Oncogenes and avian development

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ABSTRACT   In order to detect signs of oncogene activity and elucidate their possible role in avian ontogeny we implemented two different strategies. One was to detect either the protein product or messenger RNA *in situ* at various stages of development. The other was to try and disturb development with retroviruses carrying one or several oncogenes in their activated forms. Time- and tissue-specific expression of c-*myc* was apparently not related to particular phases of cell evolution, such as population amplification. Rather the presence of c-*myc* immunoreactive product at particular stages appeared to depend on cell types. c-*myb* and c-*ets* messenger RNAs were found expressed preferentially in the blood system, respectively in hemopoietic and differentiating endothelial cells. The developing embryo heart was found to be uniquely sensitive to the effect of retroviruses provided that two conditions were respected. The first was the injection of the virus or construct prior to E3.5. The second was the presence of the v-*myc* gene, whether alone or associated with one or several other v-*onc*. In such cases a large proportion (70%) of chick and all quail embryos developed multiple heart rhabdomyosarcomas within 10 days. In chickens the association of a second v-*onc* or of two others induced the formation of secondary tumors, whose type was determined by the nature of the other oncogene(s). v-*myc* + v-*mil* induced excessive proliferation of endothelial cells; v-*myc* + v-*myb*, v-*myc* + v-*ets*, or v-*myc* + v-*myb* + v-*ets* induced solid hemopoietic cell tumors; finally the association of v-*myc* with v-*erb*A induced the differentiation of ectopic cartilage nodules within the heart rhabdomyosarcomas. When the viruses were injected in E5 embryos, none of these cardiac pathologies were observed.

KEY WORDS: *avian embryo, nuclear oncogenes, developmental tumors*

Introduction

Cell multiplication and differentiation are regulated during embryonic development according to a finely adjusted balance. Growth signals and the multiple steps of their transduction through the cell membrane and cytoplasm to the genome are obviously implicated in this regulation. Many investigators are now trying to pinpoint the actions of oncogenes that code for proteins mediating these steps during embryonic development. With that aim, the expression of many of some 50 oncogenes identified to date has been analyzed in *Drosophila, Xenopus*, chicken or mouse ovogenesis or embryogenesis (Adamson, 1987; Ohlsson and Pfeifer-Ohlsson, 1987). The developmental significance of the patterns of expression thus uncovered is apparent only in a minority of instances, in which expression is restricted to a cell type and/or to a particular stage of development. Another popular approach has consisted in introducing protooncogenes under the control of ubiquitous or tissue-specific promoters into transgenic mice (review in Cory and Adams, 1988). This strategy has yielded information on the oncogenic potency of various oncogenes.

In avian embryos, we have adopted two tactics. One is a cataloguing process that others have also carried out on embryos of various species. The other consists in subjecting the developing embryo to the effects of oncogene-carrying retroviruses. The viruses we selected are leukemia viruses, because of our long standing interest in the development of the hemopoietic system in the avian embryo. The protooncogenes, whose expression we followed during development, are those that have homologues in the genome of these viruses, in particular the nuclear oncogenes *myc*, *myb* and *ets1*. The results of this latter aspect of our analysis are by necessity very patchy but they open avenues for exploration. On the other hand, the effects of retroviruses in the embryo were very different depending on the stage at which the embryo was exposed to the viruses. Thus these effects must tell us something about cell evolution during development. The background against which our
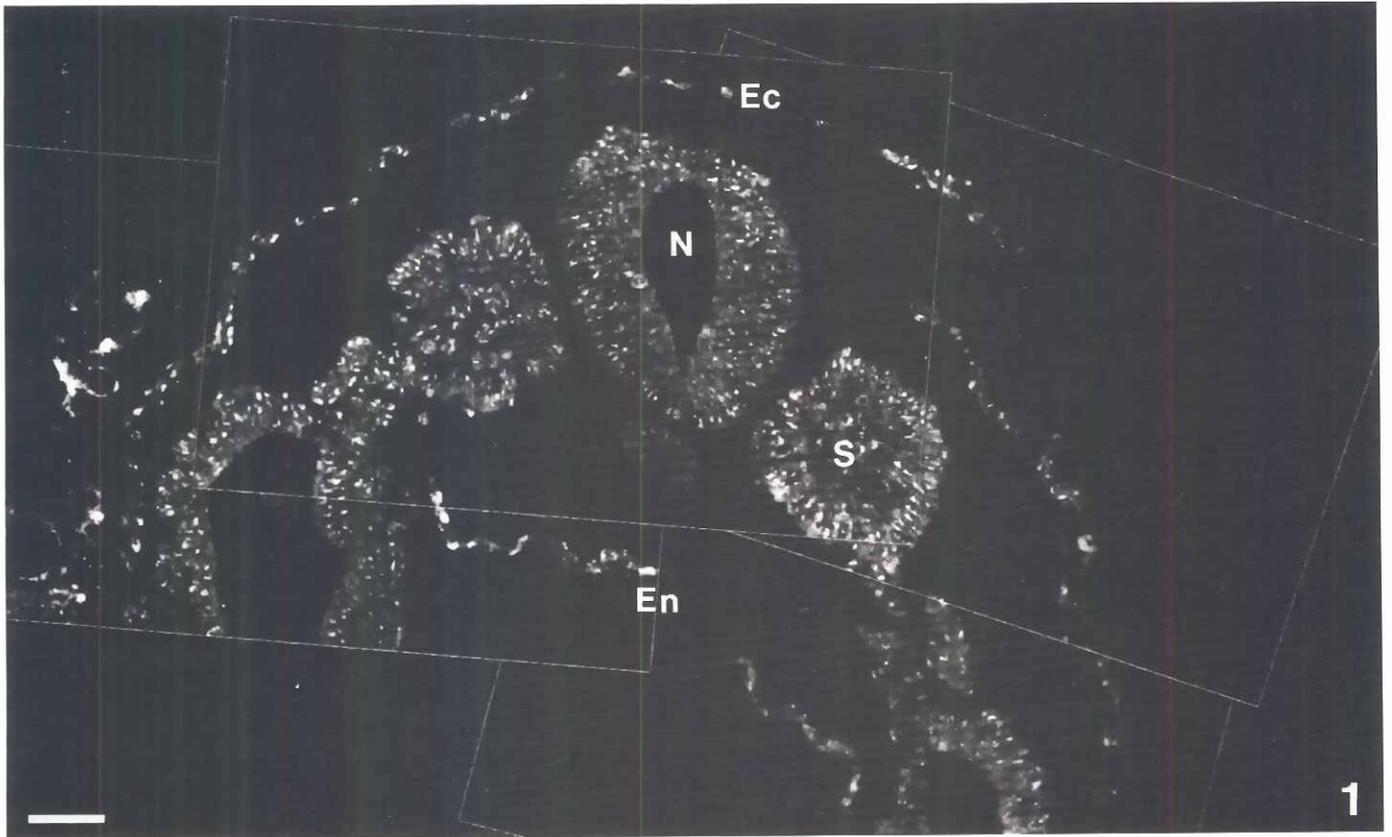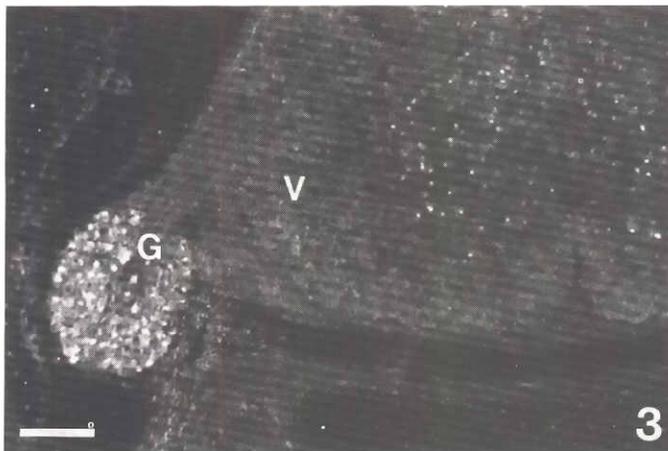
Fig. 3. Spinal cord and dorsal root ganglion (G) E13 chick embryo. *Streptavidin Biotin IF for c-myc. A number of post mitotic neurons display positivity in the two structures. V= ventral horn of the spinal cord. Bar=50μm*

Fig. 4. Same section as in Fig. 3, double-stained with Hoechst 33258 nuclear stain. *Comparison of the two figures shows that only a proportion of nuclei are myc-positive. Bar= 50μm*

previous work demonstrating that Rous sarcoma virus is innocuous for chicken embryos (Dolberg and Bissel, 1984). Injected into the limb bud of E4 embryos, the virus induced no tumor formation within the lapse of time before hatching, whereas in newborn chickens solid sarcomas developed in the alar membrane within a week.

Expression of three nuclear oncogenes during embryogenesis

Of these three oncogenes, c-myc is the best known from the viewpoints of structure, mode of regulation and pattern of expression during the cell cycle. c-myc is characterized by the very short half life of its protein product and is thought to be involved either in the regulation of transcription or in DNA synthesis (Cleveland et al., 1988). Its expression is widespread in many different types of cells. Knowledge about c-myb structure and DNA binding capacities is accumulating rapidly. It appears likely that this oncogene is also a transcription regulator. In adults, c-myb expression is more or less restricted to immature hemopoietic cells, where high levels of mRNA are present (Gonda et al., 1982; Sheiness and Gardinier, 1984). The third oncogene, c-ets1 (Boulukos et al., 1988), directs high levels of mRNA synthesis in adult lymphoid organs (Chen, 1985; Ghysdael et al., 1986).

The most extensive analysis that we have carried out deals with c-myc expression (Jaffredo et al., 1989). The presence of the protein was detected by in situ immunofluorescence at many different stages in both chick and quail embryos during the whole period of incubation, yielding a precise picture of the modulation of expression of this oncogene. The rules were found to be similar in both species. First the c-myc protein is ubiquitous in the early embryo up to E3.5, i.e. it is present in the nuclei of all three germ layers (Fig. 1). There seem to be alternate periods of expression and extinction in the neural tube (Figs. 1 and 2), but it would be necessary to carry out a finer study of stages to determine how regular this alternance is. After E3.5, a drastic decrease in the protein level occurs in all cell types, so that enhanced sensitivity of the technique is necessary to reveal a signal. Then positive groups of cells can be detected at all stages of development. Some of these are on the verge of undergoing morphogenesis, for instance precartilaginous areas, not identifiable yet through condensation of

experiments were carried out can be summarized as follows. Concerning the expression of protooncogenes, only c-src had been studied in some detail in the chick embryo (Cotton and Brugge, 1983; Levy et al., 1984; Sorge et al., 1984). Protein pp60^{c-src} was shown by immunocytology to be present at high levels in the brain, neural tube and heart at organogenesis stages and then to become restricted and selectively expressed in neurons. Thus knowledge about oncogene expression in avian development is practically a blank page.

The other aspect of our investigation was undertaken despite

TABLE 1

AVIAN DEFICIENT LEUKEMIA VIRUSES

Virus	Leukemia type	Oncogenes
MC29	myelocytomatosis	v-myc
MH2	myelocytomatosis	v-myc + v-mil
AMV	myeloblastosis	v-myb
E26	erythroid and myeloid leukemia	v-myb + v-ets
AEV	erythroblastosis	v-erbA + v-erbB

Fig. 1. c-myc expression in the early chick embryo. *The protein has been revealed with a monoclonal antibody directed against the human c-myc, followed by immunofluorescence (IF) detection. 18 pairs of somites stage. Transverse section at the level of somite 14. All nuclei of the three germ layer derivatives are positive. Bar= 50μm. Ec= ectoderm; En= endoderm; N= neural tube; S= somite*

Fig. 2. c-myc expression in the chick embryo at the 25 pairs of somites stage. *Level of somite 18. Neural tube nuclei do not display the protein, in contrast to ubiquitous positivity in other tissues. A pattern of alternate expression and extinction appears to be the rule in the E2-3 neural tube. Bar= 50μm*
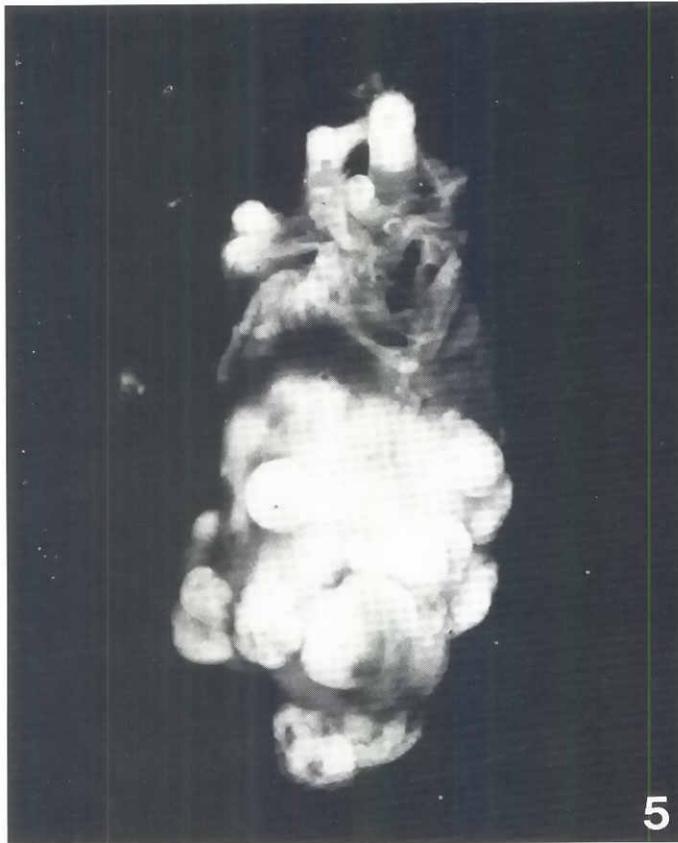
Fig. 5. **Tumoral heart in an E15 chick embryo** *injected with MC29 at E3.*

Fig. 6 . **"Bell-shaped" feathers in an E16 chick embryo** *injected at E2 with MC29. This embryo was dead at autopsy.*

mesenchymal cells. A similar pattern of expression characterizes the dermis prior to feather bud condensation. In other groups of cells, c-*myc* expression was clearly linked to a process of cell multiplication, for instance in diffuse intraembryonic hemopoietic foci (E5.5-E7). Another pattern characterizes neurons, in many of which a strong signal could be detected after their birthdate (Figs. 3-4). Finally, no protein was ever found in the endoderm or in endoderm-derived cells, in particular hepatocytes, despite the presence of a large number of mRNA copies detectable by *in situ* hybridization. In the case of other germ layer derivatives, the data from protein and messenger detection correlated well.

c-*myb* and c-*ets1* products were detected by the technique of *in situ* hybridization for mRNA, since reliable antibodies were not available at the time to identify the proteins. The messengers for these two oncogenes were interestingly correlated in the blood-forming system, where both were preferentially expressed (Vandenbunder *et al.*, 1989).

Ontogeny of the avian hemopoietic system has been our central theme of research for some years. We showed that stem cells segregated in the extraembryonic area of the blastodisc at the beginning of development are not endowed with a permanent capacity for self-renewal. Rather they give rise to the first generation of red blood cells and contribute to the first "definitive" red cell generation. Their progeny becomes extinct towards the end of the

first week of development, as was shown in quail-chick or chick-chick yolk sac chimeras (Dieterlen-Lièvre, 1975; Lassila *et al.*, 1978). Thereupon blood cell formation proceeds from stem cells emerging from the embryo proper. The emergence of these cells could be allocated to a defined area of intraembryonic mesoderm localized in the vicinity of the truncal aorta (Dieterlen-Lièvre, 1984). Interspecific grafting experiments and *in vitro* clonal assays pro-

TABLE 2

MONOCLONAL ANTIBODIES AND MARKERS USED TO IDENTIFY CELL TYPES IN THE TUMORS

|	Cell types	References
13F4	Myoblastic lineage	P.M. Rong *et al.*, 1987
QH1	Quail hemangioblastic lineage	Pardanaud *et al.*, 1987
VIA2	Chick heomopoietic cells	B. Bruner, unpublished
Anti-cytokeratin	Epidermis	(Biomedical Technologies, Inc.)
Low density lipoproteins	Endothelial cells and macrophages	(Biomedical Technologies, Inc.)
Alcian blue	Cartilage	Simons and Vanhorn, 1971
Hoeschst stain 33258	Nuclei	(Serva)

( ) Number of embryos
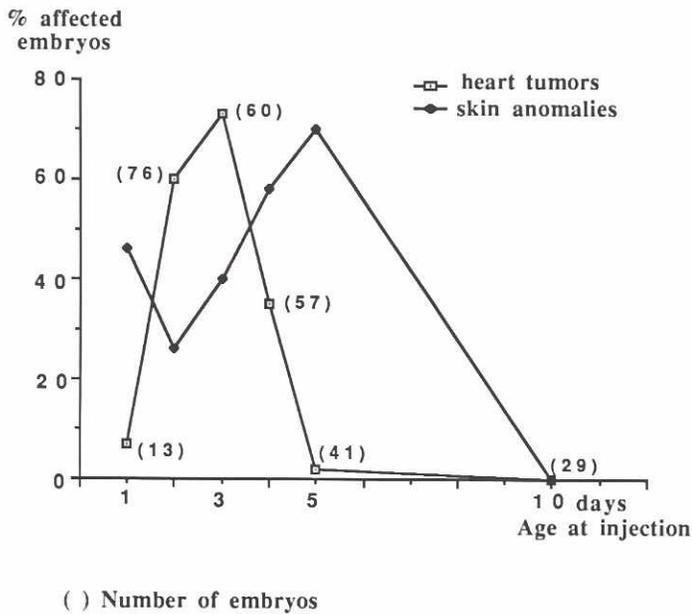
Fig. 7. Pathological incidence in chick embryos *depending on the time of MC29 injection.*

vided evidence for the central role of this region (Dieterlen-Lièvre , 1984; Cormier *et al.*, 1986; Cormier *et al.*, 1988). However, there is still some ambiguity concerning the relationship of endothelial and hemopoietic cells. The question is whether a common precursor to these two lineages exists and whether early endothelium has the capacity to produce stem cells. This problem is basic to understanding bone marrow function in adult life. Self-renewal in the bone marrow could occur at the level of this presumptive common precursor, the hemangioblast (Murray, 1932). Another aspect of this problem is whether the para-aortic stem cells are the ones that colonize the bone marrow rudiment. In the blood and vascular system, c-*myb* and c-*ets1* mRNA appeared to be expressed in complementary fashions. c-*ets1* was usually present in endothelial cell nuclei and c-*myb* in hemopoietic cell nuclei, while c-*myc* was present in both cell types. In contrast, cells of the pre-blood island condensations of the early blastodiscs all expressed c-*ets1*; this expression became restricted to the peripheral cells, i.e. those that become endothelial by virtue of their peripheral localization as the blood islands mature, while the central, hemopoietic cells began expressing c-*myc*. At these stages (5-7 pairs of somites), c-*myb* was not detected in blood-forming cells. This oncogene became expressed in intraembryonic cells undergoing erythropoiesis in the E3 intra-aortic clusters and the E6 para-aortic foci. c-*ets1*, by contrast, is expressed principally in endothelial cells of differentiating blood vessels, while the expression appears to dwindle as maturation proceeds. For instance, the aortic endothelium at E6 is no longer positive.

The complementary expression of these two oncogenes by cell types that may derive from a common precursor is especially interesting in view of the capture of these two oncogenes by E26, a virus that transforms blood cell precursors. We hope to uncover the functional significance of these expressions by isolating a population of hemangioblasts and by producing monoclonal antibodies against the relevant proteins.

Effects of viral oncogenes on avian development

Leukemia retroviruses (Table 1) were administered to embryos with the expectation that the development of the hemopoietic system might be perturbed. Among the following viruses, Avian Myeloblastosis Virus (AMV), Avian Erythroblastosis Virus (AEV) and E26, only MC29 had a clear tumorigenic effect on the embryo (Saule *et al.*, 1987; Al Moustafa *et al.*, 1988). It induced tumors in the heart of chicken or quail embryos that were infected prior to E3.5 (Fig. 5). These tumors, which grew very rapidly, were easily detected by macroscopic examination as early as E12. To diagnose cell types of origin, we used an array of specific stains and monoclonal antibodies (moab) (Table 2). The tumors induced by v-*myc* were diagnosed as rhabdomyosarcomas, the cells in the tumors having affinity for the muscle-specific monoclonal antibody, 13F4. The cells at the periphery of the tumors still displayed some muscle-specific organelles, such as myofibrils and intercalary discs. These tumors were induced when 50 infectious particles of the virus were dispensed to each embryo. In most cases the deficient virus and its helper were injected into the coelom, but it was verified that several injection routes (in brain vesicles for instance) yielded the same pathology. The only other pathology detected during embryonic life was in the skin. Hyperplasia and hyperkeratinization of the epidermis occurred and were readily visible because the feathers became bell-shaped (Fig. 6). The incidence of heart rhabdomyosarcomas was maximal after injection on E3, affecting 70% of chick embryos (Fig. 7) and 100% of quail embryos. The incidence then abruptly decreased and injection on E5 resulted in no heart tumors. Skin anomalies, in contrast, affected a higher proportion of embryos when the virus was injected at E5 (Fig. 7). When injection was carried out at E10, none of these phenotypes were observed.

The striking feature of this pathology is the unique cell target and the narrow time restriction of sensitivity to the virus. It is well established that myogenic precursors enticed to divide by the expression of one or another oncogene (for instance *myc*, *src*, *fos*...) (Falcone *et al.*, 1985; Denis *et al.*, 1987) cannot differentiate. Our interpretation of the particular tumorigenic effect of v-*myc* in early

TABLE 3

EFFECTS OF VIRUSES OR CONSTRUCTIONS ON THE CHICK EMBRYO INJECTED AT E3

Virus or construction	Oncogenes	Effects
MC29	*myc*	Heart myosarcomas
MH2	*myc* + *mil*	Heart myosarcomas + endothelial tumors
clone 25	*myc* (from MH2)	Heart myosarcomas
PA 200	*mil*	none
MAHEVA	*myc* + *erbA*	Heart myosarcomas + cartilage nodules
XJ12	*erbA* + neoR	Light erythroblastosis
E26	*myb* + *ets*	Erythroblastosis
MHE226	*myc* + *myb* + *ets*	Heart myosarcomas + hemopoietic tumors
MHE myb-	*myc* + *ets*	Heart myosarcomas + hemopoietic tumors
MHE ets-	*myc* + *myb*	Heart myosarcomas + hemopoietic tumors
MHE without gag	Δ gag *myc*; *myb*; *ets*	Heart myosarcomas + hemopoietic tumors

(Embryos were sacrificed between E7 and E21)
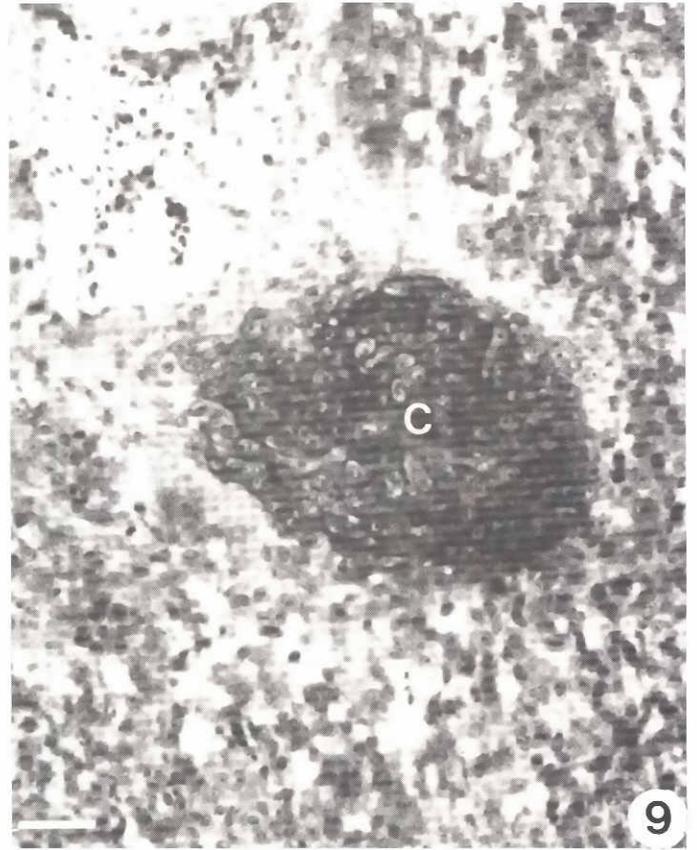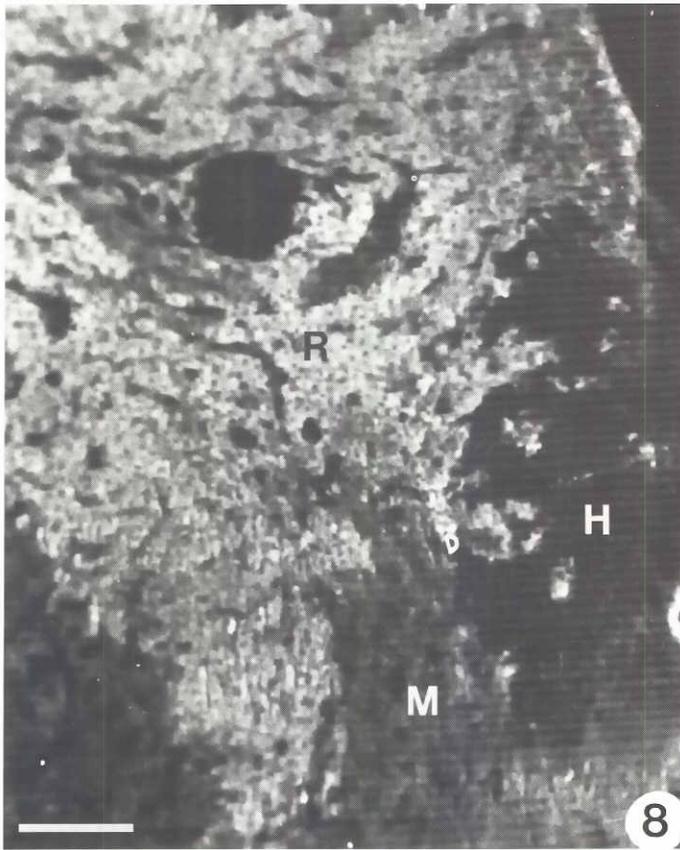
Fig. 8 . Section through a chick tumoral heart obtained after MHE226 injection (E3 → E14). 13F4 muscle specific IF. *M= normal cardiocytes; R= rhabdomyosarcoma cells intensely stained; H= hemopoietic cell tumors unstained by 13F4. Bar= 50µm*

Fig. 9. Alcian blue-positive cartilage nodule (C) in the center of a heart rhabdomyosarcoma after MAHEVA treatment of a chick embryo. *(E3 → E19). Bar= 20µm*

embryos is that cardiac myoblasts are sensitive prior to differentiation and that sensitive progenitors disappear from hearts around E4 (allowing some 24 hours for a few rounds of viral integration and replication).

We next investigated the effect of associated oncogenes on the developing embryo. While confirming the data obtained with the v-*myc* gene, these experiments uncovered *in vivo* cooperation effects between several oncogenes. Various combinations proved capable of transforming different cellular targets. The oncogene combinations used are summarized in Table 3. The cardiac cell still appears as a preferential target for v-*myc* and the time window of sensitivity is the same. Provided v-*myc* was present in the constructions and the construct was injected prior to E3.5, cardiac rhabdomyosarcomas were obtained. Furthermore, secondary tumors developed that either were or were not associated with the rhabdomyosarcomas and that were or were not subjected to the same time window restriction. Most secondary tumors were observed only in the chick species and not in the quail. The cell types of origin of these secondary tumors depended on the second oncogene associated with v-*myc*. Virus MH2 provoked an intraluminal budding of endothelial cells. c-*mil*, the cellular homologue of MH2 second oncogene, encodes a serine-threonine kinase. The budding cells were observed in the two species and could be diagnosed as being QH1-positive in the quail and VIA2-negative in the chick, and we could thus allocate these cells to the hemangioblastic lineage in the quail and to the endothelial lineage in the chick. These endothelial buddings were observed in the heart but also in other organs, notably in the chorioallantoic membrane. Cells from these overgrowths could readily be grown *in vitro* for extended periods of time (more than 2 months) and could be cloned in agar. Clones displayed several endothelial markers.

Various artificial constructs were derived by combining oncogenes from viruses MH2 and E26 (Table 3). The four constructs yielded similar pathologies. The full combination, MHE226, was more malevolent than the three others as judged from the enormous growth of the tumors (Al Moustafa *et al.,* submitted). In the quail only rhabdomyosarcomas were induced. In the chick, solid hemopoietic cell tumors appeared in the heart (Fig. 8) and in various organs. These were induced after both E3 and E5 injections.

The last construct, MAHEVA, encodes oncogenes v-*myc* and v-*erbA* from AEV. The latter is the viral homologue of a receptor for thyroid hormones (Sap *et al.,* 1986; Weinberger *et al.,* 1986). Cardiac rhabdomyosarcomas were obtained ; furthermore, from E17 onwards, alcian blue positive cartilage nodules regularly developed within these tumors (Bachnou *et al.,* submitted). These nodules (Fig. 9) did not display any of our regular lineage markers,

making it difficult to diagnose the original cell type from which they had derived. The nodules are located at the tip of the ventricles in an ectopic site by reference to chondrocytes of neural crest origin normally present in bird heart at the level of the semilunar valves (Sumida *et al.,* 1989). They most likely originate from myogenic cells, whose differentiation was blocked by the presence of the v-*myc* protein. We suggest that the expression of the second oncogene, v-*erb*A, mediates an ectopic differentiation process that can be classified as a phenomenon of transdifferentiation. Construct XJ12, which encodes oncogene v-*erb*A alone, did not elicit any cardiac anomaly.

Conclusion

Among the nuclear oncogenes that were analyzed for their expression during normal development, c-*myc* is expressed in a broad fashion. Prior to the period of organogenesis, the protein is abundant and ubiquitous. Thereafter it becomes modulated according to standard patterns, obviously related to precise though still elusive functions. c-*myb* and c-*ets1* are expressed as a tandem in the closely linked hemopoietic and endothelial cell lineages. However, their expression spreads to some other cell types, usually mesoderm-derived.

Our experiments lead us to conclude that embryos are not as completely refractory to the transforming potency of leukemia viruses as had been found previously with Rous sarcoma virus (Dolberg and Bissel, 1984). However, transformation is severely restrained. Only v-*myc* has been found capable of inducing a pathology by itself. This pathology was confined to two cell targets (cardiac myoblasts and epidermal cells). These transformation processes could be initiated only during very short time windows of development. These restrictions indicate that only definite developmental steps are prone to oncogene pathogenesis; more should be learned about these normal steps by perturbing them in this way. New targets for leukemia viruses have been found in our experiments. This should be related to previous data demonstrating that erythropoietic cells transformed by AEV are not identical in early embryos and in post-hatching animals (Moscovici *et al.,* 1983).

Our findings indicate that the embryonic microenvironment imposes stringent growth regulation constraints on most cell types. Indeed cells from most organs, developing normally in infected embryos, when explanted *in vitro* immediately displayed a transformed phenotype.

Cooperation is considered as the mechanism responsible for the development of most tumors. However it has usually been demonstrated *in vitro* (Land *et al.,* 1983). Recently an interesting cooperation process has been worked out on reconstituted embryonic mouse urogenital sinuses grafted into adult mice after transfection either with *myc* or *ras* or both (Thompson *et al.,* 1989). Several more examples of transformation through cooperation have been obtained here with *myc* + *mil*, *myc* + *myb*, *myc* + *ets1* (or both) and finally *myc* + *erb*A. Each combination (considering the two E26 oncogenes as one category) hits a different cell type.

Finally, the combination of v-*myc* with the viral equivalent of a hormone receptor appears to uncover an ectopic pathway of differentiation. We interpret this "transdifferentiation" as stemming from an interference with the normal operation of cellular oncogenes. These are generally thought of as being part of a network (Herrlich and Ponta, 1989). Several oncogenes can carry out a given step within the network. Which oncogene effectively operates depends on the cell type and a change of oncogene at one of the steps may result in a switch in phenotype. In the case of cardiac rhabdomyosarcomas, it is clear that the cells undergoing excess multiplication are blocked from differentiating into the muscle phenotype. Our hypothesis is that cell division is not totally exclusive of differentiation in the chondrocytic lineage and that the secondary oncogene is responsible for this inflexion. This hypothesis will be tested in our next experiments by transfecting cardiac myoblasts in two steps.

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