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Original Article

Lack of correlation between c-*myc* expression and programmed or experimentally-induced cell death during chick limb development

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ABSTRACT The protooncogene c-*myc* positively regulates cell death in most *in vitro* cell model systems under conditions of restricted proliferation, and it has been proposed that cell death is a physiological function of c-*myc*. To investigate possible changes in c-*myc* expression concomitant with programmed cell death, we have analyzed c-*myc* mRNA expression during chick limb development. Expression of c-*myc* was high in the premuscular masses at earlier stages and in the chondrogenic condensations at later stages of limb development, as demonstrated by *in situ* mRNA hybridization in sections and whole-mount. However, we did not detect c-*myc* expression in specific limb areas where massive apoptosis was occurring. Furthermore, when the apical ridge was removed from stage 20 wing buds, there was no increase in the expression of c-*myc* in the subridge mesoderm, despite the extensive cell death induced by this surgical manipulation. Therefore, our results show no correlation between elevated c-*myc* expression and either programmed or experimentally-induced apoptosis in the developing limb. These results argue against a role of c-*myc* in cell death occurring *in vivo*.

KEY WORDS: *c-myc, programmed cell death, apoptosis, limb development, chick embryo*

Introduction

Cell death is now widely considered as a crucial event during the process of development. Development of many organisms and systems takes place concomitantly with the occurrence of programmed massive apoptosis. Recently the study of cell death has been widened with the discovery of specific genes implicated in the death process (see review by Hurle *et al.*, 1995).

c-*myc* has been shown as one of the best characterized apoptosis-promoting genes. c-Myc is a nuclear protein that has long been implicated in the control of cell growth (Marcu *et al.*, 1992; Evan and Littlewood, 1993). Paradoxically, it has also been demonstrated that c-*myc* overexpression induces cell death under conditions of restricted cell proliferation. Examples of this are serum deprived fibroblasts (Evan *et al.*, 1992), T cell hybridomas exposed to anti-TCR antibody (Shi *et al.*, 1992), Burkitt tumor cells (Milner *et al.*, 1993), murine myeloid cells such as 32D deprived of IL-3 (Askew *et al.*, 1991) and M1 cells treated with cycloheximide or transforming growth factor-β1 (Lotem and Sachs, 1993; Selvakumaran *et al.*, 1994). To explain these results, it has been proposed that apoptosis is a physiological activity of c-Myc but normally inhibited by growth factors or by the expression of survival genes such as *bcl-2* or *bcl-*X_L (reviewed in Harrington *et al.*, 1994b; Amati and Land, 1994).

Cell death occurs in c-*myc*-transfected cell lines cultured without serum or growth factors, while in these conditions endogenous c-*myc* expression is down regulated (Amati and Land, 1994). However, it is yet unclear whether c-*myc* plays a role in programmed cell death occurring *in vivo* under physiological conditions. A broadly used model of developmentally-programmed cell death in vertebrates is the development of the chick limb. For example, the formation of the individual digits is accompanied by massive cell death in the interdigital spaces, sculpturing in this way the shape of the hand or foot (Saunders *et al.*, 1962; Hurle *et al.*, 1995).

Several studies have been reported on the distribution of c-*myc* expression during mouse and chicken embryogenesis. Elevated c-*myc* expression is observed in rapidly proliferating tissues and particularly in epithelial cells in the mouse (Downs *et al.*, 1989; Schmid *et al.*, 1989; Morgenbesser *et al.*, 1995). During avian development c-Myc protein was detected mainly in mesenchyme-derived cells (Jaffredo *et al.*, 1989). Expression of c-*myc* has also been reported in cells undergoing hemopoiesis in both extraembryonic and intraembryonic sites (Vandenbunder *et al.*, 1989). During feather germ development, c-*myc* expres-

Abbreviations used in this paper: ANZ, anterior necrotic zone; PNZ, posterior necrotic zone; OP, opaque patch; INZ, interdigital necrotic zones.

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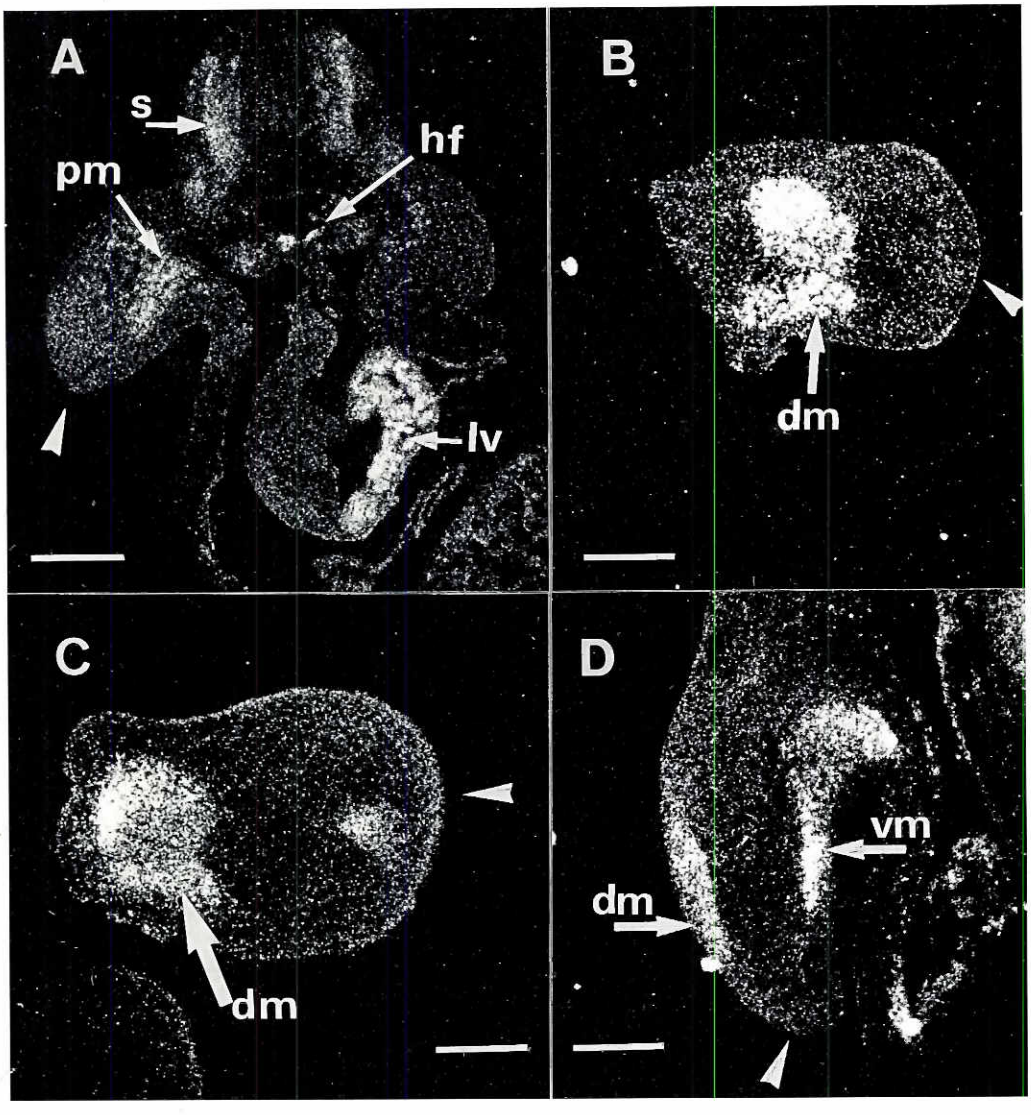

Fig. 1. Expression of c-*myc* during early stages of limb development. *The hybridization with the sense probe did not give signal over background.* **(A)** *Transverse section through a stage 20 embryo at the level of the wings. High expression of c-myc is seen in the premuscular limb masses (pm), in the somites (s), in the liver (lv) and in the hemopoietic foci of the aortic wall (hf).* **(B and C)** *Frontal sections through stage 23 and 24 limb buds, respectively. The high expression of c-myc in these sections corresponds to the localization of the dorsal premuscular mass (dm). The distal-middle expression of c-myc in the limb showed in* **(C)** *corresponds to the most distal part of the dorsal premuscular mass.* **(D)** *Longitudinal section through a stage 25 wing bud (transverse section through the embryo) where the expression of c-myc is clearly seen in the dorsal (dm) and ventral (vm) premuscular masses. In all the photographs the distal tip of the limb is indicated by an arrowhead. Bars: A,B,C, 300 μm; D, 500 μm.*

sion becomes associated with proliferation of dermal and epidermal cells (Desbiens *et al.*, 1991). However, none of the studies above were designed to analyze a possible correlation of c-*myc* expression and programmed cell death.

Here we have used the developing chick limb bud as a system to analyze the possible role of c-*myc* in programmed cell death. The chick embryo has the advantage of its easy accessibility to experimental manipulation in addition to being one of the best known developmental systems. Four areas of massive mesodermal cell death during development of the chick limb have been spatially and temporally well defined (Saunders and Fallon, 1966; see Hinchliffe, 1982). These areas are named the anterior necrotic zone (ANZ, located at the anterior margin of the bud), the posterior necrotic zone (PNZ, located at the posterior margin of the bud) and the opaque patch (OP, located between the two chondrogenic condensations of the skeletal pieces of the zeugopodium). During later development, the zones of apoptotic mesodermal cells located between the digits are called the interdigital necrotic zones (INZ). It should be noted that in these areas (despite their name given before the introduction of the term apoptosis) the dying cells show the typical morphological features of apoptosis (Zakeri *et al.*, 1993; Hurle *et al.*, 1995). Furthermore, internucleosomal DNA fragmentation, the biochemical hallmark of apoptosis, has been reported to occur in the INZ (Garcia-Martinez *et al.*, 1993; Zakeri *et al.*, 1993; Toné *et al.*, 1994; Mori *et al.*, 1995). In the present work we have performed *in situ* hybridization to detect a possible association between c-*myc* expression and the establishment of any of these areas of cell death during limb development.

To further analyze the possible relationship between cell death and c-*myc* gene expression, we have experimentally induced patterned cell death in the chick limb bud by the removal of the apical ectodermal ridge. The apical ectodermal ridge is the specialized epithelium rimming the tip of the limb bud and is indispensable for the proximodistal elongation of the bud (Saunders, 1948). The apical ridge is also necessary for subridge mesoderm survival. If it is removed from stage 18 to 22

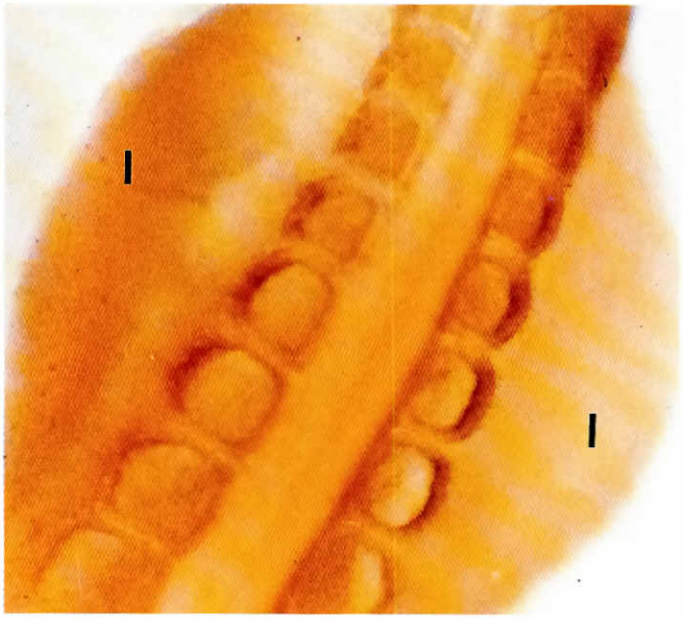

Fig. 2. Expression of c-*myc* in the somites. *Whole-mount in situ hybridization of a stage 19 embryo at the level of the leg buds. Note the elevated expression of c-myc in the lateral half of the somite. Abbreviations: l, leg buds.*

limb buds, the underlying mesoderm undergoes extensive cell death peaking at about 8 h after ridge removal (Rowe *et al.*, 1982).

Our results showed that naturally programmed and experimentally induced cell death occurred without an elevation in the level of expression of c-*myc* indicating that c-*myc* is not likely to be involved in apoptosis during chick limb development.

Results

We first analyzed the pattern of expression of c-*myc* during limb bud development from stage 17 to 33 (Hamburger and Hamilton, 1951) by *in situ* hybridization of tissue sections and whole-mounts. During the stages of early limb bud development c-*myc* was expressed in areas where the premuscular limb masses are developing (Fig. 1), as previously described (Jaffredo *et al.*, 1989). Clumps of cells expressing c-*myc* in the limb buds were first detected at stage 17 (data not shown). In addition, c-*myc* expression was detected in the somites (sclerotome and myotome, Fig. 1A) and at very high levels in the hemopoietic foci of the aortic wall and in the liver (Fig. 1A).

During stages 23 to 26 there are three areas of cell death in the wing bud (Hinchliffe, 1982). The ANZ appears at stage 23 as an area of apoptotic cells in the anterior border of the wing bud. The analysis of serial frontal sections through stage 23 wing buds demonstrated that c-*myc* transcripts exclusively colocalized with the premuscular masses. However, no c-*myc* expression was detected in the anterior border of the limb where massive apoptosis was occurring (Fig. 1B). During stage 24 apoptosis in the PNZ reaches its maximum. Again, the analysis of c-*myc* expression during this and adjacent stages failed to detect any increase in the level of c-*myc* prior to or coincident with the establishment of the PNZ (Fig. 1C). Finally, Figure 1D shows a longitudinal section through a stage 25 wing bud hybridized with the c-*myc* probe. The location of the dorsal and ventral muscular masses of the limb are clearly marked by the elevated expression of the c-*myc* gene. The establishment of the OP, a central region of cell death that spans from stage 24 to 26, was not accompanied by increased expression of c-*myc*.

Therefore, c-*myc* RNA was detected in the muscle precursors of the limb bud. Consistently, the expression of c-*myc* was also detected in the lateral half of early somites at the level of the limbs (Fig. 2). This is the region of the somite from where it has been shown that the limb muscle precursors derive (Ordahl and Le Douarin, 1992). Later, after the formation of the dermomyotome the expression of c-*myc* appeared mainly restricted to its extreme dorsomedial margin where the first myotome cells are localized (Christ and Ordahl, 1995).

At later stages of limb development c-*myc* expression was found in the chondrogenic condensations as shown in Figure 3

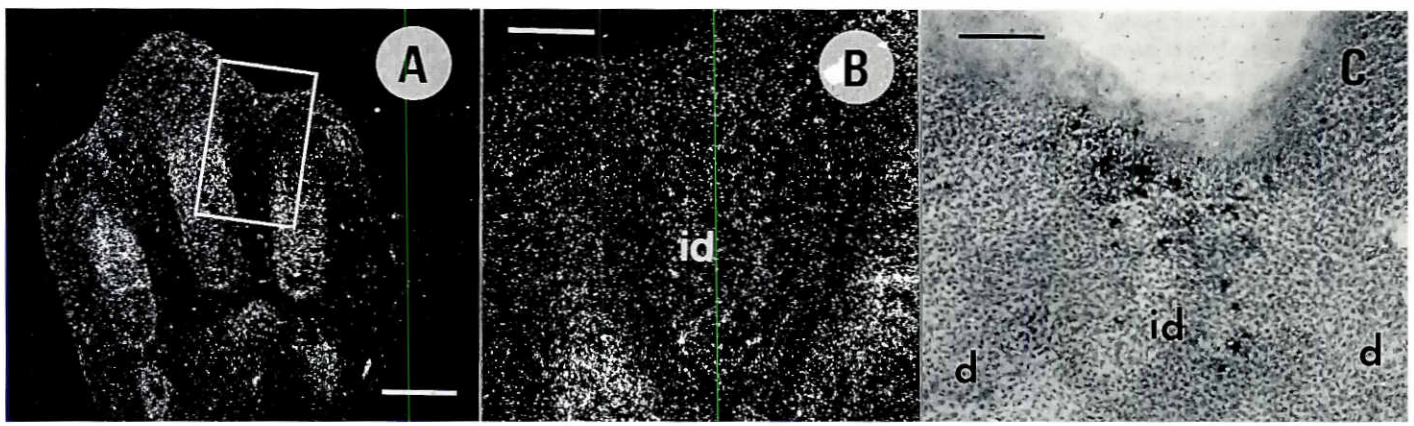

Fig. 3. Expression of c-*myc* in a stage 31 leg bud. (A) *Dark field image of the hybridization with the c-myc probe. Note the higher expression of c-myc in the chondrogenic digital rays.* **(B)** *Detailed view of the area squared in* **(A)** *showing that the level of c-myc expression is at background levels in the interdigit.* **(C)** *shows a neighboring section where the in situ end-labeling technique permits the detection of several apoptotic cells in the interdigital space. Abbreviations: id, interdigit; d, digit. Bars: A, 300 μm; B,C, 150 μm.*

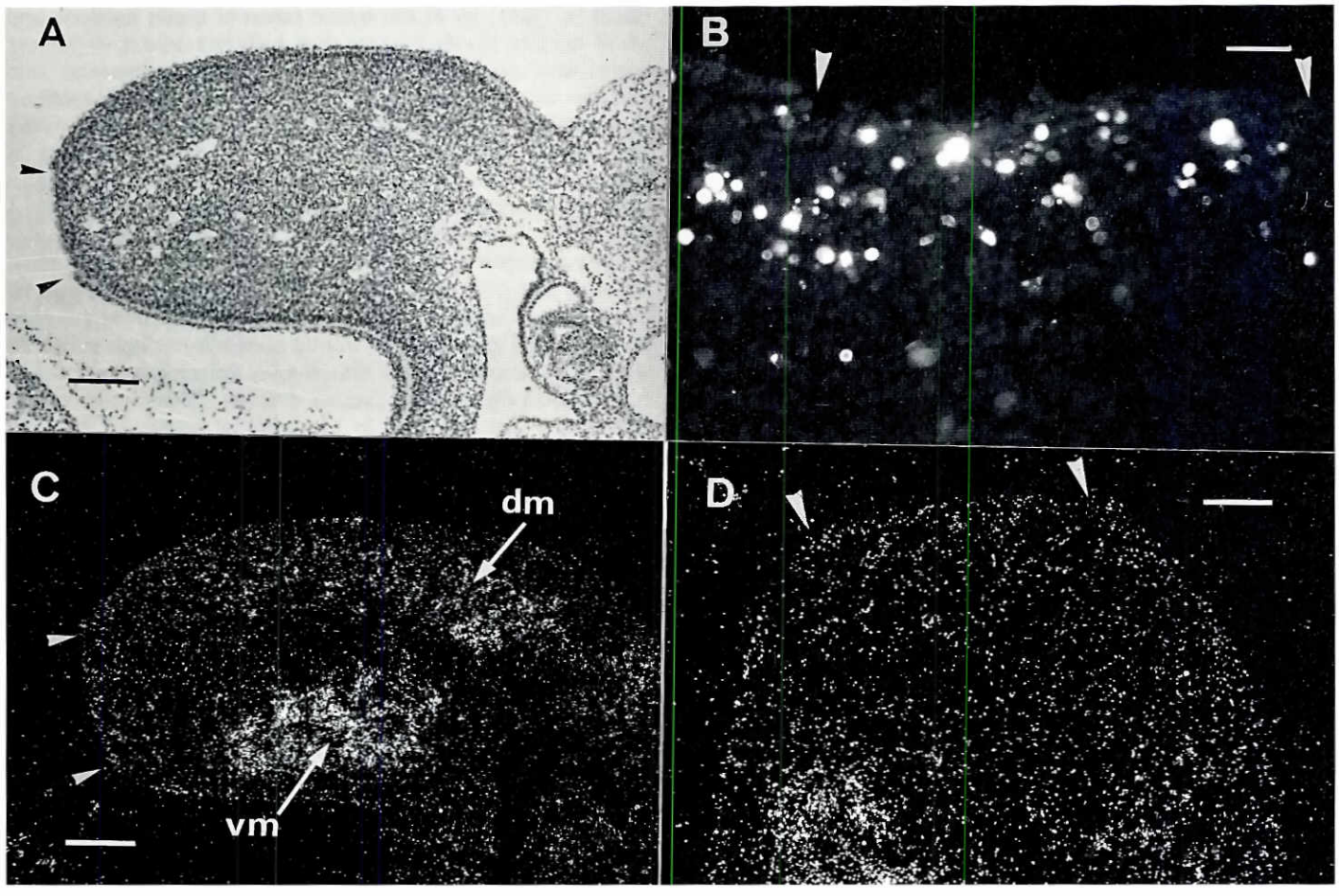

Fig. 4. Cell death induced after ridge removal is not accompanied by increased levels of c-*myc* expression. (A and C) *Bright and dark views of the same section of a wing bud 8 h after the removal of the apical ridge at stage 20. c-myc expression is detected in the dorsal (dm) and ventral (vm) premuscular masses.* **(B)** *In situ DNA end-labeling of the subridge mesoderm after ridge removal. Several apoptotic cells are clearly detected by fluorescein labeling. The detailed view of the hybridization for c-myc* **(D)** *fails to detect any increase in the expression level of this gene in the region of induced apoptosis. The arrowheads delimit the ectodermal region from where the ridge was removed. Bars: A,C, 100 μm; B, 30 μm; D, 50 μm.*

for a stage 31 leg bud. At this and subsequent stages, the INZ develop while the expression of c-*myc* remained at background levels in the interdigital areas. Figure 3B and C shows two consecutive serial sections through the second interdigital space, one hybridized with c-*myc* probe (Fig. 3B) while in the other the presence of apoptotic cells was evidenced by the *in situ* detection of DNA fragmentation. The comparison between these two images indicates that c-*myc* is not expressed in the INZ, above our detection level.

The former results indicated that c-*myc* distribution of expression did not prelude nor coincide with the known areas of cell death occurring during chick limb bud development.

Next we investigated c-*myc* expression during the experimentally-induced cell death caused by the removal of the apical ridge. The expression of c-*myc* in the subridge mesoderm was analyzed periodically from 4 to 12 h after the operation. Our results showed that cell death induced after the removal of the apical ridge was not accompanied by an increase in the level of expression of c-*myc*. A longitudinal section through a limb bud 8 h after the removal of the apical ridge at stage 20 is shown in Figure 4A. Note the absence of the apical ectodermal ridge at the tip of the bud, as marked by the arrowheads. Extensive cell death in the subridge ectoderm after ridge removal was confirmed by morphological analysis after hematoxylin-eosin staining (not shown). Furthermore, *in situ* labeling confirmed DNA fragmentation in the dying mesodermal cells (Fig. 4B). However, the hybridization with c-*myc* probe showed that the expression of this gene was not above background in the region undergoing massive cell death (Fig. 4C and D). The high expression of c-*myc* in the premuscular masses was not affected by the experimental manipulation (Fig. 4C). Therefore, mesodermal cell death resulting after ridge removal is not associated with an increase in c-*myc* mRNA expression

Discussion

The present work was aimed to investigate a possible relationship between c-*myc* expression and cell death during the development of the chick limb bud. Two different models of cell death were investigated: the normally occurring areas of pro-

grammed cell death and the ectopically induced cell death after ridge removal.

c-*myc* expression was below the detection level of our assay in the normally occurring areas of cell death during limb development. The cells in these areas die by apoptosis as shown by different criteria as morphology, *in situ* end labeling of the fragmented DNA and internucleosomal DNA fragmentation (Garcia-Martinez *et al.*, 1993; Zakeri *et al.*, 1993; Toné *et al.*, 1994; Mori *et al.*, 1995). Consequently it seems that apoptosis during limb development does not involve c-*myc* expression.

During early limb development the expression of c-*myc* was coincident with the establishment and evolution of the premuscular masses. The expression of c-*myc* in the muscle precursors cells of the limb was already detected at the somite level and remained during the period of migration and assembly into the premuscular masses up to the beginning of muscle differentiation. Previous studies have shown that c-*myc* can block myogenic differentiation (Miner and Wold, 1991). However, the close association between c-*myc* expression and the precursors of the limb muscle makes this gene a good marker of the myogenic lineage during limb development.

During later limb development c-*myc* was expressed in the chondrogenic condensations (Jaffredo *et al.*, 1989 and our data). This result is in agreement with data obtained with growth plate chondrocytes *in situ*, where higher expression of c-*myc* was found in differentiated rather than in proliferating chondrocytes (Farquharson *et al.*, 1992). So, chondrogenic cells constitute an example where high c-*myc* expression is maintained despite terminal differentiation.

c-*myc* overexpression only induces cell death *in vitro* when cells are deprived of growth factors or exposed to anti-proliferative cytokines (Amati and Land, 1994). However, growth factor deprivation in cell culture is a rather artificial condition unlikely to occur *in vivo*. Here we have tried to reproduce this situation by removing the apical ridge. The ridge maintains the subridge mesoderm in a rapidly proliferating, undifferentiated stage, forming the so-called "progress zone". Its surgical removal during stages 18 to 20 is followed by a massive cell death in the subridge mesoderm (Rowe *et al.*, 1982). This ectopically induced cell death is not accompanied by any detectable increase in the level of c-*myc* expression. It is unknown whether the induced cell death after ridge removal involves the same molecular mechanisms as physiological programmed cell death. Here we have shown that the dying cells can be detected by *in situ* end-labeling of the fragmented DNA indicating that this experimentally induced cell death has, at least, some features common to those involved in apoptosis. The probable cause of the subridge mesodermal death after ridge removal may be the sudden deprivation of growth signals produced by the ridge. We and others have shown that members of the fibroblast growth factor family (FGFs) are produced by the ridge and are able to substitute for ridge functions including the survival of the subridge mesenchyme (Niswander *et al.*, 1993; Fallon *et al.*, 1994). Also, triggering of cell death in the INZ appears to be dependent on the arrest in the local supply of FGFs associated with the physiological regression of the ridge (Macias *et al.*, 1995). However, FGF-2 was not among the growth factors able to rescue fibroblasts in culture from apoptosis induced by c-Myc (Harrington *et al.*, 1994a).

These results suggest that c-*myc* is not likely to be involved in the molecular mechanisms leading to programmed cell death in limb development. This is in contrast to its effects on many *in vitro* systems and does not preclude that c-*myc* may play a role in apoptosis in other embryonic cells. For instance, elevated expression of c-*myc* has been described during death of thymocytes in embryos and involution of rat prostate (Buttyan *et al.*, 1988; Riegel *et al.*, 1990). The different involvement of c-*myc* in the apoptotic models investigated so far may reflect a possible heterogeneity of cell death mechanisms depending on the subset of apoptosis-regulating genes expressed in each cell type.

Materials and Methods

Sample preparation

White Leghorn eggs were incubated at 38°C for 2 to 8 days. They were opened, staged according to Hamburger and Hamilton (1951) and fixed in 4% paraformaldehyde. The specimens were embedded in paraplast and serially sectioned (6 μm).

Apical ectodermal ridge removal

The apical ectodermal ridge was removed from the right wing bud of stage 18-22 embryos with a sharpened tungsten needle. After the operation, the eggs were sealed with tape and returned to the incubator for a variable period of 4 to 12 h. The embryos were processed as above.

In situ hybridization

Sense and antisense riboprobes containing the third exon of the chicken c-*myc* gene were used (Desbiens *et al.*, 1991). The hybridization of tissue sections was performed essentially as described in Wilkinson and Nieto (1993). For whole-mount *in situ* hybridization we followed the procedure of Nieto *et al.* (1995).

In situ detection of DNA fragmentation

To detect nuclear DNA breaks two methods of *in situ* end-labeling were used. In the first method, end-labeling was performed with digoxigenin-dUTP (Boehringer-Mannheim) following the procedure described by Edwards and Tolkovsky (1994), but using T7 DNA polymerase (10 U per ml of reaction mixture; Pharmacia). In the second method, we used fluorescein-dUTP and terminal transferase as labeling enzyme ("In situ cell death detection kit, Fluorescein", Boehringer-Mannheim).

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