The Chicken – a Laboratory Animal of the Class *Aves*

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Abstract. Prague inbred lines of chickens represent a unique system of *MHC(B)* congenic partners differing in the immune-based resistance/susceptibility to v-*src*-induced oncogenesis. Mapping in chickens can be facilitated by the availability of inbred lines, since many well described differences in disease susceptibility and *MHC(B)* haplotypes exist among the defined lines. Long-term intensive research on human, mouse, and rat *MHC* has established a canonical picture of this multigene complex. The chicken *MHC(B)* is clearly the best characterized outside the mammals and it was the first *MHC* clearly different from the paradigmatic structure of the above mentioned mammalian species. Chickens were in many aspects the poor relatives of mice, and they had to wait for introduction of molecular biology methods. But, when it happened, the newly gained data could be easily reconciled with classical genetic studies using available congenic chicken lines. We have established permanent tumor cell lines from *ex vivo* tumors induced by the LTR, v-*src*, LTR provirus in inbred chickens. These cells express a high level of the v-*src* oncogene and are of defined *MHC(B)* genotype. We witness a dramatic acceleration of the development of chicken (avian) genomics. The chicken is not only a good comparative model for basic science, but it is also an object of the poultry industry, which is threatened by several avian diseases. The reason for genome mapping in chickens is thus more than academic.

Despite its conspicuous differences in habitus and considerable phylogenetical distance, the chicken immune system functions in a similar way to that of humans, thus representing an important non-mammalian comparative model. Due to the availability of well defined inbred lines, the chicken is a true laboratory animal. Among avian species, the chicken immune system is the only one which has been studied in detail, but it is likely that general mechanisms identified in chickens will apply to other avian species as well.

The chicken has also contributed substantially to our understanding of (human) oncogenes. Chicken cells are predisposed to infection with numerous transforming viruses containing important oncogenes like v-*src*, v-*erb*, v-*myb*, v-*myc* etc. The main advantage is the prolonged lifespan of these chicken cells *in vitro* compared with rodent cells.

Prague inbred lines of chickens – general considerations

A survey of the breeding history and main research achievements enabled by using the Prague congenic lines of chickens has been published recently (Plachý and Hála, 1997). An up-to-date list of available congenic lines is given in Table 1.

Table 1. Congenic lines

Line	*MHC*-haplotype	Donor line of *MHC*
PR-CB	B^{12}	Inbred base
PR-CC	B^{4}	Derived from CB
PR-CB.B7	B^{7}	Iowa
PR-CB.R1	B^{12r1}	Recombinant B^{12}/B^{4}
PR-CC.R1	B^{4r1}	Recombinant B^{4}/B^{12}
PR-CC.R2	B^{4r2}	Recombinant B^{4}/B^{7}
PR-CC.B13	B^{13}	G-B1
PR-CC.B9	B^{9}	WA*
PR-CB.B15	B^{15}	WB
PR-CB.B21	B^{21}	M*
PR-CB.R4	B^{21r3}	Recombinant B^{21}/B^{15}
PR-CB.R5	B^{15r1}	Recombinant B^{15}/B^{21}

*These independent inbred lines are also available.

Three different methods have been used to prepare congenic chicken lines:

a) Residual heterozygosity: one of the first chicken lines RH-C was established in 1932 in Reaseheath, England. This line was still segregating at the chicken major histocompatibility complex (*MHC*), *MHC(B)*, after 25 generations of brother x sister mating (Gilmour, 1959) when it was imported to Prague in 1958. The immunogenetics methods were introduced for the breeding program after its arrival in Prague and the first congenic lines were developed using residual segregation (Hála et al., 1966; Hašek et al., 1966). The established sublines PR-CB and PR-CC met well the requirement of isogenicity, because they were identical for all the genes which were homozygous in the original inbred line. These consisted of almost all genes because of the high degree of inbreeding ($F_X > 99.9\%$).

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Abbreviations: LTR – long terminal repeat, *MHC* - major histocompatibility complex, RFLP – restriction fragment length polymorphism, RSV – Rous sarcoma virus.

b) Introduction of a gene by backcrossing into the base line: we have prepared several congenic lines by transfer of different *MHC(B)* alleles to the CB or CC line genetic background. The introduction of a marker gene into another inbred line takes a minimum of 10 backcross generations. After this period approximately 99.9% of genes are identical between the congenic and the base line, with the difference being in the selected marker.

c) Congenic lines from recombinant animals: this approach was employed to develop the lines CB.R1, CC.R1 and CC.R2 (Plachý and Vilhelmová, 1984), starting from recombinants within *MHC(B)* haplotypes of already established congenic lines (Hála et al., 1976, 1979, 1981a).

The genetic structure of the chicken *MHC(B)*

Recombinant congenic lines enabled the first detailed description of the structure of a non-mammalian *MHC* by means of classical genetics, biochemistry and finally molecular biology (Plachý et al., 1989a; Kroemer et al., 1990; Plachý et al., 1992b; Kaufman and Wallny, 1996; Plachý and Hála, 1997).

Analysis of the first recombinant haplotypes revealed that *MHC(B)* consists of at least three loci *F, L* and *G* (Pink et al., 1977). The tissue distributions, structures and functions of the B-F and B-L molecules appear to be very similar to mammalian classical histocompatibility class I and class II antigens, respectively. On the other hand, the product of the third highly polymorphic *B-G* gene with limited tissue distribution is of unique structure comprising an Ig V-like domain, a transmembrane domain and an intracytoplasmic domain of variable length. The B-G is only distantly related to butyrophylin and myelin-oligodendrocyte glycoprotein that are encoded by genes at the periphery of the human and mouse *MHC* (Kaufman et al., 1991). The function of the B-G molecules is not yet fully understood, but at least three immunological phenomena can be ascribed with certainty to them: the adjuvant effect, the preferential response and the presence of natural antibodies. In the adjuvant effect, B-G molecules mediate the antibody response to poorly immunogenic molecules (including the B-F) present on the same cell (Hála et al., 1981b) or liposome (Salomonsen et al., 1991). The preferential response to B-G means that antibody response is faster and higher titers are reached as compared with other erythrocyte antigens. This notion is valid for both chicken alloantibodies (Hála et al., 1981b) and mouse monoclonal antibodies (Longenecker et al., 1979). This could be explained partly by the presence of natural antibodies to B-G molecules in unimmunized chickens as well as in several mammalian species (Longenecker and Mosmann, 1980; Neu et al., 1984). For detailed discussion on the B-G function see Plachý and Hála (1997).

Two main genetic regions have been identified serologically and by means of transplantation analysis within recombinant *MHC(B)* haplotypes, the *B-F/L* and the *B-G* (Hála et al., 1981a). The *B* haplotypes of CB.R1 and CC.R1 lines arose as a result of crossing-over between B^4 and B^{12} chromosomes in (CB x CC) F_1 hybrids (see Table 1). The B^{12r1} haplotype comprises $B\text{-}F/L^{12}$ and $B\text{-}G^4$, while B^{4r1} haplotype is reciprocal, being composed of $B\text{-}F/L^4$ and $B\text{-}G^{12}$ subregions. The B^{4r2} haplotype of the line CC.R2 arose in the (CC x CB.7) F_1 hybrid and comprises $B\text{-}F/L^4$ and $B\text{-}G^7$. The distribution of restriction fragment length polymorphism (RFLP) has shown that, in fact, recombination took place either between *B-G* and *B-F/L* regions (B^{12r1} and B^{4r2} haplotypes) or within the *B-G* region (B^{4r1} haplotype) (Plachý et al., 1992a). Thus B^{12r1} and B^{4r1} haplotypes share at least one gene within the *B-G* region (for details see Plachý and Hála, 1997). The $B\text{-}F^{13}$ (class I) and $B\text{-}L^{13}$ (class II) alleles of the B^{13} haplotype are serologically identical to the $B\text{-}F^4$ and $B\text{-}L^4$ of the B^4 haplotype, while the *B-G* alleles (class IV) are different (Simonsen et al., 1982). The B^4 and B^{13} are very similar also with respect to the *B-L* RFLP, but are very different in their *B-F* patterns (Chaussé et al., 1989). Genes within the B^{12}, B^{12r1} and B^{13} haplotypes confer immunological resistance to v-*src*-induced oncogenesis, while chicken lines with B^4, B^{4r1} and B^{4r2} haplotypes suffer progressive growth of v-*src*-induced tumors. Thus, the gene responsible for resistance could be located to the *B-F* region (Plachý and Benda, 1981; compare also Tables 2 and 3). B^{15}, B^{21}, B^{21r3} and B^{15r1} chickens differ in the resistance to Marek's disease (reviewed by Plachý et al., 1992b).

Chicken *MHC(B)* is localized on one of the microchromosomes (Bloom and Bacon, 1985), and it appears to be simpler and smaller than the *MHC* of mammals. It may contain only the "minimal essential" number of genes that is required for the function of an *MHC* (Kaufman et al., 1995). The following gene arrangement of the chicken *MHC(B)* has emerged from molecular biology studies: the proper *MHC(B)* corresponding roughly to differential segments in the above mentioned recombinant haplotypes is represented, at the molecular level, by the cosmid cluster I of 130 kb. Genes of the following order were found within this cluster: *12.3* - coding for GTP-binding protein, two *B-G* genes, *B-L* - class II ß chain, *8.4.* - cell membrane molecule of unknown function, *B-L* - class II ß chain, *RINGIII* - putative transcription factor, *21.6* - complement component C4, *B-F* - class I α chain, *21.7* - transporter for antigen presentation, *B-F* - class I α chain (Guillemot et al., 1988, 1989; Kroemer et al., 1990; Kaufman et al., 1991, 1995 ; Zoorob et al., 1993; Thorpe et al., 1996). Two cosmid clusters of 60 kb and 75 kb define another region of about ten *B-G* genes with not fully determined position on the *MHC(B)* chromosome (Kaufman et al., 1991). One of the chicken peculiarities is the existence of a second *MHC*, the *Rfp-Y* locus, with two class I α and two class II ß genes of limited poly-

morphism (Miller et al., 1996). *Rfp-Y* segregates independently of the *MHC(B)*, but is located on the same microchromosome. The *Rfp-Y* locus is most probably located just on the opposite side of the chromosome at the telomeric end of the long arm, separated from the *MHC(B)* by repetitive sequences of nucleolar organizer region, which gives it genetic (recombinational) independence of the core *MHC* genes within the cluster I (for further details see Plachý and Hála, 1997).

Experimental Model for Antitumor Immunity

A simplified view of important properties of the model system of Prague congenic lines relevant to experimental tumors induced by Rous sarcoma virus (RSV) and its v-*src* oncogene is given in Table 2. This simple picture is supported, in fact, by extensive studies using also other congenic partners of the CB and CC lines as well as various crosses of these lines. Such studies have established that the *MHC(B)* genotype shows by far the largest influence on the regression of Rous sarcomas. Furthermore, a hierarchy of the *MHC(B)* haplotypes in response to RSV has been observed. The importance of other loci outside the *MHC(B)* has also been acknowledged. RSV tumorigenesis is mediated by the oncogene v-*src*. It has been demonstrated that v-*src* DNA alone can induce sarcomas *in vivo*. Good correlation between the growth of RSV-induced and v-*src* DNA-induced tumors has been observed and *src*-specific immunity has also been demonstrated. Regressors (CB line) but not progressors (CC line) mount an effective CTL reaction against RSV tumor cell targets, and this cytotoxicity is specifically blocked by means of anti-CD8 and anti-B-F (class I) antibodies, while anti-CD4 antibodies are not effective.

It has been found that certain common chicken *MHC(B)* haplotypes express only one class I molecule at a high level (Kaufman and Wallny, 1996). The level of expression vary considerably among *MHC(B)* haplotypes with a rather positive correlation to resistance in the case of RSV (higher expression – higher resistance). The opposite dependence is seen with Marek's disease (Kaufman and Salomonsen, 1997), reflecting different require-

Table 2. A simplified survey of observed phenomena relevant to v-src-induced tumorigenesis in MHC(B) congenic lines CB and CC

	CB(B^{12}/B^{12})	CC(B^4/B^4)
Basic starting points[1-5]		
The outcome of tumors induced by PR-RSV-C	Regression (protects against second challenge with PR-RSV-C or other RSV strains)	Progression (regression never observed; acceleration of primary tumor growth after second challenge)
or its oncogene alone (v-*src* DNA)	Regression (protects against second challenge with v-*src* DNA or PR-RSV-C; efficient adoptive transfer of immunity)	Progression (rare regressions do not protect against second challenge; adoptive transfer of immunity not effective)
Findings supporting explanation of these phenomena in terms of the B-F (class I)-restricted cytotoxic T lymphocytes (CTL) response[5-6]		
Presence of stringent B-F-binding motifs within RSV proteins including v-Src	Yes	No
The *B-F* gene expression	High	Low
Ex vivo CTL response	High inhibition by anti-CD8 and anti-B-F but not anti-CD4 antibody	Low
Other immunological functions controlled by *MHC(B)* by unknown mechanism[7-8]		
Phagocytic activity	High (glass adherence and zymosan phagocytosis 2-3 fold higher than that of CC; antimacrophage agents induce progression of RSV tumors)	Low
CD4 : CD8 ratio of peripheral blood lymphocytes	2.5 (45 : 18%) = help?	0.6 (25 : 40) = supression?

[1]Plachý (1984); [2]Plachý et al. (1986); [3]Plachý (1988); [4]Plachý et al. (1994); [5]Svoboda et al. (1996); [6]Kaufman and Salomonsen (1997); [7]Hála et al. (1998); [8]Malin et al. (1993).

ments for the cytotoxic- and natural killer cell-mediated antitumor immunity. Peptide motifs for dominantly expressed class I molecules were defined and it turned out that they may explain some disease associations directly. The number of appropriate antigenic peptides derived from RSV almost perfectly fits the observed degree of resistance. Experiments focused on the exploitation of certain antigenic peptides for induction of efficient antitumor immunity in this experimental model are in progress.

One of the not fully understood, but clearly *MHC(B)*-controlled traits is a characteristic ratio of the CD4 and CD8 cells in the peripheral blood of congenic lines CB, CB.R1 and CC, CC.R1 (Malin et al., 1993). From the similarity of the CD4 : CD8 ratio between parental inbred lines sharing the *B-F/L* region, the conclusion could be drawn that this trait is under the genetic control of this region with a dominant inheritance of high CD4 : CD8 ratio. Similarly, the gene regulating the adherence potential of monocyte-derived macrophages was localized within the *B-F/L* region of the chicken *MHC(B)*. The importance of cells of the mononuclear phagocyte system for the response to RSV was demonstrated using anti-macrophage agents (silica, carrageenan). We have observed progression of tumors in treated CB chickens, which are otherwise resistant, i.e. regress their tumors. Recent studies have revealed a protective effect of chicken recombinant type I and type II interferons toward Rous sarcomas in the CC chickens (Plachý et al., 1999).

New results comparing the tumorigenicity of RSV v-*src* and PR2257*src* oncogenes in the panel of inbred lines are shown in Table 3. While the fate of tumors induced by RSV v-*src* is clearly controled by the *B-F* region genes (see above), partial resistance to growth of tumors induced by PR2257*src*, the cellular c-*src* mutant oncogene, seems to be controlled rather by the *B-G* region genes. This time we observed the same response in CB.R1 and CC chickens sharing the whole *B-G* region, as defined by restriction fragment length polymorphism (see above). Recombination took part within the *B-G* region in the CC.R1 chickens; thus, CB and CC.R1 lines share part of the *B-G* region distal from the *B-F*, *B-L* regions. At least one polymorphic *B-G* gene is localized here close to a putative recombination point (Plachý and Hála, 1997). The only other gene known to reside in this part is *12.3*, coding for non-polymorphic GTP-binding protein.

While PR2257*src* posseses a newly gained sequence of potential immunogenicity (and there is indeed one stringent motif for the B12 molecule), c-*src* Y527F is fully identical with endogenous (self) antigen c-*src* (the only difference is the substitution of the regulatory tyrosine 527 for phenylalanine). c-*src* Y527F DNA induced tumor growth again more rapidly in the CB and CB.R1 chickens compared to CC and CC.R1 (data not shown). This could be considered as an indirect evidence for some different mechanism operating upon the tumor growth control than that of classical B-F-restricted cytotoxic T lymphocytes. An interesting possibility exists that the viral LTR-driven overexpression of the protein coded by c-*src* Y527F can activate potentially self reactive T-cell clones of a low avidity for autoantigen. The abolition of

Table 3. The growth pattern of tumors induced by v-src or PR2257src in a panel of congenic chicken lines

Chicken line	Genotype	v-*src*		PR2257*src*	
		Mean tumor size (mm^2) 35 days p.i.	Predominant tendency of the tumor growth	Mean tumor size (mm^2) 35 days p.i.	Predominant tendency of the tumor growth
Congenic recombinant					
CB	B^{12}/B^{12}	236[a]	>	1287[a]	<
CB.R1	B^{12r1}/B^{12r1}	288[a]	>	825[b]	<>
CC	B^{4}/B^{4}	463[b]	<	877[b]	<>
CC.R1	B^{4r1}/B^{4r1}	682[c]	<	1293[a]	<
Congenic					
CB.B7	B^{7}/B^{7}	223[a]	>	1006[a]	<
CC.B13	B^{13}/B^{13}	241[a]	>	754[b]	<>
CB.B9	B^{9}/B^{9}	215[a]	>	1325[a]	<
CB.B15	B^{15}/B^{15}	231[a]	>	1388[a]	<
Outbred					
BL		285[a]	>	854[b]	<>

>regression; <progression; <>progression or long-term persistence followed by regression. Oncogenic agents used were 0.6 μg LTR, v-*src*, LTR and PR2257*src* DNA fragments (Svoboda et al., 1996). All chicks were inoculated s.c. into the outer area of the pectoral muscle with a volume of 0.1 ml at the age of 8 weeks. The size of tumors was measured by calculating the area (mm^2) of the tumors prominent from the pectoral muscle (Svoboda et al., 1992). We used the statistical program ADSTAT 2.0 for analysis of data. Values not sharing a common superscript a-c are significantly ($P < 0.05$) different.

tolerance to self peptides of c-*src* in chickens regressing v-*src*-induced tumors has been reported (Halpern et al., 1996). The activation of T cells by peptides closely related to self could explain this crossreactivity. We observed, using Prague congenic lines, that only the CC and CC.R1, but not the CB and CB.R1 chickens are prone to autoimmunity as measured by T-cell-mediated syngeneic GvH reaction (Plachý et al., 1989b). The increase of reactivity in CC chickens compared to CB is evident even in untreated animals. Thymectomy of CC donors led to a severe reaction corresponding in strength to that of the minor histocompatibility antigen difference between the donor of lymphocytes and the host embryo. Thus, the idea of tumor rejection via self antigenic determinants of an oncogene corresponds well to our observations in the Prague congenic lines.

Permanent tumor cell lines expressing the v-*src* oncogene now available

The frequency of immortalization of cells seems to be inversely correlated to their *in vitro* lifespan. Chicken cells enter senescence after 30–50 generations in cell culture. This is only slightly less than for human cells, but significantly more than for mice, rats or Japanese quails with 10–20 generations. Indeed, chicken cells are difficult to immortalize like primate (including human) cells (Hayflick, 1965; Beug and Graf, 1977).

In our experiments we employed for cloning a provirus present in hamster tumor cell line H-19. This provirus is composed of only v-*src* flanked by long terminal repeats (LTRs) accomodating strong promoter-enhancer sequences. The LTR, v-*src*, LTR provirus was rescued from H-19 hamster tumor cells and transfected to Japanese quail cells. The cellular clone of transformed quail cells, F6K4, was used as a source of DNA (Geryk et al., 1986). For tumor induction we employed the LTR, v-*src*, LTR provirus cloned in a plasmid vector. Tumors induced in chickens have maintained unaltered LTR, v-*src*, LTR provirus, and progressively growing tumors express a large amount of v-*src* mRNA (Svoboda et al., 1992; Plachý et al., 1994). While the incidence of primary tumors is almost 100%, only about 50% of tumors escape immune surveilliance and grow progressively (depending on *MHC(B)* genotype; see above). About 1/5 of progressing tumors are also repeatedly transplantable in syngeneic hosts. The establishment of transplantable tumors expressing the LTR, v-*src*, LTR provirus indicates that v-*src*-transformed cells can be immortalized.

Tissue fragments of four different transplantable tumors stored in liquid nitrogen in the medium containing 10% DMSO were used for establishment of *in vitro* cell culture. Tumor cell lines derived from primary sarcomas of the CB.R1 and CC.R1 chickens have been growing *in vitro* for more than one year. They have overcome the crisis and the senescence period and can be therefore considered as permanent cell lines. The cells are of fibroblast-like morphology, and the integrity of the LTR, v-*src*, LTR provirus and the high level of v-*src* expression remain unaltered throughout passaging *in vitro* (Trejbalová et al., to be published). These tumor cell lines were designated F6CC-PR1259 and F6CC-PR9692, respectively. This designation conforms to the nomenclature proposed by Nazerian (1987). F6 refers to the transforming agent (see above), the third letter refers to the avian species (chicken) and the fourth designates the cell line; PR stands for Prague, the place of origin followed by the identifying number. Two other tumor cell lines of the CB and CC origin (designated F6CC-PR1186 and F6CC-PR2351) grow, after one year of *in vitro* culture, still relatively poorly. Another permanent tumor cell line was derived from a single lung metastasis in the chick No. 6286 with a growing tumor induced by inoculation with the PR9692 cells. This new cell line was designated F6CC-PR9692M. Analysis of the cytoskeleton organization and podosomal contact structures in PR9692 and PR6286M cells revealed interesting differences thought to be associated with metastatic activity (Veselý et al., 1997).

The established tumor cell lines with defined *MHC(B)* are of great advantage for reliable and reproducible *in vitro* tests in the studies of specific anti-oncogene immunity. With chicken cells (both normal and transformed), we face a generally low propensity to immortalization. Thus, both mortal clones of transformed chicken cells and immortal cell lines (derived on rare occasions from mortal clones) are available. Our tumor cell lines expressing v-*src* represent a useful model for experiments focused on the mechanism of immortalization and the possible role of the *src* oncogene in this process.

Chicken Genomics

The reason for genome mapping in chickens is more than academic. The chicken represents worldwide an important source of animal proteins for human nutrition. Thus, location and identification of genes underlying disease resistance and economically important (quantitative) trait loci are in the focus of interest of many agriculture research laboratories.

Furthermore, the chicken has been proposed as a model mapping organism alternative to the puffer fish (McQueen et al., 1996) because of a relatively small size of the genome (one-third of that of mammals) and its compactness due to smaller intronic and intergenic sequences, while the number of genes is approximately the same as in mammals. At least 11 groups of conserved synteny between chickens and humans have been identified (Burt et al., 1995; Klein et al., 1996) despite the different organization of avian genome having a large number of microchromosomes (Bloom et al., 1993).

Mapping in chickens is facilitated by the availability of defined lines, since many differences exist between

chickens of unrelated inbred lines, including susceptibility to diseases and *MHC* haplotypes. Such lines may serve as a standard for the initial establishment of appropriate methods and defining the marker loci. Chosen lines can be used as parent lines of a mapping reference population (Wain et al., 1998). Large families can be readily established in chickens. A further advantage is easy manipulation and analysis of embryonic stages.

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