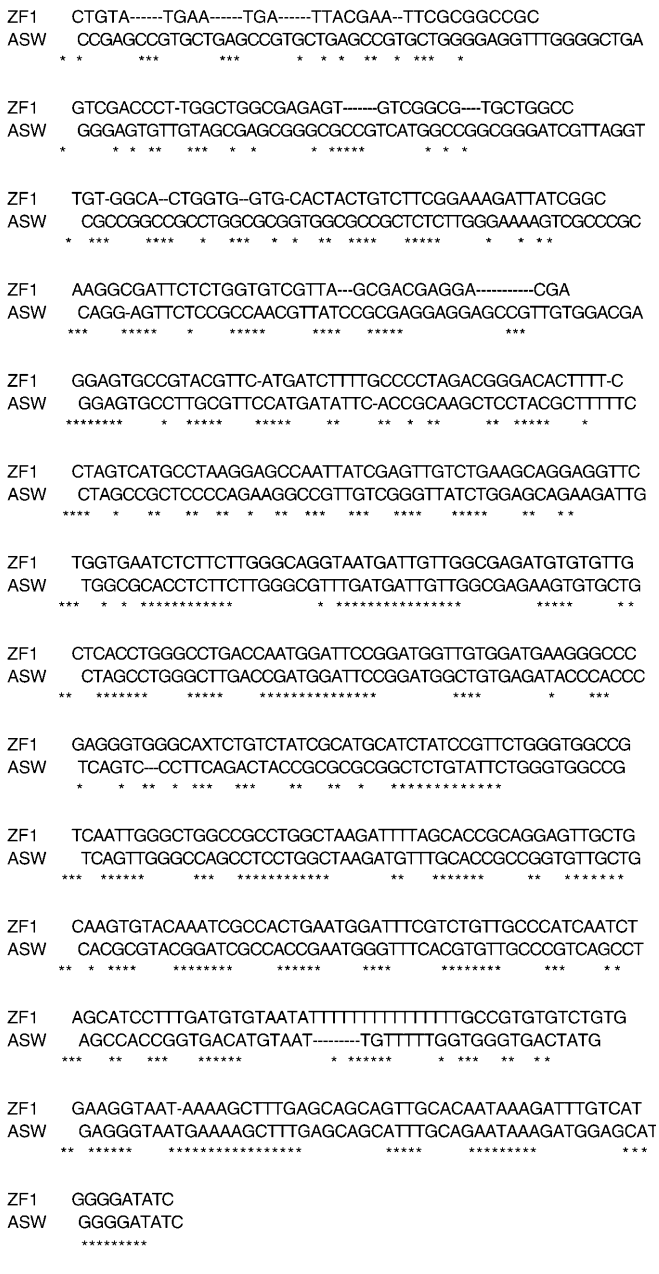

Fig. 4 Alignment (Clustal V) of ASW cDNA sequence from chicken and zebra finch (accession no. AF148456)

Even with the ORF, the function of ASW is unknown. While it has significant similarity to the ubiquitous HIT gene family, it lacks the canonical histidine triad motif which characterizes this family and has been shown to be essential for the putative hydrolase activity of HIT proteins in vitro. The in vivo function of the HIT proteins has not been determined, but they have nucleotide polylphosphate hydrolase activity in vitro (Robinson et al. 1993).

How may a gene such as *ASW*, encoding a possibly degenerate copy of a member of a family of ubiquitous cell growth regulators, function as a determinant of cell fate? We envision two possible functions of *ASW* in avian sex determination which fit either a dominant W mode or Z dose mode of avian sex determination: (a) Lack of the histidine triad in ASW could produce a dominant negative gain-of-function effect which antagonizes normal HIT protein function. It is also possible that ASW could have a novel function unrelated to normal HIT proteins. In either case, in this scenario W-borne copies of *ASW* would act as a dominant sex determining gene. (b) ASW may simply be the degenerate, non-functional W-linked form of a functional counterpart on the Z chromosome. In this scenario males would have a double dose of the functional gene while females would have only a single dose. The lack of an ORF in the zebra finch *ASW* cDNAs fits this model. Of interest then is the localization and expression of HIT genes in male birds and the status of *ASW*/HIT homologues in the ratites, which have morphologically indistinguishable sex chromosomes.

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