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Genomic Organization and Promoter Characterization of RANTES, MIP-1ß like and MCP-Like CC Chemokines of Domestic Duck (*Anas platyrhynchos*)

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Abstract: The duck immunology is gaining more interest in recent years since ducks have been identified as "Trojan horse" of highly pathogenic avian influenza. In the present study, we characterize the gene and promoter regions of three CC chemokines from domestic duck. Analysis of the gene revealed significant similarity with chicken and mammalian chemokine genes with three coding exon and two intron pattern. Though the exons are highly conserved with respect to corresponding chicken chemokine genes, introns revealed low identity. Promoter regions of duck RANTES and MCP-like chemokines were amplified by Genome walking method. Analysis of the 500 bp upstream nucleotide sequences of ATG (translational start site) of RANTES and MCP-like chemokine revealed a number of putative binding sites for transcription factors. Comparative analysis of RANTES promoter with human and chicken sequences revealed conserved TATA box, NFkB binding site and transcription start site. The predicted transcription start site and TATA box are also conserved in duck and chicken MCP-like chemokine sequences. The present study shows that the over all organization of gene and putative transcription factor binding sites in the promoter regions of these three chemokines were conserved with respect to chicken and human sequences indicating these molecules of innate immune system is conserved in the process of evolution.

Key words: CC chemokine, RANTES, MIP-1ß, MCP, duck

INTRODUCTION

Chemokines are chemotactic proteins of small (6-14 kDa) secreted molecules. They have overlapping functions and are produced by a variety of cell types (Baggiolini, 1998). Cellular response at the site of inflammation is controlled by gradients of chemotactic factors that direct leukocyte migration and movement. Based on the position of N terminal cysteine chemokines are classified into C, CC, CXC and CX3C chemokines where X represent any aminoacid other than cysteine. Interest in chemokines and their receptors has increased in recent years as they play key roles in many disease process including inflammation, autoimmune disease, infectious disease and in cancer (Zlotnik *et al*., 2006; Miller *et al*., 1988).

Avian immune response is acquiring more significance with the current threat of highly pathogenic avian influenza outbreak in Asian countries. Wild aquatic birds such as ducks, geese and swans are regarded as the principal reservoir hosts of the avian influenza viruses (Webster *et al*., 1992; Olsen *et al*., 2006). Ducks can show few or no signs of the disease even when carrying viruses that are highly pathogenic in chickens (Hulse-Post *et al*., 2005; Kishida *et al*., 2005). Ducks are also one of the extensively studied animal models for hepadna viral replication (Schultz *et al*., 2004). A comprehensive understanding of the immunological mediators in domestic duck is essential to get insight into the pathogenesis of these infections.

The role of chemokines in the avian immune system is poorly studied. When the present study was started, only six CC chemokines were reported in chicken and the genes of a few of them were mapped. However, today, based on comparative genomics, 23 chemokine ligands and 14 cognate receptors have been identified in chicken (Wang *et al*., 2005). The data on chemokines identified in ducks are still a fewer when compared to chicken. Most of the chemokine identified till now are based on subtractive hybridization analysis (Xia *et al*., 2007; Sreekumar *et al*., 2005). Complementary DNA (cDNA) sequences of three CC-chemokine homologues (Regulated-upon activation, Normal T-cell expressed and secreted (RANTES); Macrophage Inflammatory protein-1ß (MIP-1ß); Monocyte Chemoattractant protein (MCP) were identified from domestic duck in our laboratory. cDNA characterization and tissue expression profiles of these chemokines in 4 month old peking ducks were reported previously (Sreekumar *et al*., 2005). The orthologue relationship of chicken MIP-1ß and MCP with other species, including human and mouse, remain unclear. Therefore their nomenclature remains controversial. So in the present study, duck chemokines showing similarity to chicken MIP-1ß and MCP were named as duck "MIP-1ß like" and "MCP like" chemokines.

Characterization of chemokine promoters has identified their role in regulation of the protein expression and involvement in disease pathogenesis. Polymorphism of

RANTES promoter was reported to be associated with HIV-1 disease progression (Liu *et al*., 1999) and outcome of HIV associated disease (Goulding *et al*., 2005). MCP-1 promoter polymorphism was significantly associated with clearance of HBV infection (Park *et al*., 2006). Therefore analysis of promoter regions of these chemokines may help to provide details on their role in different viral infections. In this back ground, the present study was undertaken to characterize the full gene and promoter sequences of duck RANTES, MIP-1ß like and MCP-like CC chemokines. We characterize for the first time the gene sequences, exon-intron organization and promoter region of CC chemokines from domestic duck.

MATERIALS AND METHODS

Blood and tissues: Duck blood samples were collected from commercial Peking ducks (Anas platyrhynchos) and were used for mRNA expression analysis and for isolation of genomic DNA.

Genomic DNA isolation: Genomic DNA was isolated from 2 mL of venous blood as per standard protocols (Miller *et al*., 1988). DNA was dissolved in Tris-EDTA buffer, quantitated by spectrophotometry and stored at-20°C until use.

Long PCR amplification of duck CC chemokine genes: The long PCR was carried out using the Elongase Amplification System (GIBCO-BRL Life Technologies, MD, USA). Fifty microlitre reaction contained 1 unit of Elongase enzyme mix, 20 pmol each of forward and reverse primers (AB163F-R or AB 187F-R or AB330 F-R primers; Table 1), 100 ng each of duck genomic DNA, 1X PCR buffer containing 120 mM Tris-SO_4, 36 mM $(NH_4)_2SO_4$, 11.5 mM $MgCl_2$ and 0.2 mM dNTPs. PCR was done for 35 cycles with denaturation at 94°C for 30 sec; primer annealing at 50°C for 1 min and primer extension at 68°C for 4 min. The extension time was increased by 15 s per cycle. An initial denaturation at 94°C for 2 min and a final extension of 10 min were also included.

Amplification of CC chemokine promoter sequences: Twenty 5 µg genomic DNA was used for the construction of Genome Walker libraries as per the Universal Genome Walker library kit (Clonetech, Mounatain View, CA). In order to amplify the 5' flanking sequences of translational start site, PCR was done using the adapter-ligated PvuII and *EcoR*V Genome Walker libraries as the template and adapter specific forward primers (AP1 and AP2) and chemokine gene specific reverse primers (Table 2). Construction of the libraries and PCR reactions were carried out as per manufacturer's protocol.

Cloning and sequencing: All the PCR amplified sequences were cloned into pGEM-T Easy plasmid vector (Promega) and in each case positive clones were sequenced using an ABI 3730 genetic Analyzer automated DNA sequencer (PE Applied Biosystems, Foster City, CA). Vector specific and insert specific primers were used for sequencing and internal primers were designed for upstream sequencing whenever necessary.

Sequence analysis: Nucleotide sequences were aligned with 1000 bootstrap replications using CLUSTAL W (Thompson *et al*., 1994) of BIOEDIT software. Comparison of nucleotide identities were done using the same software using BLOSUM 62 matrix. Exon-intron organization was analysed using the program SPIDEY (http://www.ncbi.nlm.nih.gov/apidey) at NCBI server.

RESULTS

Structure of duck RANTES, MIP-1ß like and MCP-like CC chemokine genes: We amplified duck RANTES (1876 bp), duck MIP-1ß like (1165) and MCP-like chemokine (1295 bp) (Fig. 1 and Table 3) genes respectively from duck genomic DNA using long PCR.The products were cloned into PGEM-T easy vector and sequenced. Sequence analysis and comparison

Table 1: Primers used for amplifying cDNAs and genes

RANTES	AB163F ACCTGATCTGATCCTGCTTCTGCC		
	AB163R AACATGATTCCACAACCAGCATCCC	815	AY641435
MIP-1ß like	AB330F TACTTGCCTGCTTCAGCTCCG		
	AB330R TTTCTTGTTATTTCACCTGCTCCC	504	AY641437
MCP-like	AB187F ACAGGCCCTTTGCACATTCTAGCC		
	AB187R TCACAGAGTACCAAGAGGACAGGG	433	AY641436

Table 2: Primers used for amplifying promoter region

RANTES	AB163PMRI GAGGCCAGCCAAGCAGGAGGATGG	
	AB163PMR2 AAGCTGGAGGAGGATGGAGAGGGCTGC	Present study
MIP-1ß like	AB330PMRI GCGATGAGAACAGCCAGGGCAACC	
	AB330PMR2 ACAGCCAGGGCAACCACAGAGACC	Present study
MCP-like	AB187PMR3 GCGCCAGCAAGGGCAGCTGTGGAGG	
	AB187PMR4 CAAGGGCAGCTGTGGAGGACTTCATGC	Present study
Adapter specific primers	AP1 GTAATACGACTCACTATAGGGC	
	AP2 ACTATAGGGCACGCGTGGT	Universal Genome Walker Kit (Clonetech)

Table 3: Length of different CC chemokine gene coding exons and introns in duck/chicken

	Introns		Exons		
CC chemokine	I_1	I_2	E_1	E_2	E_3
RANTES	460/486	629/403	73/76	112/112	88/88
MIP-1ß like	340/253	326/296	73/70	115/115	86/88
MCP-like	313/282	549/393	70/72	116/118	90/100

Table 4: Percentage identity of different exons and introns of duck CC chemokines with their chicken orthologue

	Introns		Exons		
CC chemokine	I_1	I_2	E_1	E_2	E_3
RANTES	62	38	65	88	84
MIP-1ß like	35	62	85	77	68
MCP-like	35	17	47	44	42

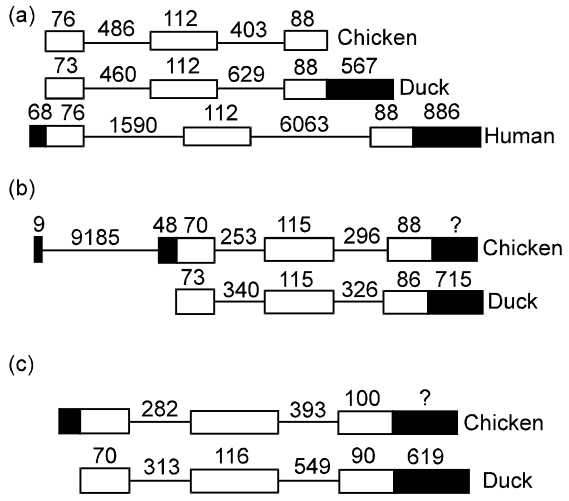

Fig. 1: (a) Exon-intron organization of avian and human RANTES gene. Numbers indicate the length of nucleotide (bp) forming different exons and introns. Figures are not drawn to scale. GenBank accession nos: NC_000017.9 (human), NC_006106.2 (Chicken) and Duck. (b) Exon-intron organization of avian MIP-1ß like chemokine gene. Numbers indicate the length of nucleotide (bp) forming different exons and introns. Figures are not drawn to scale. GenBank accession nos: NC_006106.2 (Chicken) and (Duck). (c) Exon-intron organization of avian MCP like chemokine gene. Numbers indicate the length of nucleotide (bp) forming different exons and introns. Figures are not drawn to scale. GenBank accession nos: NC_006106.2 (Chicken) and (Duck)

with the corresponding cDNA sequence revealed the presence of three coding exon and two intron in all the three chemokines under study. Though there is variation in the length of introns, duck chemokine genes exhibit typical three exon-two intron pattern. However, avian RANTES gene is shorter than human RANTES gene due to shorter introns. Since the human orthologues of avian MIP-1ß and MCP are contradictory, they are not compared with available human sequences. In case of duck MIP-1ß like chemokine, we have designed primers only from the coding region and we got the genomic region of three coding exons and introns. Sequence analysis of chicken MIP-1ß like gene revealed the presence of 4 exons and three introns with 9 nucleotide coding for 5' UTR is placed 9185 bp upstream. Therefore the data for duck MIP-1ß given in this study is partial. Nucleotide identity of different regions were compared with that of chicken chemokine sequence (Table 4). However, the intron regions revealed moderate level of identity with that of chicken intron sequences. The number of nucleotides forming the exons were comparable between duck RANTES and chicken RANTES. Exon 2 and 3 revealed maximum identity (88% and 84%) but exon 3 has only 38% identity between the sequences. In case of MIP-1ß like protein exon 2 and 3 showed maximum identity (85% and 77%) with corresponding chicken sequences however, exon 1 exhibited low identity (65%). Duck MCP-like protein gene exhibited very low (42-47%) identity with corresponding chicken homologue. The splice sites are not exactly between two codons in any of the chemokines studied and it is the same in case of other chemokines compared in the present study.

Structure of duck RANTES, MIP-1ß like and MCP-like CC chemokine promoter regions: Promoter regions of RANTES (995 bp) and MCP-like protein (500 bp) were amplified from Pvu II genome walker library. In case of MIP-1ß, 500 bp amplification was obtained in EcoR V library, however on comparison with chicken gene sequence, revealed that this region lies in the intronic region. Analysis of the 500bp upstream nucleotide sequences of ATG (translational start site) of RANTES and MCP-like chemokine revealed a number of putative binding sites for transcription factors. Comparative analysis of this region in duck, chicken and human RANTES revealed conserved TATA box, NFkB binding site and transcription start site with respect to human

sequences are conserved in all the sequences. The predicted transcription start site and TATA box are also conserved in duck and chicken MCP-like chemokine sequences. The 5'UTR region of chicken and duck RANTES are small. The promoter regions of RANTES and MCP-like chemokines of domestic duck showed 64% identity to the corresponding chicken sequences.

DISCUSSION

In the present study we characterized the gene organization of three CC chemokines of domestic duck. The gene of these three chemokines were cloned and sequenced. Sequence homology was analyzed with available chicken gene sequences. The exon regions of RANTES and MIP-1ß like chemokines revealed considerable identity with (<65%) corresponding chicken sequences, whereas the intronic regions showed a lower identity. One reason for the low identity observed in the intronic regions may be due to the difference in sequence length. In the previous report (Sreekumar *et al*., 2005) we showed the high level amino acid sequence (90 and 85% respectively) similarity of these chemokines with their chicken homologues. Like chicken chemokine, duck CC chemokines also exhibit typical three coding exon pattern (Wang *et al*., 2005). The genes are shorter than corresponding human genes due to shorter introns. The low homology of chemokine genes among different species indicates a pathogen driven selection (Hughes, 1997; Murphy, 1993).

Analysis of duck RANTES promoter region identified a number of critical sequences which are involved in the regulation of expression of RANTES in immune system related cells. Human RANTES is the best studied CC chemokine in terms of transcriptional regulation of chemokines. The RANTES promoter contains four NFκB binding sites at positions -30, -44, -213 and -579 relative to the transcription start site (Nelson *et al*., 1996). Of which NFκB at position -44 is well conserved in both human and avian sequences. Respiratory syncitial virus induced RANTES activation needs *cis* regulatory elements located in the promoter fragments spanning from -220 to +55 nucleotides corresponding to NFκB, C/EBP, Jun/CREB/ATF and Interferon Regulatory Factors (IRF) (Casola *et al*., 2001). These regions are also conserved between avian and human sequences. However, a deletion of 16 nucleotides was found in the ISRE region of duck RANTES promoter. It is also reported that mutations affecting ISRE completely abolish RANTES inducibility by RSV (Casola *et al*., 2001). Virus mediated RANTES promoter activation involves co-operative synergism between IFN regulatory factors and NFêB factors (Genin *et al*., 2000). In preliminary studies, the up regulation of RANTES in Duck Enteritis Virus infected duck splenocytes was observed (Data not shown). The relevance of the deletion in the ISRE region of RANTES promoter, in this context, needs further investigations.

Factors like ATF2/Jun stimulates expression of RANTES in influenza virus infected cells (Kujime *et al*., 2000). These factors also have been reported to be involved in the expression of RANTES in response to bacterial lipoploysaccharide (Boehlk *et al*., 2000). The binding regions for these factors are well conserved in duck and chicken RANTES promoter. It is already reported that Th-1 promoting chemokines support viral clearance and in some cases immunopathology. Expression of most chemokines to infection is regulated primarily at the level of transcription and their gene promoter region contains recognition sites for many virus activated transcription factors (Melchjorsen *et al*., 2003). Therefore better understanding of the promoter regions of avian chemokines may help to differentiate the response of these two species against avian influenza virus.

Since the avian MCP-like chemokine do not have obvious orthologue in human chemokines (Hughes *et al*., 2007), their promoter characterization is difficult based on predicted transcription factor binding site. Though, the nucleotide identity of these proteins is only 41% at gene level, the promoter sequence revealed an identity of 64% between chicken and duck sequences indicating that promoter regions are more conserved.

The present study delivers information on the full gene and promoter sequences of duck RANTES, MIP-1ß like and MCP-like chemokines of duck. This is the first report on the promoter characterization of any of the avian chemokines. Many cytokines like chicken IFN and IL-2 can be exploited as vaccine adjuvants and therapeutic agents (Hilton *et al*., 2002; Asif *et al*., 2004). Mammalian chemokines are reported to be used as effective adjuvants in vaccine preparations (Toka *et al*., 2003; 2004). Further characterization of these duck chemokines with respect to their immunomodulatory potential will be highly helpful in developing them as vaccine adjuvants.

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