

Chicken telomeres terminate in the conserved vertebrate repeat, (TTAGGG)<sub>n</sub>, on metaphase chromosomes (Meyne et al., 1989), and telomerase is constitutively expressed in somatic cells (Venkatesan and Price, 1998). Telomere repeats in chickens are, however, localized not only on the ends of chromosomes, but also in interstitial regions of several macrochromosomes and centromeres (Nanda and Schmid, 1994). Similarly, three types of telomere sequences (terminal; terminal plus centromere; whole chromosome) were identified on the chicken microchromosomes. Although the avian genome is only one-third the size of the human genome, the total amount of telomere sequences was found to be 5 to 10 times more abundant (about 4% of the chicken genome). In addition, one category of telomeres is extremely long, with 1–2 Mb arrays (Delany et al., 2000). These “mega-arrays” found in chicken and some other avian species represent the largest telomere arrays described for any organism to date. All avian species with megabase-sized telomeres carry a typical number – about 30 – of microchromosomes, while two bird-of-prey species without these large telomeres possess a karyotype with a very low number of microchromosomes – about 4. One idea seems therefore plausible, that long telomeres are localized on microchromosomes protecting these small genetic elements from telomere erosion over the long-lived natural life-span of many birds (despite higher metabolic rates and body temperature, most avian species are longer-lived than their mammalian counterparts of equal body size.) It is not, therefore, surprising that the avian model has also attracted attention of gerontologists (Holmes and Austad, 1995a, b). Another type of chicken telomeres of “normal” size (10–40 kb) with terminal localization exerted shortening in somatic tissues of adult chickens together with downregulation of the telomerase activity (Delany et al., 2000). This resembles the profiles seen in human somatic cells and contrasts with murine cells, wherein constitutive telomerase activity is found in many somatic tissues of adult mice protecting telomere erosion.

### Oncogenic transformation and differentiation of chicken haematopoietic cells

In adult vertebrates, multipotent haematopoietic precursors reside within the microenvironment of the bone marrow, where they undergo progressive maturation and commitment to one of several lymphoid and non-lymphoid cell lineages. Analysis of the mechanism of differentiation and commitment to a given lineage is difficult because of a relatively low frequency of bone marrow precursors and their limited life-span *in vitro*.

Because of the unusually high susceptibility of chicken and some other avian species to the proliferation-inducing effects of oncogenes, a large variety of acutely transforming avian retroviruses have been iso-

lated from diseased animals and several cell lines have been established (Beug et al., 1979, 1981, 1982; Moscovici and Gazzolo, 1982; Coll et al., 1983; Graf et al., 1992). The transforming specificity of different viral oncogenes for cells of a specific lineage suggests that they act as dominant alleles of genes which are involved in differentiation and lineage-specific growth control.

The avian retrovirus models have proved to be useful tools in dissecting the machinery involved in haematopoietic growth control and tumorigenesis by providing a way of selectively expanding and maintaining a homogeneous population of otherwise rare haematopoietic precursors in sufficient numbers for biochemical and molecular analyses (Graf and Beug, 1978; Moscovici and Gazzolo 1982; Beug and Graf, 1989). It is also noteworthy that with a few exceptions, avian transformants appear to closely resemble their normal counterparts (McNagny et al., 1992; McNagny and Graf 1996). Thus, these cells could also be used as a model for normal haematopoiesis.

Two different, independently isolated ALVs have transduced *v-myb* oncogenes that represent truncated, intronless and point-mutated versions of the *c-myb* protooncogene. Due to these changes, *v-myb* oncogenes code for activated proteins insensitive to some regulations that normally control the activity of the c-Myb protein (Graf and Stehelin, 1982). The E26 virus encodes a complex protein in which v-Myb is fused to a second oncoprotein, v-Ets. E26 causes a precursor type leukaemia with prevailing erythroleukaemic features and transforms multipotent and myeloid progenitors *in vitro* (Graf et al., 1992). Deletion of *v-ets* renders E26 non leukaemogenic. However, concomitant expression of the chicken myelomonocytic growth factor (cMGF) can restore myeloid leukaemogenicity and transforming capacity of E26 *v-myb*-containing, *v-ets*-deletion mutants (Metz et al., 1991). Thus, myeloid cells transformed by the E26 version of *v-myb* fail to proliferate in animals, because the E26 v-Myb is unable to provide the proliferative signal necessary for the *in vivo* accumulation of transformed myeloblasts.

In contrast to E26 *v-myb*, the avian myeloblastosis virus (AMV) *v-myb* contains more of *c-myb* sequences, especially the leucine zipper region. In addition, it has accumulated specific point mutations, with those in the DNA binding domain being crucial for the transforming specificity of this oncoprotein (Introna et al., 1990). AMV transforms monoblasts, which then grow in a cMGF-independent manner in culture and also proliferate efficiently *in vivo*, inducing acute monoblastic leukaemia in chicks. The *v-myb*-expressing monoblasts secrete small amounts of cMGF, suggesting that they grow in an autocrine fashion (Dini et al., 1995). cMGF expression in AMV monoblasts is induced by *GBX2*, a homeobox gene that was identified as a target for AMV *v-myb* (Kowenz-Leutz et al., 1997). While *GBX2* activation by c-Myb

requires signal transduction emanating from the cell surface, the leukaemogenic AMV *v-Myb* constitutively induces the *GBX2* gene. However, the expression of *GBX2* and *cMGF* genes appears to be confined only to more mature and long-term cultivated myeloid cells, as the primary *v-myb* leukaemic monoblasts analysed in short-term *ex vivo* cultures express neither *GBX2* nor *cMGF* and their growth factor independence is caused by another mechanism (Dvořáková et al., 2001). It has been shown that the sustained proliferation and leukaemic properties of *v-myb* monoblasts are dependent on the intact leucine zipper region of the *v-Myb* protein (Bartůnek et al., 1997; Dvořáková et al., 2001). Moreover, the *Myb* zipper region modulates the lineage choice of haematopoietic progenitors. The intact *v-myb* leucine zipper region was shown to bias the commitment of primitive chicken erythroid progenitors, in favour of the monocyte/macrophage lineage (Karafiát et al., 2001). Interestingly, the cooperation of *v-Myb* with the basic FGF signalling commits primitive chicken haematopoietic progenitors to erythroid lineages and strongly activates proliferation of resulting immature erythroid cells (Bartůnek et al., 2002). Thus, viral *Myb* proteins interfere with developmental programs of haematopoietic cells at several levels and this experimental model can provide valuable information about regulation of vertebrate haematopoiesis.

Three well-studied avian erythroblastosis viruses (AEV) transform predominantly avian erythroid cells. The prototype virus, AEV-ES4, contains two oncogenes *v-erbA* and *v-erbB* (reviewed in Metz, 1994). *v-erbA* encodes a mutated version of the thyroid hormone receptor  $\alpha$  (reviewed in Beug et al., 1994; Metz, 1994). The normal function of this protein in response to hormone is to transactivate genes containing thyroid hormone response elements. *v-ErbA* constitutively binds these response elements, but has lost the ability to bind the thyroid hormone and therefore acts rather as a repressor of transcription of target genes (reviewed in McNagny and Graf, 1996). *v-ErbB* represents a truncated and mutated version of the epidermal growth factor/transforming growth factor  $\alpha$  (EGF/TGF $\alpha$ ) receptor tyrosine kinase. *v-erbB* again appears to be constitutively active because of the lack of a normal ligand-binding domain, thus providing a constant proliferative signal. AEV, a natural vector of a rearranged nuclear hormone receptor, provided the first demonstration of the oncogenicity of such receptors.

*v-myc*-containing viruses are MC29, CMII, OK10 and MH2. These viruses transform macrophage-like cells *in vitro* and induce "myelocytomatosis" or "endotheliosis" *in vivo* (Graf and Beug 1978; Graf and Stehelin, 1982). The MH2 virus again contains two oncogenes – *v-myc* and *v-mil*. *c-Mil* is the avian equivalent of the mammalian *c-Raf* serine/threonine kinase and acts as a MAP kinase (Moelling et al., 1984; Suttrave et al., 1984). *v-mil* has been shown to con-

tribute to the leukaemogenicity of MH2 by induction of the chicken myelomonocytic growth factor (cMGF), thus abrogating the dependence of *v-myc* only transformed macrophages on an exogenous growth factor (Graf et al., 1986).

The oncoprotein *v-Rel* of the reticuloendotheliosis virus strain T (REV-T) is a member of the NF- $\kappa$ B/dorsal family of transcription factors (reviewed in Gilmore, 1991). The REV-T virus strain is morphologically and antigenically more closely related to mammalian retroviruses than to other avian retroviruses (Mizutani and Temin, 1973), probably representing a jump in species during the evolution of the virus. REV-T is also unusual in that most clones transformed by the virus are immortalized (Beug et al., 1981; Lewis et al., 1981).

Avian retroviruses E26 (*v-myb+v-ets*), AEV (*v-erbA+v-erbB*) and MH2 (*v-myc+v-mil*) carrying pairs of oncogenes have provided some of the first models for studying the cooperativeness of oncogenes in leukaemogenesis. In each case both oncogenes are required for the formation of aggressive leukaemias, demonstrating the multistep nature of tumorigenesis.

## Culture and differentiation of normal chicken erythroid progenitors

The ability to cultivate normal chicken erythroid progenitors *in vitro* provided a system that allows a molecular analysis of how growth factors regulate the balance between proliferation and differentiation in haematopoiesis. The avian haematopoietic system provides a unique model to investigate the multistep programming of haematopoietic stem cells into differentiated blood cells because of long-lasting "self-renewing" of committed erythrocytic progenitors, which has no analogy in vertebrates (reviewed in Müllner et al., 1996; Samarut, 1996). Activation of sustained cell proliferation – "self-renewal" in committed progenitors – may be a central mechanism that controls homeostasis in haematopoiesis and may be a likely target for leukaemogenic mutations.

The self-renewal of normal erythroid progenitors is induced by the activation of the receptor tyrosine kinase *c-ErbB* (avian epidermal growth factor receptor – EGFR) by its ligand type  $\alpha$  transforming growth factor (TGF- $\alpha$ ). Long-term growth (more than 25 "self-renewal" divisions) of these progenitors in suspension also required cooperation with the signal derived from oestradiol binding to the endogenous oestrogen receptor (Hayman et al., 1993; Steinlein et al., 1995; Müllner et al., 1996). Ligand activation of *c-ErbB* induced tyrosine phosphorylation, DNA binding, and reporter gene transcription of Stat 5b in erythroblasts (Mellitzer et al., 1996).

Stat – signal transducers and activators of transcription – are latent transcription factors, which reside in the cytoplasm, and their tyrosine phosphorylation,

dimerization and nuclear localization are induced by various ligands of receptor tyrosine kinases. Interestingly, reduction of Src by induction of antisense *src* RNA expression suppressed erythropoietin (Epo)-promoted erythroid differentiation in human erythroleukaemia cell line K562 (Okutani et al., 2001). Reduction of Src diminishes tyrosine phosphorylation of Stat 5 regardless of Epo treatment. Stat 5b enhanced the transforming potential of v-Src, v-Src-induced cell cycle progression and cell motility in stably transfected (mouse) NIH 3T3 cells. Furthermore, the dominant negative, COOH-terminal-truncated isoform of Stat 5b partially suppresses v-Src-mediated cell transformation (Kazansky and Rosen, 2001).

When TGF- $\alpha$  is removed and replaced by the differentiation-inducing factors erythropoietin (Epo) and insulin, the chicken erythroid progenitors (expressing particular receptors) are reprogrammed to terminally differentiate after 5 “differentiation” divisions. In contrast, the c-Kit receptor tyrosine kinase activation by its ligand stem cell factor (SCF) causes only transient proliferation (6–8 “self-renewal” divisions) and is unable to induce Stat 5b activation (reviewed in Müllner et al., 1996).

## DNA repair and homologous recombination

Gene constructs transfected into *Saccharomyces cerevisiae* integrate predominantly into the endogenous loci. This targeted integration has been postulated to reflect double-strand break repair mediated by the *RAD52* epistasis group of genes (reviewed in Game, 1993). Structural homologues of the *RAD51*, *RAD52* and *RAD54* genes of *Saccharomyces cerevisiae* have also been cloned in vertebrates, supporting the evolutionary conservation of these processes (Bezzubova et al., 1993a, 1993b; Shinohara et al., 1993; Bendixen et al., 1994). Targeted integration occurs, however, less frequently in mammalian cells, and most transfected DNA constructs insert at random. This may be attributed to the predominance of the so-called end-joining pathway that repairs double-strand breaks without requiring sequence homology of the DNA substrates (reviewed in Weaver, 1995).

Chicken (bursal) B cells are unique in generating a large repertoire of Ig genes by gene conversion within the bursa of Fabricius (reviewed in Reynaud et al., 1994; Funk and Thompson, 1996). The chicken, in contrast to the majority of mammals, inherits only one *V* and *J* segment. Following single recombination creating a functional *VL* gene, diversification occurs by insertion of pseudogenes, many of which occur upstream of the *V* and *J* segments. This gene conversion activity is homology-dependent and may therefore share enzymatic activities critical for gene targeting.

This has been proved in the chicken B-cell line DT40 (derived from an ALV-induced B-cell tumour). Similar to normal B cells, DT40 continues to diversify the Ig

light-chain locus by gene conversion. Of great importance is the observed high ratio of targeted to random integration after DNA transfection of DT40 cells (Buerstedde et al., 1990; Buerstedde and Takeda, 1991; Dieken et al., 1996). Elegant studies of Bezzubova et al. (1997) have shown that disruption of the chicken orthologue of *Saccharomyces cerevisiae* gene *RAD54* in the DT40 line results in a high X-ray sensitivity, 6- to 8-fold reduction in the rate of Ig gene conversion, and two orders of magnitude decrease in the frequency of targeted integration. Re-expression of the *RAD54* cDNA restored radiation resistance and targeted integration activity. The combination of X-ray sensitivity and homologous recombination defects in the DT40 *RAD54*<sup>-/-</sup> clones is remarkably similar to the phenotype of the yeast *rad54* mutants (Muris et al., 1996). The chicken cell line DT40 thus provided genetic evidence of a link between double-strand break repair and homologous recombination throughout the evolution from yeast to vertebrates.

A similar phenotype has been observed in mouse *mRAD54*<sup>-/-</sup> embryonic cells (Essers et al., 1997). Consistent with the double-strand DNA break repair defect, these cells are sensitive to ionizing radiation, mitomycin C, and methyl methanesulphonate, but not to UV light. Gene targeting experiments also showed a decrease in homologous recombination. These results imply that, besides DNA end-joining mediated by DNA-dependent protein kinase, homologous recombination contributes to the repair mechanism in mammalian cells. *mRAD54*<sup>-/-</sup> (knock-out) mice are viable and exhibit apparently normal V(D)J and immunoglobulin class-switch recombination, showing that *mRAD54*<sup>-/-</sup> is not required for the basic recombination process generating functional Ig and T-cell receptor genes in mice (Essers et al., 1997).

Where the effects of gene inactivation can be studied in cell culture, the disruption of both gene copies in somatic cells is an appropriate alternative to the production of mutant mouse strains. A possible system for such studies is the chicken B-cell line DT40. Since the description of the high targeting frequencies, DT40 has been indeed successfully used for the genetic analysis of a variety of cell biology processes (Wang et al., 1996; Bezzubova et al., 1997; Takami et al., 1997, 1999; Fukugawa et al., 1999a, b; Kurosaki, 1999).

Recently, a comprehensive database of chicken bursal ESTs to identify disruption of candidate genes and recyclable marker cassettes based on the loxP system were presented on the DT40 web site (<http://genetics.hpi.uni-hamburg.de/dt40.html>) – (Abdrakhamov et al., 2000; Buerstedde et al., 2002). Normal bursal cells from chickens of the inbred line CB were chosen as the source for the cDNA library, since the DT40 cells were originally transformed by ALV. In addition, the inbred genetic background of the CB line chickens facilitates the analysis of sequence variation