Molecular Characterization of the Smyth Chicken Sublines and Their Parental Controls by RFLP and DNA Fingerprint Analysis

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ABSTRACT The Smyth line (SL) chicken, a model for autoimmune human vitiligo, is characterized by a spontaneous posthatch epidermal pigment loss (vitiligo). Even though the immunological and morphological changes accompanying the vitiligo process have been well studied, the genetics of this phenomenon remains elusive. The SL lines have been maintained by nonpedigreed matings since their inception, and therefore, the inbreeding status is unknown. The present study was designed to provide an estimate of the inbreeding coefficients and the molecular genetic profiles of the SL sublines, each homozygous for a different MHC haplotype and their MHC-matched parental control (BL) sublines. The DNA fingerprint analysis revealed that there is a moderate level of inbreeding within the SL and BL parental sublines. Of the two SL sublines studied, SL101 had the highest level of inbreeding (0.948). Similarly, its parental control line (BL101) was more inbred than the parental subline of SL102 (BL102). The very high level of similarity between the SL sublines and their respective parental control lines is shown further by the similarity index (SI) estimates (SI between SL101 and BL101 was 0.949 and that between SL102 and BL102 was 0.932). Restriction fragment length polymorphism (RFLP) analysis of the endogenous viral genes (avian leukosis virus subgroup E, ALVE) showed that five ALVE-related *BamH*1 fragments were present in the SL101 and four in SL102 sublines, whereas the parental BL101 and BL102 sublines had five and six fragments, respectively. SL101 and SL102 shared two fragments, but the frequencies were different. Similarly, BL101 and BL102 shared two fragments. SL101 and BL101 shared three fragments, and SL102 and BL102 also shared three fragments.

(*Key words*: Smyth chicken, vitiligo, restriction fragment length polymorphism, DNA fingerprint, inbreeding coefficient)

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INTRODUCTION

The Smyth line (SL) chicken, an animal model for autoimmune human vitiligo, is characterized by a spontaneous posthatch epidermal pigment loss (vitiligo) determined to be the result of an autoimmune phenomenon (for review see Smyth et al., 1981; Smyth, 1989). An intrinsic heritable melanocyte defect, characterized by melanosomes with abnormal, irregular surfaces, predisposes SL chickens to the pigmentory disorder (Boissy et al., 1983). Both B cell (Lamont et al., 1982) and T cell (Erf et al., 1995) compartments of the immune system appear to be involved in the pathology of the disease. Melanocyte-specific SL autoantibodies that cross-react with mouse and human melanocytes have been reported (Searle et al., 1993). It was later found that mammalian tyrosinase-related protein-1 (TRP-1) is recognized by the SL autoantibody (Austin and Boissy, 1995), although its contribution to the onset and progression of the disease has not been completely determined.

The mating scheme used to develop the line from the original mutant female has been detailed (Smyth et al., 1981). Later, serotyping procedures indicated that the SL and its parental control, Brown Line (BL), were segregating for three different MHC haplotypes (Auclair et al., 1984). Three sublines, each homozygous for one of the three MHC haplotypes (SL101, SL102, and SL103), were established along with their respective MHC-matched parental control sublines (BL101, BL102, and BL103). SL101 and SL102 sublines were chosen for this study because they have similar incidences of vitiligo (70 to 90%), but SL101 chickens have a significantly earlier onset time and more extensive feather pigment loss than SL102 chickens.

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Abbreviation Key: ALVE = avian leukosis virus subgroup E; BL = parental Brown-line control subline; EAV = endogenous avian retroviral element; RFLP = restriction fragment length polymorphism; SI = similarity index; SL = Smyth line.

The two BL parental sublines have a low incidence (1 to 2%) of vitiligo and are considered parental control lines. Even though the immunological and morphological changes accompanying the vitiligo process have been well studied, the genetics of this phenomenon remains elusive. The only studies on the molecular characterization of SL chicken were made by Lakshmanan et al. (1992) and Lakshmanan (1994). They reported polymorphisms at the ALVE and MHC loci in SL102 and BL102 sublines. The present study was designed to provide an estimate of the inbreeding coefficients and the molecular genetic profiles of the SL and parental control lines of chickens. These studies complement current efforts in mapping vitiligo genes in this animal model.

MATERIALS AND METHODS

Forty animals each from the SL101, SL102, BL101, and BL102 sublines were selected for this study. All the animals were presumably homozygous for their respective MHC haplotype. These birds were obtained from pen matings of the respective sublines whose haplotypes have been previously determined based on alloantisera reactivity.

DNA Fingerprint and Restriction Fragment Length Polymorphism (RFLP) Analyses

DNA Extraction. Whole blood samples were collected by brachial venipuncture with EDTA as the anticoagulant. Blood samples were diluted in SET buffer (0.16 *M* NaCl, 0.05 *M* Tris, and 1 m*M* EDTA, pH 8.0) and were stored at –70 C. For DNA isolation, 15 μL of proteinase-K (10 mg/mL) and 10 μL of 20% SDS (wt/vol) were added to 500 μL of the diluted blood and were incubated at 56 C overnight. DNA was purified by two extractions of salt-saturated phenol followed by two extractions with phenol:chloroform:isoamyl alcohol (25:24:1) and one extraction with chloroform:isoamyl alcohol (24:1). After ethanol precipitation, DNA was stored in 1X TE (10 m*M* Tris and 1 m*M* EDTA, pH 7.0). DNA concentration and purity were determined by spectrophotometer reading at 260 and 280 nm, followed by calculation of the 260/280 ratio.

Restriction Digestion. The restriction enzymes *Hin*dIII, *Pvu*II, and *Msp*I[4] were used for the DNA fingerprint analysis, and *BamH*I was used for the RFLP analysis. Ten micrograms of DNA from individual birds was digested with 40 units of enzyme in the appropriate buffer and was incubated overnight. Small aliquots of each reaction mixture were examined in a mini gel to monitor the extent of digestion.

Electrophoresis and Transfer of DNA Fragments. After complete digestion with the restriction enzyme, genomic DNA fragments were separated on 0.8% agarose gel in 1X Tris acetate buffer (40 m*M* Tris, 20 m*M* Na acetate, and 1 m*M* EDTA, pH 7.2). λ Phage DNA, digested with *Hind*III, was used as the molecular size marker. Electrophoresis was carried out at 22 V for 48 h at room temperature. DNA was transferred to a nylon membrane (Magnagraph)[5] by capillary action in 10X SSC (1.5 *M* NaCl and 0.15 *M* sodium citrate, pH 7.0) for 16 to 18 h at room temperature according to standard protocols (Sambrook et al., 1989). Completeness of the DNA transfer from the gel was verified by staining the gel with ethidium bromide after the transfer. The DNA on the membrane was immobilized by UV cross-linking at 1,200 × 1,000 Joules/cm^2 in a CL-100, UV crosslinker.[6]

Probe Preparation and Labeling. The pRAV-2 probe, specific to the endogenous viral genes, was a gift from Eugene Smith (ADOL, East Lansing, MI). The endogenous avian retroviral element (EAV) fingerprinting probe was prepared by PCR amplification of chicken DNA with the oligomers EAV-1 and EAV-2 as per Benkel and Gavora (1993). The PCR-amplified fragments were gel purified before labeling. The DNA was labeled with α^{32}P dCTP by random primer method using the Prime-a-Gene labeling kit.[4] The unincorporated nucleotides were removed by passage through a Sephadex G-50 column. Labeled probes were denatured at 100 C for 5 min before adding to the hybridization solution.

DNA Hybridization and Autoradiography. DNA hybridization and washes were carried out as described by Smith and Crittenden (1986). Briefly, membranes were first prehybridized at 42 C overnight in 20 to 25 ml of a mixture of 50% formamide, 5X Denhart's solution, 5X SSPE (0.75 *M* NaCl, 0.005 *M* EDTA, and 0.05 *M* sodium phosphate), and 0.1% SDS. The membranes were hybridized overnight with a mixture containing 50% formamide, 5X Denhardt's solution,[7] 5X SSPE, 0.1% SDS, salmon sperm DNA (200 μg/ml), and the α^{32}P-labeled DNA probe. After hybridization, the membranes were washed, and the washing conditions were adjusted according to the amount of probe hybridization to the membrane and were optimized for a high signal-to-noise ratio. The membranes were then exposed to X-ray film with an intensifying screen at –70 C.

Reprobing the Membranes. After autoradiography, the nylon membranes were stripped with 50% formamide and 6X SSPE at 65 C for 30 min, followed by rinsing with 2X SSPE at room temperature. The stripped membranes were used for subsequent hybridizations.

Image Analysis. The autoradiographs were scanned and analyzed using Molecular Analyst software.[8]

Calculation of the Inbreeding Coefficients and Similarity Indices. The coefficients of inbreeding were calculated by the formula of Kuhnlein et al. (1990): $F_x = (b_f - 0.417)/0.566$, where b_f is the average band frequency in the population. The similarity index (SI) for individuals X and Y was the number of common bands in their fingerprint profile, divided by the total number of bands exhibited by both individuals. SI were calculated using the following formula: $S_{xy} = 2n_{xy}/n_x + n_y$ (Lynch, 1991).

[4]Promega Corp., Madison, WI 53711.
[5]MSI, Westborough, MA 01580.
[6]UVP, Upland, CA 91786.
[7]Sigma, St. Louis, MO 63103.
[8]Bio-Rad Laboratories, Hercules, CA 94547.

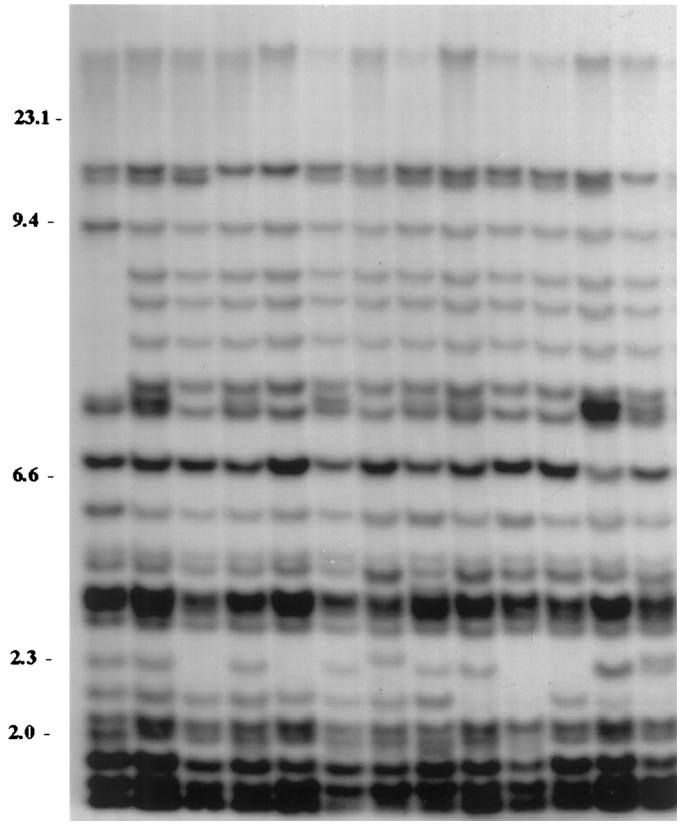

FIGURE 1. DNA fingerprint pattern of the SL101 subline. Genomic DNA was digested with the restriction enzyme *Pvu*II, transferred to nylon membranes, and probed with an endogenous avian retroviral element (EAV) probe. Molecular size markers are shown on the left margin (in kb).

Forty birds from each subline were used for the SI estimates. SL101 was compared with BL101, and SL102 was compared with BL102. Statistical significance of inbreeding coefficients and similarity indices were done by Student's *t*-test (SAS Institute, 1988).

RESULTS AND DISCUSSION

The DNA fingerprint analysis with the EAV probe revealed a moderate level of inbreeding within the SL and BL parental sublines. Of the two SL sublines studied, SL101 had the highest level of inbreeding (0.948 ± 0.015 for SL101 vs. 0.924 ± 0.032 for SL102). The parental control lines, BL101 (0.902 ± 0.071) and BL102 (0.899 ± 0.082), had lower estimated inbreeding coefficients. One representative DNA fingerprint pattern from the SL101 subline is shown in Figure 1. Because SL and BL sublines were maintained by pen mating systems, estimation of inbreeding coefficients by conventional methods were not possible. The inbreeding coefficients did not show statistically significant differences between the lines. There is a very high level of similarity between the SL sublines and their respective parental control lines, as shown by the SI estimates (SI between SL101 and BL101 was 0.949 ± 0.006 and that between SL102 and BL102 was 0.932 ± 0.031. These differences were not statistically significant).

Molecular fingerprint analysis was used for estimating relatedness at the molecular level. In chickens, this technique has been used in a variety of applications, including the measurement of genetic distance between breeds and lines (Kuhnlein et al., 1989; Dunnington et al., 1990; Siegel et al., 1992; Plotsky et al., 1995), estimation of inbreeding and homozygosity within lines of chickens (Burke and Burford, 1987; Kuhnlein et al., 1990), as a predictor of heterosis in interline crosses (Gavora et al., 1996), and for detecting quantitative trait loci in chickens (Lamont et al., 1996). The molecular probes that are currently used for fingerprint analyses are based on the bacteriophage M13 (Vassart et al., 1987) and human hypervariable minisatellite DNA elements (Jeffreys, 1987). These fingerprinting probes must be used for hybridization under conditions of reduced stringency, without the presence of blocker DNA, which will result in high backgrounds and blot-to-blot variation in banding pattern. Benkel and Gavora (1993) reported a novel molecular fingerprint probe for chicken DNA that avoids these problems. This probe is based on EAV, which are resident components of the chicken genome. The DNA fingerprints provide a set of valuable markers for linkage analysis. However, efforts to correlate specific bands to the incidence and severity of the disease, without having proper pedigree structure might result in erroneous conclusions. We have recently developed an F_2 mapping panel with the SL101 and BL101 sublines. Mapping experiments with this panel are expected to provide more insights into the molecular mechanisms underlying vitiligo in this animal model.

Results of the frequency of the *BamH*I ALVE fragments are presented in Table 1. The results of a Southern blot analysis with the pRAV-2 probe for the SL102 and BL102 sublines are shown in Figure 2. The smallest *BamH*I fragments (1.0 and 1.5 kb) are internal ALVE gene fragments and, therefore, are not included in this discussion. Five ALVE-related *BamH*1 fragments were observed in the SL101 subline and four in SL102 subline, whereas BL101 and BL102 showed five and six fragments, respectively. SL101 and SL102 shared two fragments, but the frequencies were different. Similarly, BL101 and BL102 shared

TABLE 1. Frequency of *BamH*I ALVE[1] fragments in the Smyth line (SL) and parental control (BL) sublines

Size (kb)	SL101	SL102	BL101	BL102
24.9	0	0	0.214	1.0
23.5	0	0	0	0.9
23.0	0	0.938	0	0
15.0	0.526	1.0	0.214	0.40
9.0	0.895	0	0.286	0
7.0	0.420	0.812	0	0.30
5.5	0	0	0	0.9
5.0	0	0.625	0	0.3
4.0	0.520	0	0.357	0
3.5	0.632	0	0	0
3.0	0	0	0.429	0
1.5[2]	1.0	1.0	1.0	1.0
1.0[2]	1.0	1.0	1.0	1.0

[1]Avian leukosis virus subgroup E. Endogenous virus (ALVE) loci are defined by the restriction enzyme *BamH*I.

[2]*BamH*I internal fragments of ALVE.

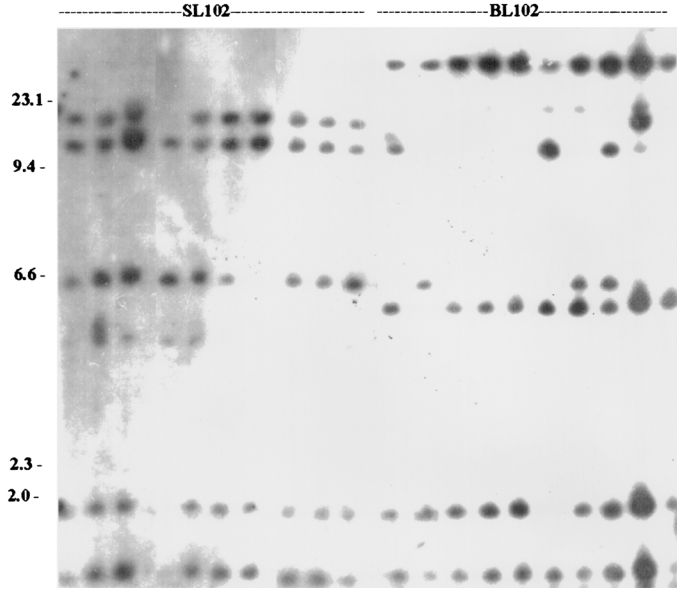

FIGURE 2. Restriction fragment length polymorphism analysis of endogenous avian leukosis virus subgroup E (ALVE) in SL102 and BL102 sublines. Genomic DNA from SL102 and BL102 chickens was digested with the restriction enzyme *BamHI*, transferred to nylon membranes, and probed with the pRAV-2 probe. Lanes 1 to 17 are from SL102, and lanes 18 to 27 are from BL102. Molecular size markers are shown on the left margin (in kb).

two fragments. SL101 and BL101 shared three fragments, and SL102 and BL102 also shared three fragments. The only fragment shared by all four lines was the 15.0-kb fragment. The frequencies of the bands were calculated under the assumption that the lines were randomly mated and were in Hardy-Weinberg equilibrium. However, as the SL and BL sublines were divergently selected for the development of autoimmune vitiligo and were maintained as small closed populations, these assumptions might not be valid in these cases.

An important observation from the present study is the degree of polymorphisms shown by the ALVE genes in the SL and BL. Only a few fragments were observed at high to moderate frequencies. Many of those found in high frequency in one line were found to be low in frequency in the other lines. Iraqui et al., (1991) reported the lack of fixation of ALVE genes in chicken populations. This finding is interesting, considering that the ALVE genes have apparently been resident in the chicken genome since the origin of the Red Jungle Fowl, from which the domestic chicken is thought to have evolved and is similar to the situation of mice, in which hundreds of endogenous viral genes can be present in a single individual (Stoye et al., 1988). The lack of fixation of the ALVE genes in the SL and BL populations suggest that ALVE genes are not neutral but, rather, are subject to selective forces that tend to prevent fixation and to maintain intermediate frequencies. Because all four lines in this study were derived from a single base population about 15 generations ago, it is quite unlikely that the polymorphisms observed at the ALVE locus are due to the presence of different ALVE genes. We hypothesize that the differences in the restriction fragment lengths observed at the ALVE locus are due to mutational events, generating the different RFLP patterns. The results of fluorescent in situ hybdridization analyses with the pRAV-2 probe support this hypothesis (Sreekumar et al., 2000).

Endogenous viral loci have been reported to be associated with many autoimmune diseases in human and animal models (Krieg et al., 1989). The first direct connection between endogenous retrovirus, immune dysregulation, and autoimmunity was found in *MRL/lpr* mice, in which an integration of a retrotransposon sequence into the *Fas* gene led to abnormal apoptosis, which contributes to the autoimmunity and lymphocyte accumulation in this model (Adachi et al., 1993). Later, it was shown to be involved in a number of autoimmune diseases including Type I diabetes and multiple sclerosis (Anderson et al., 1998). Treatment with DNA methylation inhibitors has been reported to induce the gene expression of ALVE genes in chickens (Groudine et al., 1981). We previously demonstrated that treatment with 5-azacytidine induces vitiligo in the genetically susceptible BL parental controls (Sreekumar et al., 1996). Recent results from our laboratory also show that ALVE genes are expressed in vitiliginous SL chickens and also in 5-azacytidine-induced vitiliginous BL parental control chickens (Sreekumar et al., 2000). The exact mechanism by which the ALVE genes induce autoimmune vitiligo in the SL chicken is not known at the present time. The possible explanations are 1) viral integration near immunologically important genes and their phenotypic modification through the dominant insertion at promoter or enhancer elements, 2) recessive insertional mutagenesis through gene disruption, and 3) direct effect of viral gene expression, or superantigen-induced molecular mimicry, or both. We are currently investigating these issues in an F_2 mapping panel involving SL101 and BL101 sublines.

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