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Original Article

An identical effect mediated by thyroid deficiency or oncogene v-*erb*A in the chick embryo

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ABSTRACT We have shown earlier that the association of v-*myc* and v-*erb*A (MAHEVA construct) is responsible for the appearance of a specific phenotype in chick embryos inoculated at E3. This phenotype comprises rapidly growing heart rhabdomyomas (induced by v-*myc* alone) and within these tumors secondarily appearing cartilage nodules (Bachnou *et al., Oncogene 6:* 1041-1047, 1991). Here we report that v-*erb*A can be replaced by thyroid deficiency. When decapitated embryos were inoculated with virus MC29 (v-*myc* alone) or when v-*myc* inoculated embryos were treated with thiourea, 100% of the embryos reaching E17 to E19 displayed tumoral hearts bearing cartilage nodules. We thus report *in vivo* evidence that v-*erb*A acts by antagonizing the effects of thyroid hormones. Remarkably, thyroid deficiency rendered embryos more sensitive to the effect of v-*myc*, since 100% developed heart rhabdomyomas and cartilage nodules, versus about 70% affected when either v-*myc* or MAHEVA were inoculated. Thyroid deficiency did not alter the species-specific character of transdifferentiation, since only chick but not quail embryos developed cartilage nodules after thyroidectomy or MAHEVA infection.

KEY WORDS: *transdifferentiation, oncogenes v-erbA, v-myc, hypothyroidism*

Introduction

Thyroid hormone receptors (TR's) are members of a family of nuclear receptors for steroid and thyroid hormones (thyronine, T_3 and T_4), for retinoids (RA) and for vitamin D3 (Green and Chambon, 1986; Evans, 1988). These receptors function as ligand-dependent transcription factors and elicit cellular responses to hormonal signals by direct control of gene expression. Several regions can be distinguished in these proteins. Among these a cysteine-rich, conserved region is responsible for binding of the receptor to specific DNA sequences (thyroid hormone response elements, TRE's) and a highly hydrophobic carboxyterminal region is the ligand binding domain. The v-*erb*A protein is the viral homolog of the c-*erb*Aα (T_3Rα) gene, present in the genome of the avian erythroleukemia and sarcomas inducing virus AEV (Graf and Beug, 1978; Sap *et al.*, 1986; Weinberger *et al.*, 1986). v-*erb*A differs from its progenitor in the following respects: it has retroviral *gag* structural sequences fused to its N-terminus; it has sustained small N- and C- terminal deletions including the ligand binding domain; and there are 13 internal amino-acid residue differences (Sap *et al.*, 1986). v-*erb*A alone is non-oncogenic *in vivo* but it blocks the terminal differentiation of erythroblasts which are induced to multiply by tyrosine-kinase encoding oncogenes, like the second oncogene v-*erb*B derived from the receptor of the epidermal growth factor, present in the genome of AEV (Kahn *et al.*, 1986; Gandrillon *et al.*, 1989; Schroeder *et al.*, 1990). V-*erb*A induced the proliferation of several type of cells contrasting with c-*erb*Aα which instead inhibited their proliferation (Zenke *et al.*, 1990; Carnac *et al.*, 1993; Iglesias *et al.*, 1994).

In contrast to steroid receptors which bind to DNA as homodimers, RAR (receptors for all *trans*- and 9-*cis*-retinoic acid) and T_3R bind DNA with high affinity in the form of heterodimers with RXR, the receptor for 9-*cis*-retinoic acid (Yu *et al.*, 1991; Leid *et al.*, 1992; Zhang *et al.*, 1992). In animal cells the v-erbA protein binds to the TRE, prevents the action of *bona fide* thyroid hormone receptors at these sites and is a dominant negative inhibitor of c-erbAα function (Damm *et al.*, 1989; Sap *et al.*, 1989; Bonde and Privalsky, 1990). The v-erbA protein can also repress the functions of the RARs and the estrogen receptors, and repression of RAR appears to play an important role in the establishment of the neoplastic cell phenotype (Desbois *et al.*, 1991; Sharif and Privalsky, 1991; Chen and Privalsky, 1993; Yen and Chin, 1994). In the absence of thyroid hormone, c-*erb*A itself acts as a transcriptional repressor (Damm *et al.*, 1989; Sap *et al.*, 1989) acting through the basal transcription machinery (Fondell *et al.*, 1993), and sup-

Abbreviations used in this paper: AEV, avian erythroblastosis virus; TRE, thyroid hormone responsive element.

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presses RAR-dependent transactivation by titrating out RXR (Barettino *et al.*, 1993).

We have recently documented an *in vivo* cooperative effect between v-*erb*A and v-*myc* using the MAHEVA retrovirus, an artificial construct encoding both oncogenes (Bachnou *et al.*, 1991). V-*myc* alone, which encodes the viral version of a transcription factor, causes heart rhabdomyomas in avian embryos infected with the MC29 retrovirus before day 4 of incubation (E4) (Saule *et al.*, 1987; Al Moustafa *et al.*, 1988, 1992), and so transforms a specific cell type that is different from the post hatching targets (myelocytes, epithelial cells). When coupled with v-*myc*, v-*erb*A is responsible for the appearance of cartilaginous nodules within the heart rhabdomyomas, never seen with v-*myc* alone (Bachnou *et al.*, 1991). These nodules became first detectable at E17, they were located at the tip of the ventricle, always within a muscle tumor; they had no affinity for the muscle specific mab 13F4 and were composed of cells with a typical chondrocytic appearance that had affinity for alcian blue. Such nodules were present in 81% of tumoral hearts at E17-E19. They were interpreted as the result of a transdifferentiation phenomenon affecting the myoblasts, whose normal differentiation potentialities were blocked by the expression of v-*myc*. To check whether the transdifferentiation process was due to the inhibitory effect of v-*erb*A on gene(s) expression, we studied the effects of depriving the infected embryos of thyroid hormones, a manoeuvre that should have the same effect on v-*myc* induced rhabdomyomas as does v-*erb*A in MAHEVA- infected embryos. We report here that cartilage nodules appeared in heart rhabdomyomas of hypothyroid chick embryos infected with MC29 alone.

This suggests that repression of a subset of genes regulated by a c-erbAα product is involved in the chondrogenic transdifferentiation of v-*myc* transformed cardiomyocytes.

Results

To formally exclude the possibility that cartilage nodules induced by MAHEVA in the rhabdomyomas arose from transformed neural crest cells (giving rise to cartilage cells from the heart, Le Lièvre and Le Douarin, 1975), we transplanted quail neural folds into E1.5 chick embryos and followed with MAHEVA infection at E3. Ten embryos were examined at E16-17 (Table 1). Quail cells were detected, as expected, in the arterial walls and valvular cartilage, whereas the cartilage nodules usually occur at the tip of the ventricle (Bachnou *et al.*, 1991). None of the 8 tumor-bearing embryos exhibited the quail specific heterochromatin marker in either the rhabdomyomas or the transdifferentiated cartilage nodules (data not shown). Therefore, we can be certain that these nodules did not arise from neural crest cells, because all the neural crest-derived cells present in the heart are of quail origin in these transplanted embryos.

To study the effect of thyroid hormone deprivation in MC29 infected embryos, we either decapitated E1.5 chick embryos according to Fugo's method (1940) or injected thiourea at E7 and E15 in the yolk sac (see Materials and Methods). Most of the embryos were examined at E17 or later since the experiments were designed to detect late appearing cartilage nodules. The decapitated embryos, which survived until the day before normal hatching (Fig. 1, E17 embryo), were poorly developed and showed an important lag in yolk sac internalization. The thyroid glands were so hypoplastic that they were barely visible: histology showed flat follicular epithelium with no colloid and the follicles separated by large blood vessels. Mortality was high (42.8%) whether decapitated embryos received no further treatment or were injected with v-*myc* one day later. Death rate increased with age of the embryos.

TABLE 1

EFFECTS OF MAHEVA IN NEURAL CREST CHIMERAS

Age at autopsy	Total number of MAHEVA infected chimeras	Number with cardiac tumors	Number with cartilage nodules	Number with skin anomalies
E12-E15	9	3 (33%)	0	2 (22%)
E16-E17	10	8* (80%)	5* (50%)	6 (60%)

*No quail cells could be found in the tumors or the nodules.

The death rate was lower in thiourea-treated embryos (20%). These did not internalize their yolk sac whereas, in normal embryos, this process has begun at E19 and is complete by E20. The thyroids of these E18 embryos displayed a striking hyperplasia (Fig. 2a compare with control, Fig. 2b), due to absence of the negative feedback of thyroid hormone on the hypophysis.

The two phenotypes described after MAHEVA infection, namely heart tumors and skin anomalies (Bachnou *et al.*, 1991) were present in embryos infected with MC29 either after decapitation or before thiourea administration (Fig. 3, Tables 2, 3). The heart tumors were positive with 13F4 (Rong *et al.*, 1987) and MF20 (Bader *et al.*, 1982), two monoclonal antibodies that are specific for cells of the myogenic lineage. When the embryos reached E17, the first cartilage nodules could be observed (Fig.

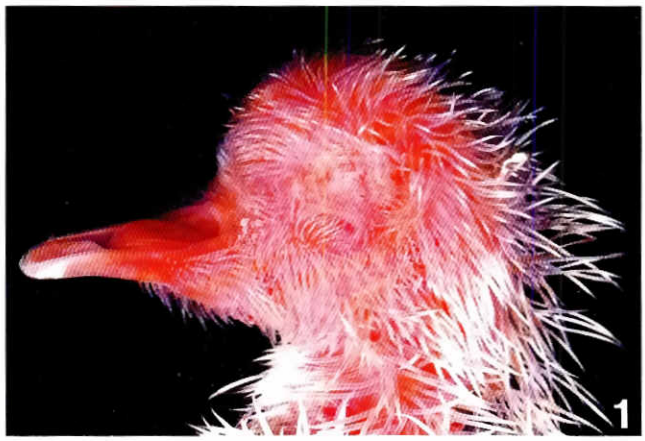
Fig. 1. E17 chicken embryo decapitated surgically according to Fugo (1940). *The telencephalon and half of the mesencephalon were severed at 36 h of incubation, yielding an embryo without eyes and upper beak. (x3).*

Fig. 2. Thyroid glands from E18 embryos. (2a) *From two MC29-infected, thiourea-treated, E18 embryos;* **(2b)** *from an E18 control embryo. (x30).*

Fig. 3. Heart region of an E19 embryo, infected with MC29 at E3 and thiourea-treated at E7 and E15. *The thyroid glands (arrows) are enlarged and hyperemic, the heart is studded with large tumors (T). (x15).*

Fig. 4. Large rhabdomyomas with alcian blue staining nodules. *These tumors are located at the tip of the ventricle in the hearts of E17 decapitated chick embryos infected with MC29 at E3.* **(4a)** *Two alcian blue staining nodules within the rhabdomyoma. The largest is typically associated with a blood vessel. Feulgen-Rossenbeck/alcian-blue. (x164).* **(4b)** *Another typical rhabdomyoma with an alcian blue positive nodule. This tumor is continuous with normal heart muscle (light staining tissue in the left corner).*

Fig. 5. Blood smears from MC29-infected (5a), *AEV-infected* **(5b),** *MAHEVA-infected* **(5c),** *hypophysectomized and MC29-infected* **(5d),** *MC29-infected and thiourea-treated* **(5e)** *E18 embryos. (x459). Leukemic cells are very numerous in these last three protocols, while the MC29 blood smear is identical to a normal one.*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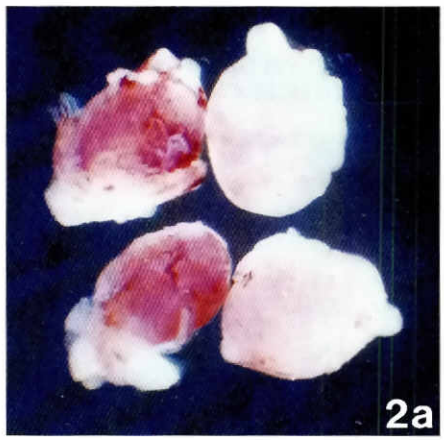
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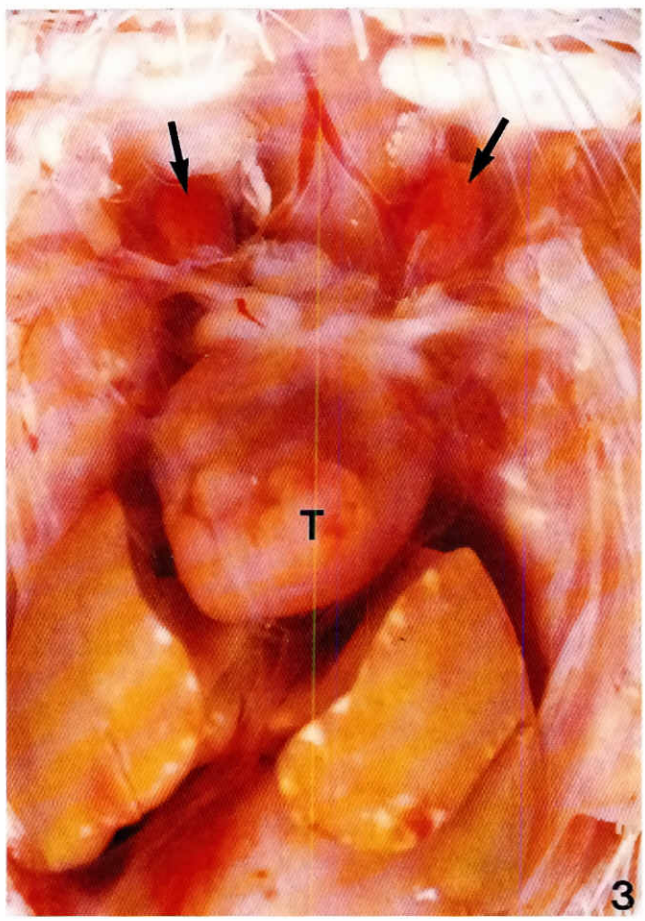
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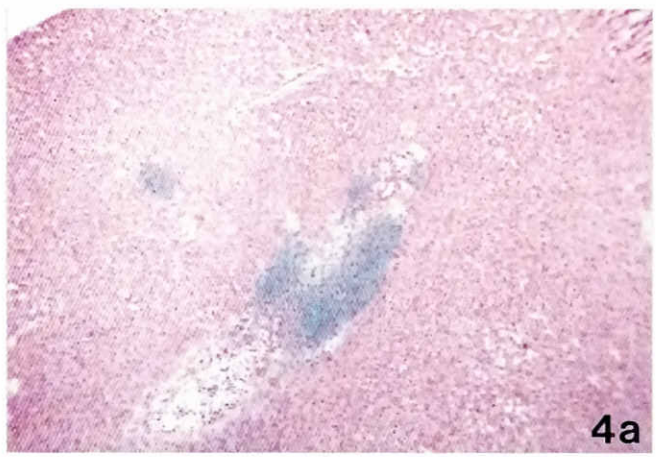
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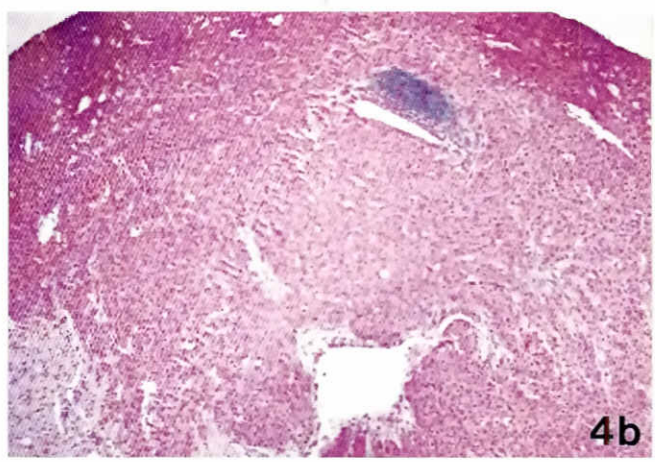
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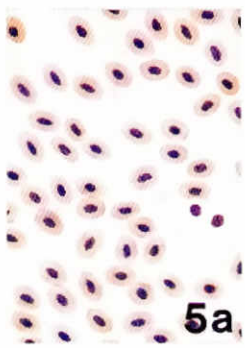
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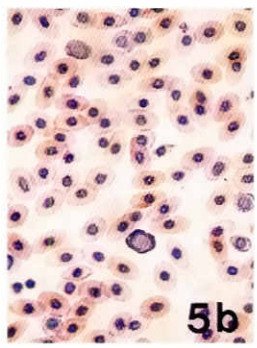
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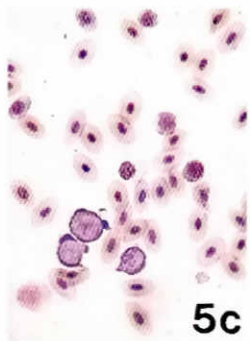
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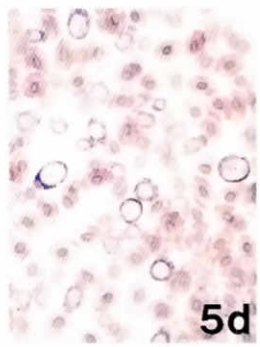
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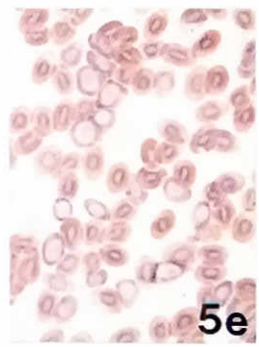
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TABLE 2

TUMOR INCIDENCE IN CHICK EMBRYOS DECAPITATED AT E2 AND INFECTED WITH MC29 AT E3

number of embryos	Age at sacrifice (days) E14-15	E17	E18	E19
total	7	20	19	12
with heart tumors	2 (28%)	16 (80%)	16 (84%)	12 (100%)
with cartilage nodules	0	8/16 (50%)	15/16 (94%)	12 (100%)
with skin anomalies	1 (14%)	15 (75%)	15 (79%)	12 (100%)

4a and 4b). These nodules were seen as patches of 13F4-negative cells inside the 13F4-positive tumor cells, and were stained blue when alcian blue was applied. Alcian blue labels the chondroitin sulfate extracellular matrix deposited by the chondrocytes. Interestingly, 100% of the decapitated embryos that reached E19 bore heart tumors with cartilage nodules (Table 2). Among non-decapitated embryos injected with v-*myc* or with MAHEVA, only 70% developed heart rhabdomyomas (Bachnou *et al.*, 1991; Al Moustafa *et al.*, 1992). None of the E3 embryos bearing functional c-erbAα injected with MC29 developed a cartilage nodule in the rhabdomyoma (studied on sections or *in toto* preparations of tumoral hearts stained with alcian blue, Bachnou *et al.*, 1991, and data not shown).

In the decapitated quail, heart rhabdomyomas and anomalous feathers were observed (Table 4) but cartilage nodules were not found. Again these observations are parallel to those after MAHEVA infection in this species. No thiourea protocol was carried out in the quail embryo.

Finally we wish to mention that all these treatments have an important incidence on the blood of the embryos. Avian Erythroblastosis Virus (AEV) infection of early embryos, which was lethal before E15, induced the expected appearance of erythroblasts (Fig. 5b, compare with normal embryos, Fig. 5a). A highly abnormal blood picture was found in 30% of MAHEVA infected embryos that bore heart tumors with cartilage nodules (Fig. 5c). The numerous blastic cells in the blood of these embryos or of hypothyroid embryos infected with v-*myc* (Fig. 5d and 5e) are not identical to those in AEV infected embryos (Fig. 5b). Their larger size and numerous pseudopods indicate a very immature myeloid character rather than an erythroleukemic phenotype. The frequency of thiourea-treated/v-*myc* infected embryos affected with this blood anomaly is two-fold increased as compared to MAHEVA infected embryos (Table 5).

Discussion

In this paper we have shown that the ablation of thyroid development in chicken embryos by either of two techniques, reproduces an effect that we have previously documented with the viral oncogene v-*erb*A: it mediates the transdifferentiation of v-*myc*-transformed cardiomyocytes into cartilage cells. We also observed that v-*erb*A or thyroidectomy both cooperate with v-*myc* to induce the proliferation of blood progenitors in chick embryos. In the quail, v-*erb*A is unable to cooperate with v-*myc* to induce the transdifferentiation event (Bachnou *et al.*, 1991) and we observed that thyroidectomy did not modulate v-*myc* transforming properties. Differences in chick and quail susceptibility to various viruses transforming properties have been previously reported (Graf *et al.*, 1979; Biegalke *et al.*, 1987; Al Moustafa *et al.*, 1992). For example, the hemopoietic tumors induced by the *myb-ets-myc* combination developed in the chick embryo are never observed in the quail, even if both species exhibit similar heart rhabdomyomas (Al Moustafa *et al.*, 1992). The molecular basis of this different sensitivity is still unknown.

v-*erb*A alone is incapable of transforming cells, either *in vivo* or *in vitro,* and its contribution to oncogenesis becomes apparent only when it is linked to another oncogene, or associated with a growth factor (Gandrillon *et al.*, 1987; Garrido *et al.*, 1993; Iglesias *et al.*, 1994). This is seen in the case of AEV (Sealy *et al.*, 1983) or co-infection experiments between v-*erb*A and v-*Ha ras* or several tyrosine kinases (v-*sea*, v-*fps*, v-*src*) (Kahn *et al.*, 1986). V-*erb*A was shown to arrest the differentiation programme of erythroid precursors at the CFU-E stage (Gandrillon *et al.*, 1989). In the absence of a secondary oncogene this arrest, affecting only a proportion of erythroid precursors, has no detectable effect at the level of the organism. Added tyrosine-kinase activity ensures the emergence of leukemia by promoting the overmultiplication of the maturation-arrested precursors. The differentiation arrest was expected to be due to the antagonistic effect played by v-*erb*A on c-*erb*Aα, one of the thyroid hormone receptors (Damm *et al.*, 1989; Sap *et al.*, 1989). However, it has also been proposed that v-*erb*A may act in neoplasia by promiscuously interfering with a retinoid-mediated differentiation process (Desbois *et al.*, 1991; Sharif and Privalsky, 1991; Chen and Privalsky, 1993). In post-hatching animals, the v-*myc* oncogene is able to transform myeloid cells (Graf and Beug, 1978) but is, by itself, without effect on erythroblast differentiation (Graf *et al.*, 1980). In embryos, hemopoietic precursors accumulate in the blood when v-*erb*A or thyroid hormone deprivation is added, suggesting that repression of the c-*erb*Aα gene and presence of the v-*myc* protein cooperate in inducing transformation *in vivo.* It is not surprising however that cooperation between v-*erb*A and v-*myc* induces the appearance of a different blood cell pheno-

TABLE 3

TUMOR INCIDENCE IN MC29 INFECTED, THIOUREA-TREATED CHICK EMBRYOS

number of embryos	Age at sacrifice (days) E15	E17	E18	E19
total	8	15	12	16
with heart tumors	0	10 (66%)	10 (83%)	15 (94%)
% with cartilage nodules	0	6/10 (60%)	7/10 (70%)	11/15 (73%)
with skin anomalies	0	11 (73%)	10 (83%)	14 (87%)

type than v-*erb*A with v-*erb*B. It is to be expected that maturation should be arrested at two distinct stages by these different oncogene combinations.

In the absence of its ligand, the c-*erb*Aα product exerts a negative regulatory effect on T3-responsive-genes. This feature provides an explanation for our observation that the induction of a state of hypothyroidectomy substitutes for the action of v-*erb*A as a mediator of the cooperation effect with v-*myc* in the cartilaginous transdifferentiation of the transformed cardiomyocyte. However, v-*erb*A also recognizes a different DNA sequence than c-*erb*A, this difference being determined by amino acids outside the zinc finger domain (Chen *et al.*, 1993). Since thyroid hormone deprivation replaces v-*erb*A in the cartilage induction, this indicates that transcriptional repression of c-*erb*A regulated genes is involved in this event. It is thus possible that negative regulators of cartilage-inducing genes are turned off after thyroid hormone deprivation. It remains to be understood whether this induced transdifferentiation effect involves RA-receptors or not. Such a mechanism may have been envisaged since RA is known to be critically involved in heart developmental processes (Kastner *et al.*, 1994) and v-*erb*A or unliganded c-erbAα has been shown to interfere negatively with the RAR responses, either by competing with RAR's or by forming inactive heterodimers with RAR and RXR (Barettino *et al.*, 1993; Chen and Privalsky, 1993; Yen and Chin, 1994). Alternatively some reports show that, depending on the TRE target, in the absence of its ligand, the c-*erb*Aα (Saatcioglu *et al.*, 1993) or c-*erb*Aβ (Hollenberg *et al.*, 1995) products exert a positive regulatory effect on T3-responsive-genes. Therefore, it remains possible that, instead of a negative repression, a direct activation of cartilage-inducing genes could lead to the transdifferentiation phenotype.

The way in which v-*erb*A or thyroid hormone deprivation cooperate with v-*myc* to mediate transdifferentiation of v-*myc* transformed heart myocytes (or myoblasts) is not known. In all cases, the development of cartilaginous nodules was significantly delayed with reference to the appearance of the rhabdomyomas. This sequence of events was unchanged, irrespective of the way in which transdifferentiation was achieved (i.e. by the action of v-*erb*A or by ablation of thyroid function) or whether infection with v-*myc* preceded or followed the induction of hypothyroidism. The blockade of the thyroid hormone response thus appears to be a late factor in the transdifferentiation effect. Thyroid hormone receptors play an clear role in heart development: the level of *c-erbAα* transcripts increases 6-fold between days 9 and 19 of chick embryonic development (Forrest *et al.*, 1990). Thyroid hormone modulates the developmental transitions between myosin heavy chain isoforms (Izumo *et al.*, 1986). Thus, a critical event in heart differentiation may be missing after hormone deprivation and under the mitogenic pressure of v-*myc* a shift of differentiation may take place in the transformed myocytes. Among the potential cartilage-inducing genes that may be induced are the genes encoding the bone morphogenetic proteins (BMP), able to induce a chondrogenic transdifferentiation from myoblasts or de-differentiated myocytes (Kawamura and Urist, 1988) or the factor produced by the notochord and the floor plate, able to inhibit the differentiation of the dorsal somitic derivatives, converting the entire somite into cartilage (Pourquié *et al.*, 1993).The involvement of thyroid hormone in the regulation of these genes remains to be tested.

TABLE 4

TUMOR INCIDENCE IN DECAPITATED, V-MYC INFECTED QUAIL EMBRYOS

number of embryos	Age at sacrifice (days) E9-10	E14	E16	E19
total embryos	9	14	19	17
with heart tumors	0	8 (57%)	14 (74%)	17 (100%)
with cartilage nodules	0	0	0	0
with skin anomalies	4 (44%)	10 (71%)	16 (84%)	17 (100%)

TABLE 5

BLOOD ANOMALIES IN CHICK EMBRYOS WITH CARTILAGE NODULES IN HEART RHABDOMYOMAS

MAHEVA (E3)	MC29 (E3) Thiourea (E7/E10) +	Decapitation (E2)
9/30* (30%)	15/23 (65%)	12/33 (36%)

*Number of embryos with blood anomaly/number of examined embryos with normal blood

Materials and Methods

Embryos

Outbred Leghorn chicken and quail eggs were incubated until the stages required, when a window was opened in the shell.

Viruses

Supernatant was obtained from quail embryo cells (QEC) transfected with pMC38, (Vennström *et al.*, 1981) a molecular clone of MC29 (20 μg), or MAHEVA, the v-*erb*A, v-*myc* containing virus (Bachnou *et al.*, 1991) and pRAVI DNA (10 μg). 10 μl of the transformed QEC supernatant was injected into the heart or the coelomic cavity of E3 embryos before or after the induction of hypothyroidism, depending on whether a surgical or chemical procedure was used.

Neural tube transplantation

The operation was performed on embryos with 7 to 10 pairs of somites, both for the chick host and the quail donor, according to the technique described by Le Lièvre and Le Douarin (1975). The transplants involved the level from the anterior limit of the rhombencephalon to the level of the 5-6th pair of somites.

Ablation of thyroid function

Two procedures were used. In the first, embryos that had been incubated for 36 h were decapitated surgically according to the method of Fugo (1940). The telencephalon and half of the mesencephalon were severed to yield, in successful cases, embryos without eyes, upper beaks, or hypophyses. The second procedure involved two injections of 0.5 ml of a solution of thiourea (0.07 M CS NH_2 in 0.05 M NaCl) into the yolk sac at E7 and E15 (Grossowicz, 1946; Adams and Bull, 1949; Tixier Vidal, 1958; Daugéras-Bernard and Lachiver, 1983).

Histology and immunocytology

Hearts retrieved from experimental or control embryos were cut into 5 µm sections and double stained with monoclonal antibody 13F4, which is specific for the myogenic lineage (Rong *et al.*, 1987) or MF20 that recognizes the sarcomeric myosin heavy chain (Bader *et al.*, 1982) and with Hoechst nuclear stain 33258 (Serva). Mab binding was revealed with a goat antimouse antibody coupled to fluorescein isothyocyanate (Biosis). Alternate sections were stained with alcian blue or glychemalaun-picroindigocarmine (Gabe, 1968). Some hearts were stained *in toto* with alcian blue according to Simons and Vanhorn (1970). Hearts from neural crest chimeras were stained with the Feulgen-Rossenbeck technique (Gabe, 1968).

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