In the course of vertebrate development, totipotent cells in the early embryo yield cell types specialized for function in tissues. This process entails a progression of stages during which the fate of a cell is influenced by endogenous and exogenous factors. Soon after blastula formation, cells are generated for each of three distinct embryonic layers: endoderm, ectoderm and mesoderm. Eventually, an array of differentiated cell types emerges. Although the lineage, or progression, from totipotent cell to differentiated cell is not as well defined as in Drosophila or C. elegans, at some point a mammalian cell is destined to specialize along a given pathway, such as erythropoiesis or myogenesis. The final step, when tissue-specific genes are expressed, is often referred to as terminal differentiation.

How fixed and irreversible is the commitment of a differentiated cell? Perhaps the earliest suggestion that the destiny of a differentiated cell could be altered was provided by the experiments of Gurdon\(^1\). When nuclei from amphibian intestine were introduced into enucleated eggs, feeding tadpoles developed. However, since the frequency was relatively low, this dramatic change in nuclear function could have been characteristic of a subpopulation of cells, possibly residual stem cells. Later experiments by Gurdon et al.\(^2\) and DiBerardino and Hoffner\(^3\) ruled out this possibility by clearly showing that the nuclei of well-differentiated keratinocytes and even noncycling erythrocytes could display multipotentiality upon transplantation. Moreover, the frequency of obtaining feeding tadpoles was increased to 75% if the nuclei were initially injected into oocytes and conditioned by oocyte cytoplasm before transplantation into enucleated eggs (Ref. 4 and reviewed in Ref. 5). These experiments provided strong evidence that genetic material was not lost or permanently inactivated during vertebrate differentiation and that, in general, the specialized state of a cell was achieved by regulating the activity of its genes. The possibility remained that this degree of reactivation of dormant genes was contingent upon nuclei being exposed to a sequence of cues that accompanies the progression from undifferentiated zygote to specialized tissue.

Several types of experiments have confirmed that the differentiated state of a cell can be altered. Injury sometimes resulted in transdifferentiation, or the generation of one or more distinct cell types from a differentiated cell, for example upon regeneration of medusa striated muscle, axolotl limb, or lens tissue in several species\(^6-9\). When implanted outside the placenta in an ectopic site, an embryo often gave rise to malignant tumors, or teratocarcinomas (reviewed in Refs 10, 11); conversely, upon injection into blastocysts, cells derived from teratocarcinomas participated in normal embryogenesis and made a substantial contribution to the differentiated tissues of adult mice\(^12\). Finally, after exposure to 5-azacytidine, the fibroblast 10T1/2 cell line yielded three mesodermal cell types including myoblasts, adipocytes and chondrocytes\(^13\).

Since in each of these cases the changes in differentiation were accompanied by cycles of cell division, it seemed possible that DNA replication was a prerequisite. Thus, the results were compatible with the hypothesis proposed by Holtzer et al.\(^14\), which suggested that the decision to differentiate was regulated by a quantal mitosis, or critical cell division, that made available to transcription specific sets of genes that were previously inaccessible. In addition, the results could be explained by molecular models that suggested that cell specialization was achieved by stably repressing genes through protein–DNA complexes that could only be displaced by a round of DNA replication\(^15,16\). However, cell fusion studies have raised alternative possibilities. As described below, heterokaryon experiments indicate that the differentiated state of a cell can be altered in the absence of DNA replication or cell division. This plasticity is likely to result from a dynamic interaction of the combination of proteins the cell contains.

**Cell fusion studies indicate the role of trans-acting regulators in differentiation**

Cell fusion experiments provided a means by which two differentiated cell types could be combined so that the influence of one on the function of the other could be studied. Somatic cell hybrids yielded early evidence that mammalian gene expression could be altered by diffusible trans-acting regulators. Upon fusion with another cell type, the expression of differentiated functions frequently ceased (Ref. 17 and reviewed in Ref. 18). This repression could be reversed upon chromosome loss and, in some cases, was ascribed to specific genetic loci that act in trans\(^19,20\). Trans activation of genes was also reported\(^21-23\). Finally, studies with hybrids provided evidence that the malignant state is, in some cases, recessive to the differentiated state (Refs 24, 25 and reviewed in Ref. 26), a prediction that was recently confirmed at the molecular level for retinoblastoma\(^27\).

Although these hybrid experiments demonstrated that the activity of some tissue-specific genes could be regulated in trans, they did not address the question of whether the differentiated state of a cell was changed. This was not possible, since the hybrids were inherently unstable. Chromosomes were frequently lost and rearranged in the course of genetic selection.
Heterokaryons provide a stable cell fusion system for studying changes in differentiation

A heterokaryon system was developed that permitted an analysis of changes in gene activity in the context of the whole cell (Fig. 1). The key feature of this system was that it was stable for the duration of the experiment. There was no nuclear fusion or cell division after cell fusion; consequently, all genetic material remained intact within its own nucleus. The advantage of such short-term nondividing fusion products was that they made possible an assessment of the influence of two or more sets of cytoplasmic and nuclear components on gene expression with minimal disruption. Moreover, since growth and genetic selection were not required to obtain the fusion product of interest, changes in gene expression could be monitored immediately after the fusion event and at well-defined time intervals thereafter.

Harris and Ringertz pioneered the use of interspecies-specific heterokaryons in the study of regulation of gene expression. After fusion with human cells, differentiated chick erythrocyte nuclei swelled, resumed RNA synthesis, and contained human nuclear proteins (Ref. 28 and reviewed in Ref. 31). Either coexpression or extinction of genes contributed by both cell types were frequent outcomes of heterokaryon experiments produced with cells of different species and differentiated states, including muscle29-35.

The first evidence that previously silent genes could be activated in heterokaryons was provided by Blau et al., who showed that after fusion with mouse muscle cells, muscle gene expression was induced in human amniotic fibroblasts. The success of these experiments was probably due to a combination of three features: (1) the choice of cell type - primary diploid cells, rather than aneuploid transformed cells were used; (2) culture conditions - heterokaryons were maintained in media that promoted differentiation, not proliferation (This was important for two reasons. First, conditions that stimulate proliferation are antagonistic to muscle differentiation. Second, the heterokaryons did not divide for up to two weeks, permitting an analysis of gene expression over much longer periods of time than had previously been possible.); (3) the differentiated state - the muscle cells used were multinucleated, well-differentiated myotubes. These findings were soon corroborated for muscle cells by Wright37,38 and extended to other cell types such as erythroid cells by Baron and Maniatis39.

Using species-specific assays for ten different human muscle gene products, Blau et al. demonstrated that a range of muscle functions could be induced in the non-muscle cell type including structural proteins of the contractile apparatus, membrane components and enzymes39 (Table 1). These gene products were produced in the relative amounts and with a time course typical of human myogenesis. Moreover, the production of proteins and transcripts typical of the non-muscle cell type was repressed and the cell rapidly assumed a cytoarchitecture characteristic of muscle. Within hours of cell fusion, the Golgi apparatus and the microtubule-organizing center changed from a polar to a circumnuclear location and the centrioles were dispersed. These complex changes suggested that fusion with muscle cells in heterokaryons induced a fundamental alteration in the differentiated function of non-muscle cells.

Cell origin influences gene expression in heterokaryons

A particular advantage of heterokaryons in the analysis of differentiation is that changes in gene expression can be induced and studied in cells that would normally never express those genes. This contrasts with most studies of differentiation in tissue culture, which typically use precursor cells such as myoblasts, adipoblasts or erythroblasts which are already destined for the differentiated state they ultimately express. Such precursors are likely to have undergone a substantial number of the changes in protein composition and DNA conformation required by their particular cell type. Thus, by creating a novel differentiated state in cells previously committed to a different fate, heterokaryons allow an analysis of a more complex series of regulatory steps.

Heterokaryon experiments were designed to determine to what extent the origin of a cell influenced its ability to express previously silent muscle genes. Representatives of the three embryonic lineages - fibroblasts (mesoderm), keratinocytes (ectoderm) and hepatocytes (endoderm) - were each fused with...
Table 1. Cell types and muscle functions assayed in heterokaryons

<table>
<thead>
<tr>
<th>(A) Cells activated</th>
<th>(B) Cells not activated</th>
<th>(C) Muscle function activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesoderm</td>
<td>HUT-12 (mutagenized KD fibroblast)</td>
<td>Enzyme</td>
</tr>
<tr>
<td>Amniotic fibroblast</td>
<td>HT1080 (fibrosarcoma)</td>
<td>Creatine kinase: M subunit</td>
</tr>
<tr>
<td>Fetal skin fibroblast</td>
<td>CV-1 (monkey kidney line)</td>
<td>Contractile</td>
</tr>
<tr>
<td>Adult skin fibroblast</td>
<td>UC (EBV transformed, B lymphoid line)</td>
<td>Myosin light chains: fetal</td>
</tr>
<tr>
<td>Muscle tissue fibroblast</td>
<td>HeLa (cervical carcinoma)³</td>
<td>1s</td>
</tr>
<tr>
<td>Fetal lung fibroblast (MRC-5)</td>
<td></td>
<td>2s</td>
</tr>
<tr>
<td>Fetal lung fibroblast (IMR-90)</td>
<td></td>
<td>2f</td>
</tr>
<tr>
<td>Adult lip fibroblast (KD)</td>
<td></td>
<td>Myosin heavy chain: slow</td>
</tr>
<tr>
<td>Chondrocytes</td>
<td></td>
<td>Actin mRNAs: α cardiac</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α skeletal</td>
</tr>
<tr>
<td>Endoderm</td>
<td></td>
<td>Membrane</td>
</tr>
<tr>
<td>Primary fetal hepatocytes</td>
<td></td>
<td>Cell surface antigens:</td>
</tr>
<tr>
<td>HepG2²</td>
<td></td>
<td>24,1D5</td>
</tr>
<tr>
<td>Ectoderm</td>
<td></td>
<td>N-CAM</td>
</tr>
<tr>
<td>Keratinocytes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

³Activates after 5-Aza C treatment.
²HepG2 is the only immortal cell activated in heterokaryons.

muscle cells and analysed in heterokaryons⁴⁰,⁴²,⁴³. As a control, chondrocytes, keratinocytes and hepatocytes were shown to express products typical of their differentiated state when fused to themselves in homokaryons and cultured in the same dish as heterokaryons. Thus, expression of muscle functions was not induced by cell fusion per se; cell contact, or soluble factors in the media, but was restricted to cells fused with muscle cells in heterokaryons.

There were marked differences among cell types in the time course and efficiency of muscle gene expression in heterokaryons (Fig. 2). The average frequency of expression of human neural cell adhesion molecule (N-CAM) on the cell surface was 95, 60 and 25% for heterokaryons containing fibroblasts, keratinocytes and hepatocytes, respectively. In addition, the onset of expression of N-CAM differed by at least two days. These differences in muscle protein accumulation were paralleled by differences in the accumulation of muscle actin transcripts⁴³, which suggested that they resulted from regulation at the transcriptional level.

An analysis of a number of heterokaryons containing different cell types (Table 1) revealed that muscle gene activation was generally observed in primary diploid cells, but not in transformed aneuploid cell types. In one case, the HeLa cell, it was shown that the lack of response could be reversed⁵⁹. If HeLa cells were treated with 5-azacytidine (5AC) before and continuously after cell fusion⁴⁰,⁴²,⁴⁵ and assayed for the novel activation of several muscle genes. As shown in Fig. 3, no significant differences in the expression of the muscle gene encoding human N-CAM were observed in the presence or absence of DNA replication. These results indicate that structural or conformational changes in chromatin either do not accompany gene activation in heterokaryons or do not require extensive DNA replication. Experiments are currently in progress to distinguish between these possibilities.

DNA replication is not required for gene activation

Experiments were designed to determine whether the primary diploid cells in which the time course of activation of muscle genes was slower had to undergo changes in chromatin structure that require DNA replication. The fibroblasts, keratinocytes and hepatocytes shown in Fig. 2 were each exposed to a DNA synthesis inhibitor, cytosine arabinoside, before and continuously after cell fusion⁴⁰,⁴²,⁴⁵ and assayed for the novel activation of several muscle genes. As shown in Fig. 3, no significant differences in the expression of the muscle gene encoding human N-CAM were observed in the presence or absence of DNA replication. These results indicate that structural or conformational changes in chromatin either do not accompany gene activation in heterokaryons or do not require extensive DNA replication. Experiments are currently in progress to distinguish between these possibilities.

The role of positive and negative regulators

Gene dosage, or the relative genetic contribution of the two fused cell types, is critical to the suppression of malignancy in hybrids (reviewed in Ref. 26). Similarly, an analysis of gene dosage, or the relative contribution of muscle and non-muscle nuclei within heterokaryons, suggested that the concentration of positive regulators was critical to gene activation⁴⁰. In addition, dosage experiments suggested the existence of negative regulators that could account, in part, for muscle genes are more readily induced in fibroblasts, which are from the same embryonic lineage as muscle (mesoderm), than in keratinocytes (ectoderm) or hepatocytes (endoderm). To gain insight into how these different specialized cell types arise and how their differentiated state is maintained, it would be useful to know about the molecular events that constitute the lag period before muscle gene expression. Toward this end, the roles of DNA replication and gene dosage were analysed.
the delay and reduced efficiency of gene activation in certain cell types. For example, irrespective of nuclear ratio, 95% of heterokaryons containing fibroblasts from three different sources (fetal lung, fetal skin and adult skin) ultimately expressed human muscle genes\(^3,6\). These results suggest that if fibroblasts produce inhibitors of muscle differentiation, like the well-documented extinguishers of liver gene activation\(^19,20\), they are readily overridden by the positive regulators contributed by differentiated muscle cells, due to differences in their nature or concentration. In heterokaryons produced with hepatocytes, on the other hand, the ultimate probability of activating muscle genes was dependent on the proportions of muscle and hepatocyte nuclei\(^42\). Thus, when hepatocyte nuclei outnumbered muscle nuclei, muscle gene activation was rarely observed even after long time periods; the outcome is presumably determined by the relative levels, or balance, of negative and positive regulators contributed by each cell type.

**Conclusion**

From the studies described in this review, it is apparent that the differentiated state can be altered by nuclear transplantation, tissue regeneration, or cell fusion. Heterokaryons, multinucleated cell hybrids, have shown that tissue-specific genes typical of muscle can be readily induced in numerous cell types that normally never express them, including representatives of all three embryonic lineages. In addition, genes typical of the non-muscle cell phenotype are repressed. Most striking is the finding that the intracellular location of organelles such as the Golgi apparatus, microtubule-organizing center and centrioles is altered. This implies that a major change in cytoarchitecture accompanies muscle gene activation in heterokaryons. Although the heterokaryon state is typical of differentiated syncytial muscle cells, the findings are not limited to muscle cells\(^39\). The conclusion from these diverse studies is clearly that the differentiated state is not fixed.

Future prospects

Given the stability of the differentiated state in vitro, an understanding of the regulation of cell differentiation by mechanisms that allow the type of plasticity observed in heterokaryons is of particular interest. How is the change in differentiated state achieved? Molecular models that suggest that DNA replication is required for the activation of previously silent genes\(^14-16\) are not supported by heterokaryon experiments. This does not rule out the possibility that changes in chromatin structure such as the creation of DNase hypersensitive sites, removal of histones or changes in DNA methylation occur. However, if they occur, these changes are mediated by mechanisms that are independent of DNA replication. The elucidation of these mechanisms is now possible.

How, then, is a heritable differentiated state achieved? Heterokaryon studies suggest that the differentiated state is largely governed by the dynamic interaction of the combination of proteins a cell contains. This is particularly clear from the striking effect on gene activation observed when the relative contribution of components, or dosage, of the two fused cell types is altered. How does this pertain to differentiation
in vitro? The protein composition of a cell is part of its heritage, the product of a history of responses to cues in the course of development. The cell transmits these proteins to progeny through division. Some proteins may activate the expression of their own promoters, like c-fjun. Positive autoregulation and feedback loops are levels of control that operate during the commitment of phage lambda to lysogeny (reviewed in Ref. 48) and are likely to be characteristic of early-acting regulators in mammalian cell differentiation. Protein–protein interactions provide another level of control. Recent findings that transcriptional regulators with 'leucine zipper' or 'helix–loop–helix' motifs bind DNA sequences as heterodimers suggest that a range of protein–protein combinations is possible. A family of myc proteins with the helix–loop–helix motif, including MyoD, myogenin and myf-5, has been shown to regulate the expression of muscle genes (Refs 51–53 and reviewed in Ref. 54). It will be interesting to determine whether these proteins are sufficient to mediate the changes in gene expression seen in heterokaryons produced with disparate cell types. Moreover it seems likely that novel heterodimers are generated upon cell fusion that are either positive or negative regulators. These heterodimers could act on the same or on different promoters from those recognized by the protein complexes originally present in each of the parental cells. It should now be possible to elucidate the nature of the types of heterodimers present in heterokaryons.

Acknowledgements
I am grateful to Corey Goodman, Bob Schimke, Bruce Wallace and Keith Yamamoto for critical review of this manuscript and to Karen Bird for expert secretarial help. I thank, in particular, the members of my laboratory group whose efforts have contributed to the work cited here, which was supported by grants from the NIH, the NSF, and Muscular Dystrophy Association.

References
26 Harris, H. (1988) Cancer Res. 48, 3502–3506
53 Braun, T. et al. (1989) EMBO J. 8, 701–709