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GEORGE KLEIN

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INTERACTION OF RETROVIRAL ONCOGENES WITH THE DIFFERENTIATION PROGRAM OF MYOGENIC CELLS

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I. Introduction

It is generally agreed that *in vitro* transformation of differentiating cells is frequently accompanied by the appearance of two remarkable events, namely, an altered control of proliferation and perturbations in the expression of a given cell-specific differentiation program. The loss of growth control after transformation with oncogenic viruses has been studied mostly in fibroblastic cells, i.e., mammalian established cell lines or early passage avian and rodent fibroblasts. These studies have allowed the identification of several properties of transformed cells that distinguish them from their normal counterparts. On the other hand, the relationship between transformation and expression of differentiated traits has remained an elusive and poorly understood phenomenon. The reasons for the slow progress in this field can be attributed to the complexity of the regulatory mechanisms underlying tissue-specific gene expression, to the heterogeneity in the coordinate regulation of proliferation and terminal differentiation exhibited by different cell types, and to the existence of more than one mechanism of transformation by the oncogenic agents utilized. Indeed, the formulation of relevant hypotheses and their experimental verification may be facilitated by the use of *in vitro* differentiating systems of relatively low complexity. Muscle cells are a particularly attractive model, in view also of the considerable background of information available (Pearsons and Epstein, 1982).

The aim of this article is to review recent data on the interference exerted by various retroviral oncogenes on the expression of the differentiation program of skeletal muscle cells, and, by comparison with the effects exerted on other "simple" systems, to subsume the various experimental observations into a more coherent framework. In the following sections our intention is to emphasize work performed with nonestablished cells using retroviruses as transforming agents, because differentiation of continuous cell lines may not be representative of the normal regulation of differentiated functions and transformation by other agents is not adequately efficient.

II. Myogenic Differentiation

Myogenesis certainly provides the best known model for studying the mechanisms governing the transition from a determined to an overtly differentiated state, defined at the molecular level by the activation and expression of cell type-specific gene products and, at the cellular level, by formation of tissue. All the principal features of myogenesis can be faithfully reproduced and studied *in vitro* (Fig. 1). Commitment of primitive mesenchymal cells to the presumptive myoblast stage (determination) is an event most likely happening in the embryo and still not fully characterized. The subsequent developmentally distinct stages can be readily recognized in essentially pure cultures of myogenic cells, established from avian embryos of various ages. Primary cultures derived from embryonic muscles consist of both cycling precursors and terminally differentiated, postmitotic muscle cells. Presumptive myoblasts are highly replicating cells, do not appreciably express any muscle-specific function or gene, with the possible exception of desmin (Devlin and Emerson, 1978), and their identity is recognized only retrospectively, when they terminally differentiate. Morphological differentiation is characterized by the acquisition of the unique competence to fuse into long, multinucleated syncytia called myotubes (Figs. 1 and 2). It is fundamental to stress here that a definitive withdrawal from the cell cycle, as suggested by Holtzer and co-workers (Stockdale and Holtzer, 1961; Holtzer *et al.*, 1975b), is now recognized as a *prerequisite* for fusion and expression of muscle-specific genes in both primary cells and mammalian myogenic cell lines (Nadal-Girard, 1978; Turner, 1978). During skeletal muscle differentiation all myogenic precursors are considered equivalent, and it is commonly accepted that they will differentiate into postmitotic muscle cells in response to environmental stimuli, such as low mitogen concentrations (Konisberg, 1977).

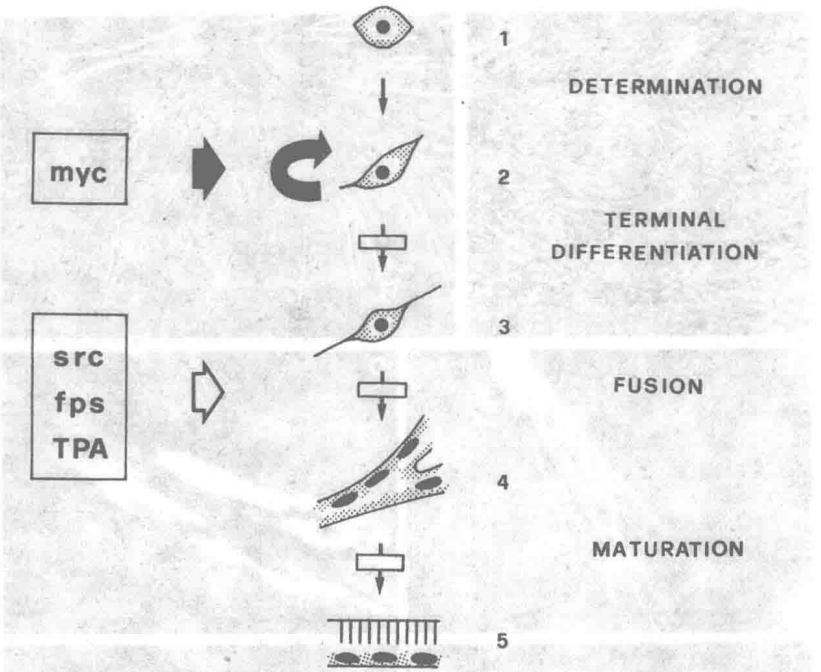

FIG. 1. A schematic representation of developmentally distinct stages of myogenic differentiation and putative steps affected by oncogenes and tumor promoters. 1, Primitive mesenchymal cell; 2, self-renewing presumptive myoblast; 3, postmitotic myoblast; 4, multinucleated myotube; 5, cross-striated myofiber; TPA, tetradecanoylphorbol acetate.

In terms of cell population the regulation of myogenesis is probably more complicated than it appears from the foregoing formalization. So, for instance, a simple inductive model (mitogen concentration-driven expansion of, or exit from, the postdetermination compartment of the lineage) (Fig. 1) does not account for the asynchronous time course of terminal muscle differentiation observed both *in vivo* and *in vitro*. Nor does it help to introduce a stochastic dimension to this model, with data derived from experiments with continuous cell lines, which, unlike nonestablished myogenic cells, can be induced to differentiate synchronously (Nadal-Girard, 1978; Linkhart *et al.*, 1981). Recent findings have been interpreted as consistent with the existence in the end phases of the chick myogenic lineage of a self-renewing stem cell that gives rise to a fixed number of committed precursor cells (Robinson *et al.*, 1984; Quinn *et al.*, 1985). The model raises the

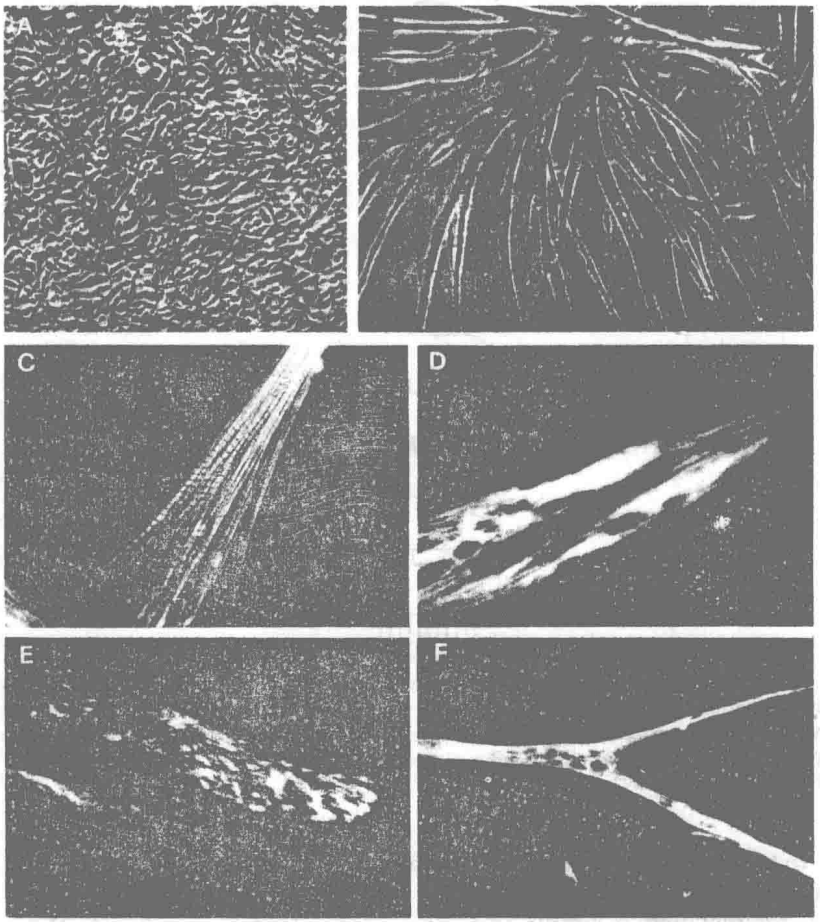

FIG. 2. Fusion and myosin expression in clonal strains of quail myogenic cells transformed by various oncogenes. (A,B) Phase contrast micrographs of *ts* RSV-transformed cultures grown at 35°C (A) or 41°C (B). (C) Direct immunofluorescence staining of muscle-specific myosin in *ts* RSV-infected cells grown at 41°C: phenotypically normal myotubes show extensive cross-striation. (D) *wt* RSV-transformed cells grown in differentiation medium at 37°C: atypical organization of myosin in a representative "revertant" myotube. (E, F) Myosin distribution in "revertant" myotubes from *wt* AEV- (E) and *td* 10H- (F) transformed cells grown in differentiation medium at 37°C.

possibility that genetic or cytoplasmic regulatory events controlling muscle gene expression in end-stage cells may occur at the stem cell level (Quinn *et al.*, 1985).

The irreversible exit from the proliferative state is followed by a several hundredfold increase in the coordinate synthesis of myofila-

ment proteins such as skeletal muscle actin, myosin, and tropomyosin (Devlin and Emerson, 1978) and in the activity of intracellular enzymes such as creatine phosphokinase (MM-CPK) and glycogen phosphorylase (Lough and Bishoff, 1977; Shainberg *et al.*, 1971). Acetylcholinesterase (AChE) and acetylcholine receptors (AchRs), which later will be used for neuromuscular transmission, are synthesized *de novo* and inserted at the cell surface (Vogel *et al.*, 1972; Fluck and Strohman, 1973). The available evidence indicates that this activation is based at the molecular level on the accumulation of specific mRNAs, which in turn is due to a transcriptional activation of specific genes (Caravatti *et al.*, 1982; Hastings and Emerson, 1984; Paterson and Eldridge, 1984) (see also Section V,B). The final step in the myogenic pathway is maturation: myotubes containing hundreds of nuclei start to exhibit cross-striations which are due to assembly of myofibrils and then to the building of sarcomeres (Figs. 1 and 2). Finally, after a few days in culture, myotubes become excitable and spontaneously contract. A review by Pearsons and Epstein, (1982) should be consulted for a more detailed account of myogenesis *in vitro*.

III. Properties of Avian Retroviral Oncogenes and Their Products

Transforming retroviruses represent excellent tools for the study of cell transformation *in vitro*. They carry in their genome specific sequences, known as oncogenes, that are responsible for tumor formation *in vivo* and cell transformation *in vitro*. Each of the dozen different oncogenes so far found in avian retroviruses cause tumors in only a limited set of tissues, while the same oncogenes show a much more relaxed pattern of selectivity *in vitro* (Graf and Beug, 1978; Weiss *et al.*, 1982). Recent findings imply that the mechanism of transformation by at least some retroviruses depicts with reasonable fidelity one of the steps involved in the conversion of normal cells to tumor cells during natural oncogenesis (Weinberg, 1985; Zarbl *et al.*, 1985). In addition, carefully characterized viral strains are available that permit an efficient and rapid en masse transformation *in vitro*, thus avoiding selection and cloning of rare transformants. The conditional temperature-sensitive (*ts*) and nonconditional transformation-defective (*td*) mutants available for many viral oncogenes enable a genetic approach to identifying the relationship between transformation and differentiation, not always feasible with other oncogenic agents. *ts* mutants appear particularly suited to this aim since they allow unambiguous identification of cellular phenotypes upon shift to the restrictive temperature. This approach can easily discriminate between a fully re-

versible altered control of differentiation, solely due to the transformed state, and more complex derangements in the control of specific gene expression that, beside the transforming event, are dependent on unknown cellular or environmental factors. The only caveat in the use of *ts* mutants is the residual presence of transforming ability at the restrictive temperature that may vary among different mutants as well as among different host cells, and thus generate potentially ambiguous results.

Retroviral oncogenes are derived from a restricted set of cellular genes christened *protooncogenes* (Duesberg, 1985; Bishop, 1985). The transduction of these cellular genes into the viral genome usually results in the loss of one or more viral structural genes and the appearance of deletions and point mutations in the captured genes. Genetic modifications and the acquisition of an autonomous regulatory system provided by the viral enhancer–promoter sequences contained in the long terminal repeats (LTRs) are believed to be the molecular mechanisms required to convert nontransforming protoncogenes into transforming oncogenes (Weiss *et al.*, 1985). Protooncogenes can also be activated to oncogenes while still residing in the cellular genome by such mechanisms as various as point mutation, translocation, enhancer–promoter insertion, and gene amplification (Klein and Klein, 1985). Cellular and viral oncogenes code for specific proteins that can be provisionally allocated into various groups according to their known biochemical properties and their intracellular localization. A summary restricted to avian isolates is shown in Table I (for a recent and comprehensive review, see Bishop, 1985).

The largest group includes oncogene products endowed with protein kinase activity that may be further divided into three groups:

1. Tyrosine-specific kinases, such as v-*src*, v-*fps*, and v-*yes*. v-*src*, the oncogene of Rous sarcoma virus (RSV), encodes a phosphorylated protein of M_r 60,000 denoted pp60$^{v\text{-}src}$ that has the intrinsic activity of protein kinase specific for tyrosyl residues (for review, see Weiss *et al.*, 1985). pp60$^{v\text{-}src}$ is myristylated at the NH_2 terminal and is localized at the plasma membrane of infected cells and at specialized structures such as adhesion plaques (Weiss *et al.*, 1985). v-*fps* is the oncogene of the Fujinami sarcoma virus (FSV). In this strain the oncogene is fused to a deleted viral structural gene, the *gag* gene, and this results in a fusion protein of M_r 140,000 known as P140$^{gag\text{-}fps}$ whose biochemical properties and intracellular location are very similar to those of pp60$^{v\text{-}src}$ (Moss *et al.*, 1984).

2. Tyrosine-specific kinases homologous to growth factor receptors

TABLE I
PROPERTIES OF AVIAN RETROVIRAL ONCOGENES[a]

Oncogene	Virus	Product	Localization	Function
v-*src*	RSV	pp60^{src}	Membranes	P-tyrosine kinase
v-*fps*	FSV	P140$^{gag-fps}$	Membrane/cytoplasm	P-tyrosine kinase
v-*ros*	UR2	P68$^{gag-ros}$	Membranes	P-tyrosine kinase
v-*yes*	Y73	P90$^{gag-yes}$	Membranes	P-tyrosine kinase
v-*erbB*	AEV	gp74^{erbB}	Membranes	Truncated EGFR/ P-tyrosine kinase
v-*sea*	S13	gp155^{sea}	Membranes	P-tyrosine kinase
v-*mil*	MH2	P100$^{gag-mil}$	Cytoplasm	P-serine kinase
v-*erbA*	AEV	P75$^{gag-erbA}$	Nucleus/cytoplasm	Thyroid hormone receptor
v-*myc*	MC29	P110$^{gag-myc}$	Nucleus	DNA binding
v-*myb*	AMV	P45^{myb}	Nucleus	DNA binding
v-*ski*	SKV	P110$^{gag-ski-pol}$	Nucleus	?
v-*ets*	E26	P135$^{gag-ets-myb}$	Nucleus	?

[a] For review, see Weiss *et al.* (1985).

such as v-*erbB*. Avian erythroblastosis virus (AEV) strain ES4 carries two separtate oncogenes, v-*erbA* and v-*erbB*. Genetic analysis has shown that the main transforming capacity resides in *erbB* and that *erbA* alone has no detectable effect (Frykberg *et al.*, 1983; Sealy *et al.*, 1983). v-*erbB* codes for a glycoprotein of M_r 74,000 known as gp74^{erbB} (Hayman and Beug, 1984) that represents a truncated version of the epidermal growth factor receptor (EGFR) (Downward *et al.*, 1984). Like the EGFR it has an intrinsic tyrosine-specific protein kinase activity (Kris *et al.*, 1985), and its localization to the plasma membrane is required for erythroid cell transformation (Beug and Hayman, 1984).

3. Cytoplasmic serine-specific kinases such as v-*mil* (Moelling *et al.*, 1984).

Other groups include nuclear proteins such as v-*myc*, v-*myb*, and v-*erbA*, which is a mutated form of the thyroid hormone receptor (Sap *et al.*, 1986). v-*myc* is the characteristic oncogene of various avian myelocytomatosis virus (AMV) strains, whose prototype is the MC29 strain. In the MC29 genome v-*myc* is fused to a deleted *gag* gene and thus encodes a fusion protein of M_r 110,000 denoted P110$^{gag-myc}$. v-*myc* encoded proteins, with or without fusion to *gag* peptides, are local-

ized mainly in the nucleus and are able to bind DNA *in vitro* (Moelling, 1985).

From a functional standpoint oncogenes may be classified according to their ability to cooperate in the tumorigenic conversion of early passage rodent fibroblasts. Full transformation of these cells requires the concerted action of two oncogenes possibly belonging to different complementation groups: nuclear or *myc*-like and cytoplasmic or *ras*-like oncogenes (Land *et al.*, 1983; Ruley, 1983). Avian cells, however, would appear to differ considerably from nonestablished rodent cells, as they are efficiently transformed by single members of either complementation group (see Section IV).

IV. Effects of Retroviral Oncogenes on Myogenesis *in Vitro*

It is almost a decade since it was independently reported (Holtzer *et al.*, 1975a; Fiszman and Fuchs, 1975) that primary cultures of chick embryo muscle cells could be transformed by RSV and that transformation prevented the formation of multinucleated myotubes. Instrumental for this discovery was the use of *ts*-mutants of v-*src*. Thus, at the permissive temperature (35°C) the majority of replicating transformed myogenic cells in standard culture medium fail to (1) withdraw from the cell cycle, (2) initiate the synthesis of muscle-specific products, (3) assemble striated myofibrils, and (4) fuse into multinucleated myotubes. However, when these cells are shifted to the nonpermissive temperature (41°C), many withdraw from the cell cycle, assemble myofibrils, and form multinucleated, spontaneously contracting myotubes (Fig. 2) which express muscle-specific proteins (Fiszman, 1978). It was concluded from these early studies that RSV does not irreversibly cancel, but only suppresses the muscle-specific terminal differentiation program and that this block is under the continuous control of the transforming oncogene.

A. Spontaneous Differentiation of RSV-Transformed Myoblasts

The above-mentioned data and the absence of biochemical differentiation at the permissive temperature (Moss *et al.*, 1979) were also interpreted as consistent with the notion of a stable block exerted by v-*src* on the *in vitro* differentiation of transformed myoblasts. However, single cell analysis of RSV-transformed myoblasts has shown the unequivocal presence of terminally differentiated, mono- and multinucleated cells in uniformly transformed cultures of *ts* RSV-infected

myoblasts (Tató *et al.*, 1983). The medium composition was found to play a role in allowing or preventing this spontaneous differentiation. Thus, upon cultivation in growth medium (GM), only a very small percentage (1–2%) of the population expresses a significant level of muscle-specific myosin; in sister cultures cultivated in differentiation medium (DM), a larger fraction (up to 20–30%) can differentiate and fuse into small, atypical myotubes that synthesize muscle-specific myosin, desmin, AChR, and MM-CPK (Tató *et al.*, 1983). We shall refer to these myotubes as "revertant myotubes," in order to distinguish them from bona fide myotubes arising from either uninfected cells or *ts* RSV-transformed cells at the restrictive temperature (Fig. 2). Although revertant myotubes express a muscle-specific gene repertoire qualitatively similar to that of normal cells, they exhibit peculiar characteristics: they often have a flattened, irregular shape with nuclei confined to a centrally located area of the sarcoplasm. Immunofluorescence studies show that myosin is not organized into the muscle-specific sarcomeric, striated myofibrils and is rather diffuse or preferentially located in the perinuclear region of the myotube (Fig. 2). It is possible to manipulate the system further, by using dimethyl sulfoxide (DMSO) or hexamethylenebisacetamide (HMBA), typical inducers of erythroid differentiation (Friend, 1979). These compounds prevent in a reversible manner spontaneous differentiation of wild-type (*wt*) RSV-transformed muscle cells promoted by DM. The inhibitory effect is not observed in uninfected muscle cells and is similar to that exerted by the same compounds on rat myogenic cell lines (Blau and Epstein, 1979).

The development of revertant myotubes is also observed in homogeneous cell populations represented by clonal strains of quail myoblasts transformed by *wt* RSV, selected for anchorage independence, the most stringent criterion for *in vitro* transformation (Kahn and Shin, 1979). The incidence of spontaneous differentiation in independent clones is variable, ranging from 5 to 50% of nuclei in revertant myotubes after cultivation in DM (Tató *et al.*, 1982; Falcone *et al.*, 1985). A comparison between two clones selected as representative of high and low tendency to differentiate indicates that the poorly differentiating clone expresses the RSV provirus better than the highly differentiating one, as suggested by a 3-fold higher level of $pp60^{v\text{-}src}$ kinase activity and a 6-fold higher production of progeny virus (S. Alemá and F. Tató, unpublished). Albeit limited this observation suggests a correlation between levels of transforming protein and proneness to spontaneous differentiation.

While it is clear that *ts* RSV-transformed myoblasts hardly express

biochemical markers of differentiation at the permissive temperature, these cells can still undergo two distinguishable processes of terminal differentiation: (1) upon shift to restrictive temperature the suppression of the transforming activity of $pp60^{v\text{-}src}$ allows the expression of the differentiation program in all the myogenic cells; (2) upon cultivation in DM at the permissive temperature, a minor but sizable fraction of transformed myogenic cells initiates the expression of the program by forming revertant myotubes that do not progress to acquire the functional capacity of muscle fibers. In conclusion, expression of v-*src* prevents differentiation in muscle cells by blocking the transition from the replicating precursor cell compartment to the terminally differentiated one. This block, however, is unstable and is sensitive to environmental cues; thus a variable proportion of the transformed population may escape this constraint and express its program.

The mechanism by which revertant myotubes arise remains largely obscure. The finding that they continue to express high levels of $pp60^{v\text{-}src}$ (S. Alemá and F. Tató, unpublished) rules out the possibility that they originate from cells that either have lost the provirus or selectively do not express the *src* gene. To a first approximation, spontaneous differentiation might be explained by postulating that *src*-transformed cells normally experience a transient attenuation of the transformed phenotype. During this postulated brief period of normalcy, myoblasts would withdraw from the cell cycle and commit themselves to terminal differentiation. Since proliferation and differentiation are antithetic processes in muscle cells, a transient event of this sort would be capable of generating a long lasting effect, which is likely to be underscored in other cycling cell types. A possible origin of revertant myotubes from a heterogeneity in the cell population prior to establishment of transformation seems to be ruled out by their presence in both uncloned and cloned transformed cells.

B. *ts* Mutants of *src*

Avian myogenic cells have been transformed by a variety of different transformation mutants that include *ts* LA24 (Holtzer *et al.*, 1975a; Falcone *et al.*, 1984), *ts* NY68 (Fiszman and Fuchs, 1975), *ts* LA25, *ts* LA29 (Moss *et al.*, 1979), *ts* LA30, *ts* LA32, *ts* LA33, *ts* GI201, *ts* GI251 (Tató *et al.*, unpublished), and clonal strains were derived from some of them (Montarras and Fiszman, 1983; Falcone *et al.*, 1985). In spite of scattered reports of residual transformed properties exhibited by some of these mutants at the restrictive temperature in fibroblasts (Wyke and Linial, 1973; Wyke, 1975; Weber and Friis, 1979), genetic