Extinction of gene expression in somatic cell hybrids – a reflection of important regulatory mechanisms?

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Extinction in somatic cell hybrids is a multifactorial process that leads to loss of cell-type-specific gene expression. The underlying mechanisms are thought to mirror, at least in part, the repertoire of regulatory mechanisms controlling mammalian cell differentiation.

Somatic cell hybridization was among the first tools used for genetic analysis of mammalian cell differentiation. In intertypic hybrids, cell-type-specific functions, but not housekeeping functions, are usually repressed. This phenomenon is called extinction[1,2]. In some systems, cell-type-specific genes are activated rather than extinguished after fusion[3–5]. The pioneering studies of M.C. Weiss and collaborators have established hepatoma × fibroblast hybrids as the best-characterized system[6,7]. Remarkably, extinction of the hepatic phenotype is observed in hybrids made with many other different cell types. Whereas extinction is regularly seen in hybrids that have one complement of chromosomes derived from each parent, hybrids that have two sets of hepatic chromosomes continue to express liver-specific functions and often activate the fibroblast-derived alleles encoding those functions[8]. This might reflect a delicate balance of positive and negative transcriptional regulators. The combinatorial action of regulatory molecules implies that small changes in the concentration of a particular regulator may cause changes in the rate of transcription[9]. This may lead to a switch in genetic programs, as exemplified by the lysogenic and lytic phases of bacteriophage λ.

Analysis of gene expression in somatic cell hybrids may provide valuable insights into fundamental mechanisms of cell differentiation. The extensive body of literature detailing the basic observations on extinction in somatic cell hybrids has been recently reviewed[10]. Three general principles emerge[1–3,5,10]:

(1) Cell differentiation is controlled, at least in part, by diffusible *trans*-acting factors.

(2) The remarkable plasticity of the differentiated state demonstrates that its maintenance is an active, ongoing process requiring the continuous expression of regulatory factors.

(3) Negative regulatory mechanisms play an important, if not dominant, role in somatic cell hybrids.

With the cloning of numerous cell-type-specific transcription factors over the past ten years, overwhelming evidence has been provided for the first two of these principles. However, relatively few examples of negative regulatory factors involved in cell-type-specific gene expression have been documented[11]. Thus, identification of the factors that mediate extinction may reveal a previously unrecognized class of players involved in the mechanisms that regulate cell differentiation. This premise has greatly stimulated the genetic analysis of extinction.

The genetic basis of extinction

Most progress in the genetic analysis of extinction has been made in the hepatoma hybrid system developed by M.C. Weiss, R.E.K. Fournier and their colleagues. In karyotypically complete hybrids between rat hepatoma cells and a mouse fibroblast cell line, virtually all liver-specific genes are silent[10]. If chromosomes of the non-hepatic parent are lost, a given liver function may be re-expressed[7,8]. Thus, re-expression of individual liver-specific markers, and activation of the fibroblast alleles for those markers, can be correlated with the loss of specific chromosomes from the fibroblast parent (Fig. 1). Microcell fusion has been used to construct hybrids that retain a single fibroblast chromosome, tagged by a selectable marker, on the hepatoma background (Fig. 1). Killary and Fournier[12] have used this approach to define the tissue-specific extinguisher locus 1 (*Tse1*) on mouse chromosome 11, and its human homolog (*TSE1*) on chromosome 17. This locus mediates extinction of several liver-specific markers, including the gene that encodes tyrosine aminotransferase (*TAT*). However, other liver-specific functions remain unaffected. This work has demonstrated a genetic basis for extinction: a single regulatory locus mediates repression of a relatively small subset of genes that are normally expressed in liver but not in other tissues. Interestingly, all the genes repressed by *TSE1* are normally inducible by the cAMP signal transduction pathway[13,14].

In monochromosomal hepatoma hybrids that contain only the fibroblast-derived chromosome 17, or a fragment of this chromosome that carries *TSE1*, target genes are repressed about 20-fold. This phenotype is fully reversible by addition of exogenous cAMP[13,14]. The phenotype of complete hybrids is qualitatively and quantitatively different: repression is of the order of 1000-fold, is not reversible by cAMP, and appears to affect all liver-specific genes[10]. Thus, additional extinguisher loci must contribute to the complete repression of *TSE1*-responsive genes and to extinction of other genes whose expression is independent of *TSE1*. This hypothesis was supported by the identification of *Tse2*, on mouse chromosome 1, which represses expression of the albumin and alcohol dehydrogenase genes[15], and of a locus on mouse chromosome 3 that extinguishes synthesis of albumin, but not of alcohol dehydrogenase (Ref. 16; C. Deschatrette, S. Cereghini and M.C. Weiss, pers. commun.).

Mechanisms involved in extinction

Extinguisher loci act as dominant negative regulators of the transcription of target genes. Their genetic definition does not place constraints on the types of mechanisms by which they might act[10]. Direct repression or silencing via interaction with regulatory

elements of target genes can be envisaged, as well as indirect action by down-regulation of the synthesis or activity of cell-type-specific transcription factors. On the assumption that the mechanisms responsible for extinction in hybrids are part of the normal mechanisms that regulate cell-type-specific gene expression, the activity of *cis*-acting elements and *trans*-acting factors involved in cell-type-specific transcription of target genes has been analysed in a variety of hybrid systems. For example, expression of the growth hormone gene is extinguished in pituitary × fibroblast hybrids. The pituitary-specific growth hormone promoter is inactive in these hybrids as a consequence of the down-regulation of the pituitary-specific transcription factor GHF-1/Pit1 (Ref. 17). Extinction of immunoglobulin genes in B cell × fibroblast hybrids is accompanied by lack of transcription of the lymphoid-specific factor Oct-2 (Refs 18, 19). In hepatoma × fibroblast hybrids, the liver-specific promoters of the genes for albumin and α_1-antitrypsin are inactive owing to down-regulation of the mRNA for the transcription factor HNF1α (Refs 20, 21). Other examples are listed in Table 1.

In most systems analysed so far, extinction is correlated with the absence or inactivity of crucial cell-type-specific transcription factors. However, Table 1 also includes systems in which extinction has been correlated with the function of negative regulatory elements, for example silencer-like elements in the enhancer of the gene for the immunoglobulin heavy chain[34] and the promoter of the growth hormone gene[35]. Although we are beginning to understand the final steps of the regulatory pathways used by extinguishers, we still need much more information about the nature and mode of action of the extinguisher loci themselves. The mechanism of action of one extinguisher locus, *TSE1*, has recently been elucidated (see below), but general conclusions await the genetic definition and cloning of more of these interesting regulatory loci.

Detailed analysis of *TSE1* function was made possible by the generation of hepatoma microcell hybrids that differ by just a small fragment of the fibroblast-derived chromosome carrying *TSE1* – such cells are called deletion hybrids[22]. In this defined experimental

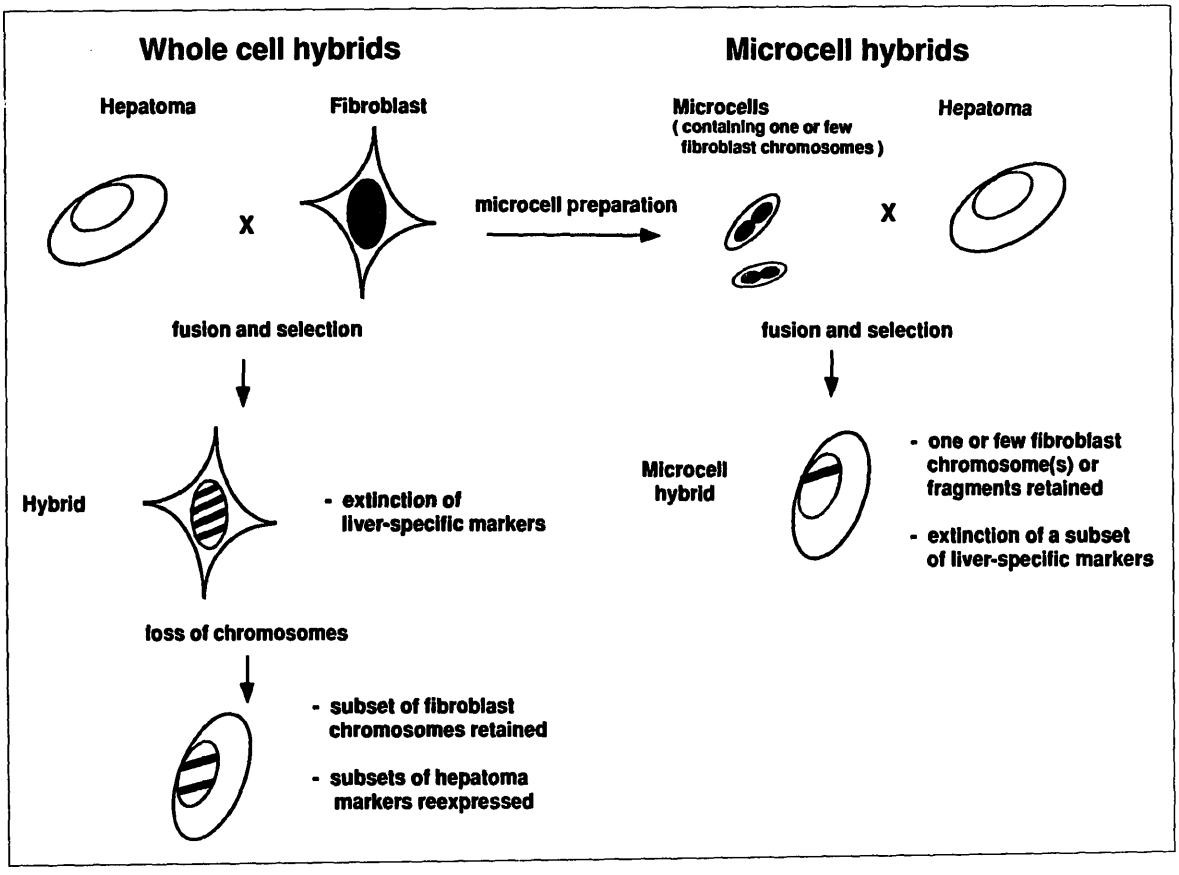

FIG 1

Somatic cell hybrids as tools to study cell differentiation. Whole-cell hybrids are generated by co-cultivation and polyethylene glycol (PEG)-mediated fusion, followed by selection. Microcell hybrids are generated by centrifugal enucleation of donor cells arrested in mitosis and PEG-mediated fusion of the resulting microcells (membrane-enveloped chromosomes) with intact recipient cells. In some cases, endogenous markers can be used for selection, e.g. human chromosome 17, which carries the *TSE1* locus, also carries the selectable thymidine kinase gene[12]. In general, the chromosome to be selected must be tagged with a selectable marker in the cell line used as the donor for microcells[22]. In the hepatoma × fibroblast fusions depicted, the relative contribution of genetic material from the fibroblast is indicated: hybrids may contain a full set of fibroblast chromosomes or a subset, whereas microcell hybrids contain just one or a few fibroblast chromosomes.

TABLE 1. Extinction mechanisms in selected somatic cell hybrids

Target locus	Hybrid type	Extinguisher locus	Target sequence	Mechanism	Ref.
Immunoglobulin (Ig)	Myeloma × fibroblast		Heavy-chain enhancer Light-chain promoter (octamer-binding site)	↓Oct-2/OTF2 mRNA	18,19
	Myeloma × T cell		Heavy-chain enhancer (μE4 and octamer site)	Silencer activity	34, -[a]
Growth hormone (GH)	Pituitary × fibroblast		GH promoter	↓GHF1/Pit1 mRNA	17
	Pituitary × fibroblast		GH promoter	Silencer activity	35
Albumin (ALB)	Hepatoma × fibroblast		ALB promoter (HNF1α-binding site)	↓HNF1α/LFB1 mRNA	20
	Hepatoma × fibroblast	*Tse2*	ALB promoter (−340 bp to +10 bp)		15,16
Tyrosine aminotransferase (*TAT*)	Hepatoma × fibroblast	*TSE1*/RIα	−3.6 kb enhancer (CRE)	↓PKA activity ↓Phosphorylation of CREB	23,24,27
	Hepatoma × fibroblast		−3.6 kb enhancer (HNF4 site)	↓HNF4 mRNA	28
	Hepatoma × fibroblast		−11 kb enhancer (−11/2 site)	↓*in vivo* binding of −11 binding protein	28
	Hepatoma × fibroblast		−11 kb enhancer −2.5 kb enhancer (HNF3 sites)	↓HNF3β and HNF3γ mRNA	28
α_1-antitrypsin (α_1AT)	Hepatoma × fibroblast		α_1AT promoter (HNF1/LFB1 site)	↓HNF1α/LFB1 mRNA	21
Retinol binding protein (RBP)	Hepatoma × fibroblast		RBP promoter (−334 bp to 130 bp	↑Putative repressor activity	36
	Hepatoma × HeLa				
Insulin	Insulinoma × fibroblast		Insulin gene enhancer (IEB2 element)	↓Activity of β-cell-specific transcription factor IEF1	37

[a]L. Eckhardt, pers. commun.

system, phenotypic differences between these hepatoma cell lines can be attributed to *TSE1* with a high degree of confidence. *TSE1* affects a subset of liver-specific genes, including the *TAT* gene. Gene transfer experiments have shown that while a liver-specific enhancer some 3.6 kb upstream of the transcription start site of the *TAT* gene responds to *TSE1*, the other known regulatory elements of the *TAT* gene do not[23]. *TSE1* represses the activity of a cAMP-responsive element (CRE), which is essential for the function of

this enhancer, and this repression is reversible by cAMP induction[23]. This finding provided an explanation for the observation that all *TSE1*-responsive genes known to date are normally inducible by the cAMP signal transduction pathway[13,14]. *TSE1* was shown to antagonize binding of the CRE-binding protein[24] (CREB) to the CRE *in vivo*. Phosphorylation of the Ser[133] residue of CREB by the cAMP-dependent protein kinase (PKA) is required for transcriptional activation and stimulates binding of CREB to its target site[23,25,26]; this phosphorylation was repressed in the presence of *TSE1*. These observations suggested that *TSE1* might directly interfere with cAMP signalling.

It became clear that *TSE1* acts by inhibiting the basal activity of PKA, and this information was the key to identifying the product of the *TSE1* locus[24]. Among the activities known to antagonize PKA (e.g. regulatory subunits, phosphatases and phosphodiesterases), the RIα regulatory subunit of PKA was shown to be identical to TSE1, and all the characteristics of extinction by *TSE1* could be reproduced by overexpressing RIα in hepatoma cells. An alternative approach to the identification of the *TSE1* gene product used differential cDNA hybridization: a collection of candidate clones was isolated that included RIα (Ref. 27).

The pathway of TSE1-mediated extinction is illustrated in Fig. 2. PKA is a tetramer of two catalytic and two regulatory subunits, drawn for simplicity as a dimer. Binding of cAMP to the regulatory subunits triggers dissociation of the tetramer and release of two active catalytic subunits. The level of RIα/TSE1 determines the concentration of 'free' catalytic subunits of PKA, which are responsible for basal kinase activity. PKA-mediated phosphorylation enhances both binding of CREB to DNA and transcriptional activation by CREB. Reduced phosphorylation of CREB leads to decreased activity of the CRE, which is an essential element of the liver-specific enhancer situated −3.6 kb of the *TAT* gene. Thus, RIα/TSE1 acts by post-translational modification of an ubiquitously expressed transcription factor, and *TSE1*-mediated extinction results from lack of activation. The fact that the first molecularly characterized extinguisher turned out to act via inhibition of a common signal transduction pathway was quite unexpected. Molecular characterization of other extinguisher loci may uncover even more tantalizing surprises.

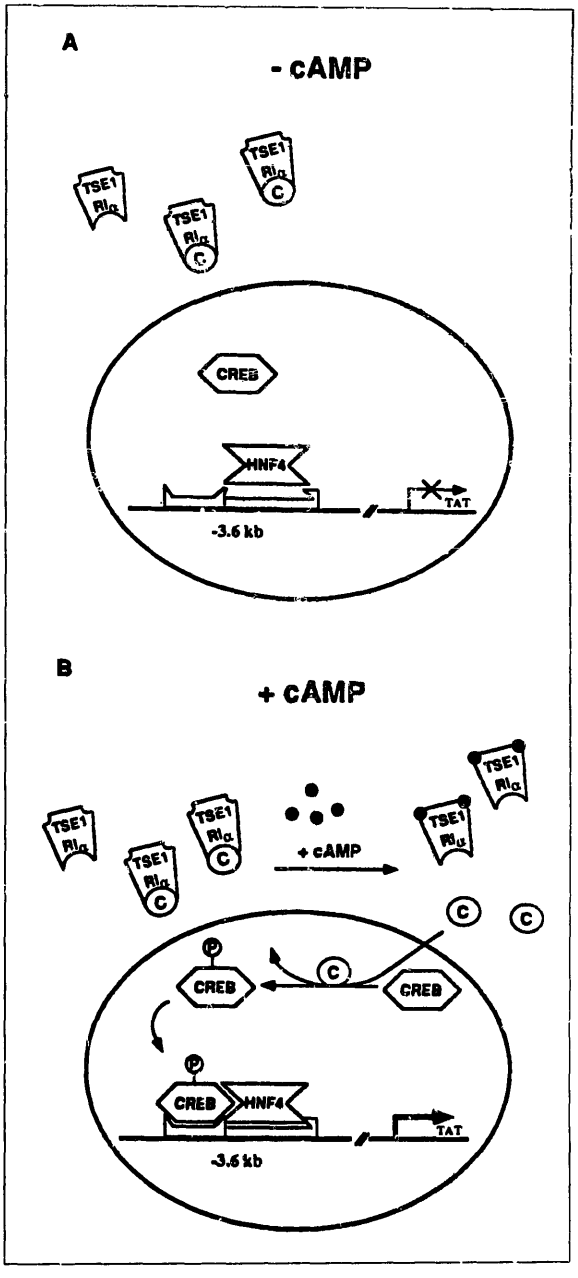

FIG 2

The mechanism of action of the tissue-specific extinguisher locus 1 (*TSE1*). TSE1, the regulatory subunit RIα of the cAMP-dependent protein kinase[24] (PKA), complexes with and inactivates the catalytic subunit (C) in the cytoplasm. For simplicity, the diagram depicts PKA as a dimer rather than a tetramer of two C and two R subunits, and shows only the liver-specific enhancer of the *TAT* gene at −3.6 kb, which has binding sites for the essential transcription factors CREB and HNF4. (A) *TSE1*$^+$ monochromosomal hybrids, which contain only the part of the fibroblast-derived chromosome 17 that encompasses the *TSE1* locus: because PKA is inhibited, CREB is mostly unphosphorylated. This leads to reduced binding of CREB, and consequently to inactivity of the enhancer and extinction of the *TAT* gene. (B) Reversal of *TSE1*-mediated extinction after addition of exogenous cAMP. The dissociated catalytic subunit (C) of PKA enters the nucleus to phosphorylate CREB, which then cooperates with HNF4 to activate the liver-specific enhancer[23,24,28].

Extinction: a multifactorial process

Extinction in complete hybrids must result from the action of multiple genetic loci. For example, as mentioned previously, expression of the *TAT* gene is repressed about 20-fold by *TSE1* alone, but repression of *TAT* in complete hybrids is about 1000-fold[14]. Three enhancer regions that are located within 11 kb upstream of the transcriptional start site are known to contribute to cell-type-specific transcription of the *TAT* gene; the activity of all three is inhibited in complete hybrids[28]. Moreover, in each of the two liver-specific enhancers at −3.6 kb and −11 kb, at least two separate elements are subject to down-regulation. Among these elements are binding sites for the liver-specific transcription factors HNF3 and HNF4

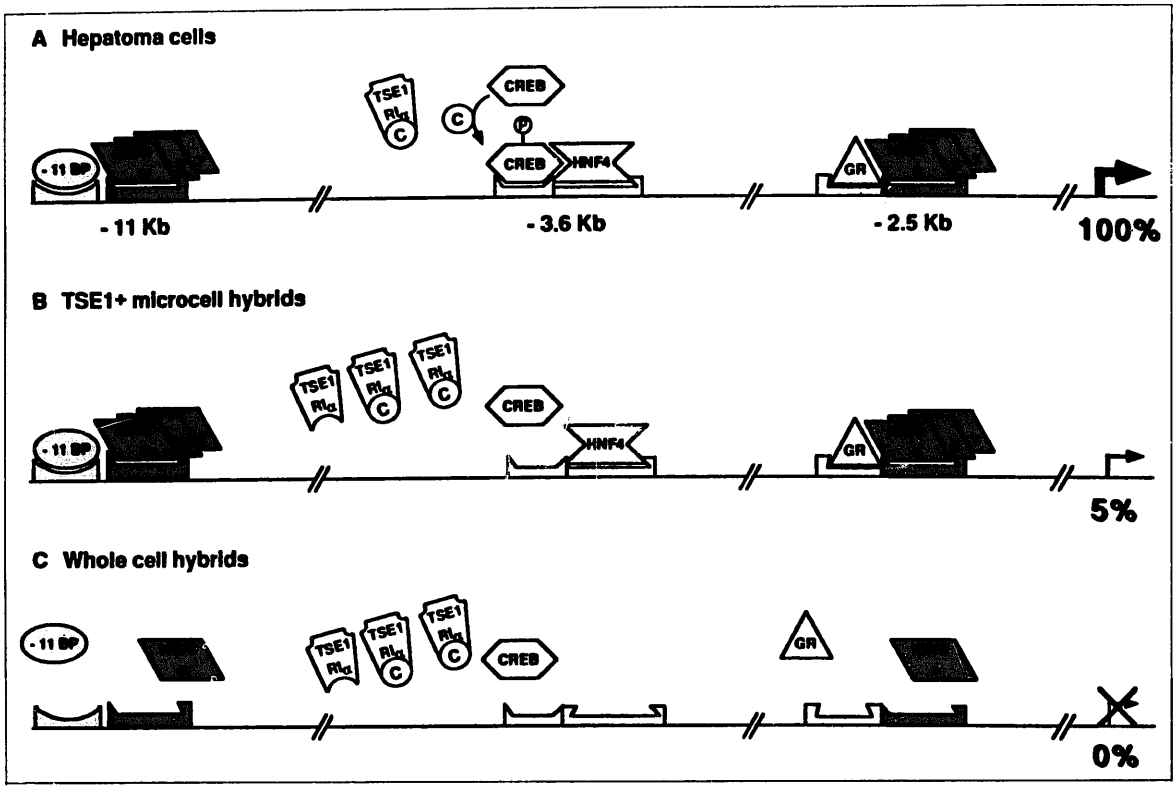

FIG 3

Extinction in whole-cell hybrids, caused by the inactivity of multiple regulatory elements of the *TAT* gene. (A) The regulatory region of the *TAT* gene with the three enhancers known to contribute to liver-specific transcription in hepatoma cells. At the glucocorticoid-inducible enhancer at −2.5 kb, the glucocorticoid receptor (GR) cooperates with members of the HNF3 family of liver-specific transcription factors. Cooperation of CREB and HNF4 is essential for the function of the liver-specific and cAMP-inducible enhancer at −3.6 kb (see legend to Fig. 2). The −11 binding protein (−11 BP) and proteins of the HNF3 family bind to sequences at −11 kb that confer liver specificity. (B) In *TSE1*$^+$ microcell hybrids, high-level production of RIα/TSE1 (as compared to expression in hepatoma cells) lowers the basal PKA activity, thereby preventing binding of and activation by CREB. The enhancers at −2.5 kb and −11 kb are unaffected by RIα/TSE1 and ensure residual (~5%) expression of the *TAT* gene. (C) In whole-cell hybrids all binding sites are unoccupied, as shown by analysis of DNase-I-hypersensitive sites and *in vivo* footprinting. HNF4 and HNF3β and γ are absent because of down-regulation of their mRNAs. Expression of the *TAT* gene is not detectable.

(Fig. 3). It has been shown that the mRNAs for HNF4, HNF3β and HNF3γ are virtually absent in hybrids, but the level of that for HNF3α is unaffected. A factor that binds to the −11 kb enhancer is present in hybrids, but fails to bind *in vivo* to its target sequence in these cells. The inactivity of all three of these enhancers is also reflected by the loss of the cell-type-specific DNase-I-hypersensitive sites that mark these elements in hepatoma cells[28].

Extinction of the albumin gene has been related to loss of the liver-enriched transcription factor HNF1α (Ref. 20). However, expression of HNF1α is independent both of the *Tse2* locus[10,15] and of the locus identified by Petit and her colleagues[16]; these loci were defined as negative regulators of albumin expression in microcell hybrids (M.C. Weiss, pers. commun.). This indicates that extinction of albumin gene activity is mediated by at least two regulatory elements. The involvement of multiple loci in extinction of an individual gene is also suggested by the fact that expression of extinguished genes in a particular hybrid cannot be rescued by transfer of a gene for a transcription factor that is repressed in that hybrid. For example, exogenous expression of Oct-2 in lymphoid × fibroblast hybrids activates a transfected immunoglobulin promoter, and HNF1α expression restores activity of a transfected α_1-antitrypsin promoter in hepatoma × fibroblast hybrids, but in each case extinction of the endogenous gene is maintained[18,21].

The cell-type specificity of transcription is the result of the combinatorial action of multiple transcription factors. Analogously, the degree of extinction in hybrids is determined by the combined action of multiple extinguishers, some of which act indirectly by down-regulating or inactivating transcription factors. This is further evidence that extinguishers are part of the complex regulatory network that controls cell differentiation. Although we have yet to discover an extinguisher that acts as a repressor of transcription, the recent characterization of the WT1 Wilms' tumor gene product, which functions in the developing kidney as a tissue-specific direct transcriptional repressor of genes that are activated by the mitogen-inducible factor EGR-1 (Ref. 29), illustrates a mechanism by which extinguishers may act directly.

One step up the regulatory hierarchy

Cell-type-specific activation and extinction may involve hierarchies of regulatory interactions. For example, HNF4 is required for expression of HNF1, placing HNF4 upstream of HNF1α in a hierarchy of liver-specific transcription factors[30,31]. In extinguished hybrids, down-regulation of HNF4 contributes to down-regulation of HNF1α and consequently of all genes dependent on HNF4 or HNF1α (Ref. 30). Analysis of the regulation of transcriptional activators, such as HNF3 and HNF4, may reveal factors that mediate active repression of these cell-specific activators and that have the properties of extinguisher loci.

The question of what regulates the regulator is particularly interesting because of the dominant action of extinguisher loci. For example, the transcriptional activity of the RIα/*TSE1* gene in human fibroblasts is preserved when the chromosome 17 on which it is carried is transferred by microcell fusion into the nucleus of a hepatoma cell that expresses RIα/*TSE1* at a very low level[24]. Thus two mitotically stable, epigenetic states of gene expression coexist in the same nucleus and are inherited *in cis*, possibly via mechanisms similar to those commonly referred to as imprinting. Clearly, this kind of locus-autonomous regulation is a prerequisite for the dominant action of extinguishers in experimentally generated cell hybrids.

The activity of extinguisher loci is maintained over many cell generations. What is the molecular basis for this stable inheritance of the extinction phenotype? One possibility is that regulatory cascades and autoregulatory loops, which have been observed in several systems and which are crucial for determining cell fate, will be disrupted if a response to an extinguisher induces alterations in the concentration or activity of the regulatory molecule that is responsible for a particular control circuit. This model implies that extinguishers have the potential to act as key regulators that control on/off switches. Once such a switch has been turned by a particular extinguisher, the extinguished state may be maintained by mechanisms common to all cells. It has recently been shown in *Drosophila* that the *hunchback* gene silences *Ultrabithorax* (*Ubx*) expression at late embryonic stages when hunchback protein can no longer be detected. This was interpreted as indicating that hunchback acts in a 'hit and run' fashion by initiating a particular chromatin structure or by generating a stable silencing complex at the *Ubx* locus[32]. An activity similar to that of hunchback might regulate the *TSE1*/RIα gene in response to a developmental signal, and expression of the gene might then be maintained by mechanisms independent of the inducing signal. The activity might be imprinted by a particular chromatin structure, as hypothesized for the *Polycomb* group genes of *Drosophila*: these act in a dosage-dependent manner and are required to repress gene expression in differentiated cells[33]. Such a mechanism could account for the remarkable stability of the extinguished phenotype in hybrid cells, for so long as the relevant chromosome is retained.

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