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Regulation and Patterns of Endogenous and Exogenous Gene Expression during Differentiation of Embryonal Carcinoma Cells

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Embryonal carcinoma (EC) cells offer an interesting model system for evaluating differentiation because the cells are pluripotent, thus resembling germ cells and embryonic stem cells, and because a number of agents have been defined that are capable of promoting the differentiation of these cells. This chapter examines how EC cells might be triggered to differentiate, with emphasis on retinoic acid because this compound is a potent, naturally occurring inducer that has been studied extensively in this system. The nature of alterations in gene expression during EC cell differentiation is reviewed from the perspective of evaluating whether these changes are likely to be responsible for, or a result of, the differentiation event. Finally, we consider in molecular terms why EC cells, but not their differentiated derivatives, are refractory to the expression of many viral genomes following infection. Based upon these studies, we propose that fundamental changes in gene expression that are observed when differentiation is triggered in EC cells are likely to be due to the disappearance or neutralization of strong repressor elements.

Introduction

Embryonal carcinoma (EC) cells have been extensively used for several years as a model system for the study of differentiation. Several reviews on this subject have considered the pluripotent nature of these cells and their similarity to early embryonic cells and germ cells (*1,2*), their biology and cell biology (*1-4*), the nature of the agents that can induce EC cell differentiation (*4*), and the antigenic, biochemical and molecular biological markers that characterize the differentiation event (*4,5*). Rather than reiterate much of the material that has already been reviewed, we have attempted to focus upon an examination of how exposure to inducers of differentiation initiates changes in gene expression and how alterations in gene expression in EC cells might trigger and/or subsequently imprint upon the cells a differentiated phenotype. It is important to note at the outset that the differentiation of EC cells is a complex process which assuredly involves qualitative or quantitative alterations in the expression of large numbers of genes. The issue of EC cell differentiation is greatly complicated by the facts that some of these cells, by virtue of their pluripotent nature, can give rise to several final phenotypes (and thus progress along any of a number of differentiation pathways) and that cells from different EC lines have their own peculiarities, including propensity for, and patterns of, differentiation. In fact, the EC line F9, which has probably been used more extensively than any other, differs from other EC lines in several respects (*6*). Indeed, one should exercise caution in drawing any general conclusions about EC cell behavior from studies with a single cell line (something which has been done all too often with EC cells in the past).

The following section considers how exposure to an inducer of differentiation, particularly retinoic acid (RA), might be translated into triggering of new patterns of gene expression characteristic of the differentiated phenotype. In the subsequent section, we describe changes in gene expression during EC cell differentiation with emphasis upon more recent studies that have not already been extensively reviewed. In accordance with our stated objectives, we have attempted to consider critically how

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closely these alterations are related to the differentiation event. In the final section, we examine how studies of exogenous (predominantly viral) gene expression might provide insight into regulation of endogenous gene expression during EC cell differentiation.

Triggering of Differentiation of Embryonal Carcinoma Cells

EC cells can be triggered to undergo differentiation in several ways. In early studies it was demonstrated that physical manipulations of the cultures (e.g., growth at high density or as nonadherent multicellular aggregates) would result in differentiation of cells from some EC lines (*7,8*). A number of small organic molecules have been found to promote differentiation of these cells, albeit with differing potencies. Included in this list of active agents are RA, retinol, other synthetic retinoids, hexamethylenebisacetamide (HMBA), sodium butyrate, dimethylacetamide and α-difluoromethylornithine. Proposals concerning mechanism of action of some of these agents have been considered elsewhere (*9–12*). It is probably fair to state that there is no unequivocal and unanimously accepted explanation of the molecular mechanism by which any one of these agents promotes differentiation of EC cells. In lieu of reviewing all of the proposed mechanisms of action for all of these differentiation inducers in detail, this discussion will focus upon RA, as a great deal of information is available about this inducer.

Considerable evidence has been presented to support the view that the cellular RA-binding protein (CRABP) mediates the induction of differentiation of EC cells by RA. There is in general a good qualitative correlation between the ability of acidic retinoids to compete for binding sites on CRABP and to promote EC cell differentiation (*13,14*). Mutant EC lines that have little or no CRABP activity are differentiation-defective (*15–17*). Cell fusion between differentiation-defective EC cells leads to the ability to respond to RA only when the resulting hybrids reacquire CRABP activity (*18*), and treatment of CRABP-deficient EC cells with sodium butyrate and RA restores CRABP activity and simultaneously causes a significant proportion of the cells in the culture to undergo differentiation (*19*).

Although CRABP seems to participate in RA-induced differentiation of EC cells, its mode of action remains to be elucidated. Following early reports that the RA-CRABP complex could interact with nuclei, it was suggested that, by analogy with steroid-receptor protein complexes, the retinoid holoprotein might influence gene expression by direct interaction with DNA (*20*). In this way, the RA-CRABP holoprotein complex could be viewed as a master switch, initiating a cascade of gene expression changes leading ultimately to the final differentiated phenotype. However, it has become apparent that there are significant differences between the steroid and retinoid systems; for example, at least some steroid receptors appear to be resident of the nucleus whether or not they possess ligand (*21,22*), whereas the studies of Takase et al. (*23*) and others (*24,25*) are consistent with the view that CRABP delivers its ligand from the cytoplasm to the nucleus but does not itself remain associated with nuclear components. In fact, there is little evidence to suggest that RA-CRABP or RA alone interacts directly with DNA (*26*).

There have been reports of nuclear proteins that bind RA (*27,28*), but their specificities and physiological relevance have not been established. Very recently, however, two groups have independently identified mRNA that encodes a putative and new binding protein for RA (which has been called a receptor and which we shall refer to as RetR) that is clearly a member of the family of nuclear proteins that bind biological response modifiers (hormones, growth factors) in one domain and DNA in another (*29,30*). It is too early to evaluate whether this RetR in fact serves as a master switch because nothing is known about the DNA sequences with which this protein specifically interacts. However, the presumed existence of a nuclear RetR, together with recent results concerning CRABP, merit a reappraisal of the role of CRABP and its involvement in the differentiation of EC cells.

The simplest and most straightforward alternative is that CRABP could be critical for EC cell differentiation because it must deliver RA to RetR. This being the case, any mutation which interfered with CRABP function could preclude transfer of RA to the RetR and thereby block alterations in gene expression. At the other extreme, however, one could imagine a scenario, also consistent with existing results, in which CRABP would play no direct role in EC cell differentiation. Barkai and Sherman have reported that CRABP levels increase when EC cells are treated with RA for as little as 2 hr (*25*). Furthermore, exposure of EC cells to RA for 4 hr or more leads to the induction of a cytochrome P-450 enzyme system that efficiently metabolizes RA, but only if CRABP is present in the cells (*31*). There is evidence to suggest that both of these events are regulated at the level of transcription (N. H. Chi, personal communication; Gubler et al., in preparation). If these transcriptional alterations are under the control of a master switch (e.g., RetR), then they might be elicited in parallel to, but independently of, the activation of the differentiation pathway. The absence of CRABP transcripts and protein in differentiation-defective mutants could thus be due to a mutation that eliminated a master switch function. Silencing of the master switch would in turn prevent the expression of both the CRABP gene and those genes involved in the triggering of the differentiation cascade.

If CRABP were not directly involved in the differentiation pathway, this could explain why other cells such as HL-60 promyelocytic leukemia cells differentiate in response to RA even though they appear to lack CRABP (*32*). It would also lead to the prediction that one could modulate CRABP activity by direct action upon that gene without interfering with the ability of the cells to differentiate in response to RA (since the RetR-triggering mechanism would be unaffected). In fact, we have obtained preliminary evidence to suggest that in some cases CRABP activity can be restored to differentiation-

defective (originally CRABP$^-$) EC cells in such a way as to allow the cells to metabolize RA but not to differentiate in response to the retinoid.

The preceding discussion makes clear that CRABP could be important for differentiation of EC cells by modulating accessibility of RA to RetR. This could be achieved in a positive way (by transfer of RA to the nucleus and RetR) and/or in a negative fashion (via promoting activation of the RA-metabolizing enzyme system, thereby reducing cellular RA levels). The latter function could be required generally for inactivation and detoxification of RA (*9*), even in cells that do not differentiate in response to RA. Modulation of RA levels could well be critical in embryonic cells. RA can be a potent teratogen (*33*). Also, evidence is beginning to accumulate that RA is a morphogen during embryogenesis and that concentration gradients of this retinoid influence tissue pattern formation (*34,35*). One might, in fact, hypothesize that CRABP and the RA-metabolizing enzymes are prominent in EC cells because of their germ cell/embryonic cell origins. On the other hand, CRABP might not be critical for the differentiation of cells in which the concentration of RA after induction of differentiation would be unimportant (e.g., HL-60). If the latter alternative is correct, then there would have to be a way (not involving CRABP) in which RA could be transported to the nucleus and/or RetR.

Whereas we might now be in a position to learn more about the mechanism by which RA elicits differentiation of EC cells, it will remain for us to determine whether other inducers of differentiation operate in identical, parallel, or completely unrelated ways. In other words, is there a single master switch or multiple approaches to eliciting differentiation of EC cells? Retinol has no documented ability to compete with RA for CRABP sites (*13,15*), and there is no evidence to implicate the cellular retinol-binding protein (related to, but distinct from, CRABP) in retinol-induced differentiation of EC cells. It is possible, but not yet resolved, whether retinol acts via metabolic conversion to RA [for conflicting views, see Gubler and Sherman (*36*) and Williams and Napoli (*37*) and discussion by Barkai and Sherman (*25*)]. Retinol does appear to compete weakly for binding to RetR (*29,30*). HMBA has been proposed to act at the level of protein kinase activity (*10*). Interestingly, a number of differentiation-defective mutants selected by lack of responsiveness to RA are also refractory to HMBA (*16*). HMBA does not compete with RA for binding to CRABP (*16*) and has yet to be tested for ability to interact with RetR.

Information is equally limited regarding mechanistic relationships between RA and other low molecular weight inducers of EC cell differentiation. As mentioned above, it has been known for some time that physical manipulations such as aggregation can influence differentiation of EC cells; although combinations of aggregation and small molecules such as RA can influence the extent and direction of EC cell differentiation (*38*), we know essentially nothing about the molecular mechanisms governing such physical influences. In summary, there is insufficient information to indicate whether or not there is a single master switch for differentiation of EC cells.

Finally, the physiological relevance of all of the above studies remains to be determined. It is clear that cells from different EC lines differ in their propensity for differentiation in tumor form, and to some extent we can mimic this differential behavior in culture (*39*). It has also been demonstrated that RA administered to animals bearing EC tumors can promote differentiation of the cells within the tumors (*40,41*). However, this does little to establish the nature of the endogenous agents that normally promote differentiation of EC cells *in vivo* in animals not receiving dietary supplements of RA. Since RA circulates at very low levels in animals on normal diets (*42*) and since EC cells can convert retinol (which circulates at much higher concentrations) to RA very poorly at best (*36,37*), we have attempted to modulate differentiation of EC cells *in vivo* by increasing dietary retinol or, conversely, by placing animals on retinoid-deficient diets. Although we were able to elicit increases or decreases in circulating retinol with such dietary regimens, we had little success in modifying the extent of differentiation of cells in the EC-derived tumors (*43*). We must, therefore, leave open the possibility that other factors, perhaps hormones, are responsible in large part for the induction of differentiation of EC cells *in vivo*.

Endogenous Gene Expression and Differentiation of Embryonal Carcinoma Cells

Homeobox-Containing Genes

The homeobox is a 180-nucleotide protein-encoding DNA sequence present as a highly conserved region in sets of genes known to control embryonic development and differentiation in Drosophila (*44,45*). The homeobox sequence has also been found in the genome of several other species, including mammals, supporting the notion that homeobox-containing genes similar to those found in Drosophila could be universally involved in controlling certain aspects of development and differentiation (*46–49*).

mRNs containing the antennapedia-related human homeobox sequence Hu-1 are detected at relatively high levels in the human teratocarcinoma cell line NT2/D1 only after induction of differentiation by RA. It is notable that even though the cells are presumably irreversibly differentiated with RA, expression of Hu-1 requires the continuous presence of the inducer. The mouse homolog Mu-1 (subsequently named H24.1) was not found in this study to be expressed in the murine cell line PSA-1 at any stage of differentiation induced by aggregation (*50*).

mRNAs containing the mouse homeobox sequences Mo-10 and m6-12 are both observed in 01A1 cells (a ouabain- and thioguanine-resistant clone of the murine EC line P19). When aggregate cultures are induced to differentiate either into glial cells by RA or into cardiac muscle cells by DMSO, a strong but transient increase in Mo-10 transcripts is observed. On the other hand, expres-

sion of m6-12-containing genes does not seem to be differentiation-specific in these cells (*51*). The homeobox sequence m6-12 is also expressed in F9, P19, PC13, and EK cells. In F9 the level of expression is higher than in the other cell lines and increases upon RA-induced differentiation (*52*).

Recently Deschamps et al. (*53*) have proposed that induction of expression of genes containing the mouse homeobox sequences H24.1 and m6-12 in EC cell lines is a direct effect of RA treatment and does not correlate with cell differentiation: induction of high levels of expression of H24.1 and m6-12 in three different murine EC cell lines (C17S1, PCC7.S AzaR1 and PSA-1) occurs only in the presence of RA but not following induction of differentiation by aggregation. By contrast, in Swiss 3T3 cells treatment with RA produces an increase in the synthesis of extracellular matrix components but not in the expression of H24.1, indicating that RA-induction of homeobox sequences is not a generic phenomenon, but may be restricted to EC cells. In summary, some studies of homeobox sequence expression in EC cells provide hints of a relationship with differentiation, whereas other investigations warrant cautious interpretation of such results.

Structural Proteins

Components of the extracellular matrix appear to be involved in cellular functions such as adhesion, migration, and cell-cell interaction. Together with cytoskeletal components, the extracellular matrix strongly influences cellular morphology. When EC cells are induced to differentiate, they undergo dramatic morphological alterations, and many changes occur concurrently in the expression of genes that are associated with the production of an extracellular matrix.

Fibronectin is a glycoprotein synthesized by a wide variety of cells *in vitro*. Cultured EC cells synthesize fibronectin and release it into the medium but do not retain it on their surface. Only upon formation of embryoid bodies is there accumulation of fibronectin under the endoderm layer (*54*). In F9 cells the synthesis and secretion of fibronectin during embryoid body formation increase on day 2, followed by a slight decrease after day 6, suggesting that fibronectin may play a role in the early events of aggregate formation and may trigger the organization of a basement membrane (*55*).

Sherman and colleagues (*19,56,57*) have demonstrated that surface-associated fibronectin is often characteristic of differentiated derivatives that result from treatment of EC cells with retinoids. In one of these studies, Sherman et al. (*57*) demonstrated that Nulli-SCC1 cells treated with RA display readily detected levels of surface-associated fibronectin. Retinol, albeit a poor inducer of differentiation of Nulli-SCC1 cells, also generates ample amounts of this surface antigen. Cells from a variant line of Nulli-SCC1 cells undergo a clear morphological change in response to retinol, but this phenotypic alteration is transient: the cells revert to a typical EC-like morphology following removal of the retinoid from the medium (*57*). Thus, although it is clear from many other studies that several differentiated cell types possess surface-associated fibronectin, whereas undifferentiated EC cells generally do not, the report by Sherman et al. (*57*) suggests that in certain EC cells, retinoids can promote surface deposition of fibronectin, even though those cells fail to undergo terminal differentiation.

Type IV collagen is synthesized by undifferentiated OC15S1 and PC13 EC cells. Following differentiation into endodermlike cells, there is suppression of the synthesis of collagen I. However, in F9 cells the production of collagen IV increases slightly during RA-induced differentiation (*55,58*). The secretion of laminin and entactin is also increased during differentiation of F9 cells (*55,59*). Northern analysis of RNA from F9 cells revealed a decrease in the level of mRNA encoding laminin and collagen IV 3 to 6 hr after the addition of RA and dibutyryl cAMP followed by an increase over the next 12 to 72 hr (*60*). This increase is regulated at the level of transcription (*61*).

Vinculin is a 130 KD protein associated with the sites of contact between actin and the cell membrane and has a role in cell attachment. Undifferentiated F9 cells show vinculin-specific staining without fibrillar organization. Upon differentiation into endodermal cells vinculin is organized into plaques. This change precedes the formation of actin-containing fibers and the appearance of keratin (*62*). Undifferentiated PCC3 cells display vimentin, whereas the endodermal derivatives differentiating from these cells show both vimentin and keratin (*63,64*).

Both differentiated and undifferentiated PCC3 EC cells contain similar amounts of actin, but it is only upon differentiation that organized microfilament bundles can be detected (*65*). Based upon studies such as these, it might be expected that the amount of actin mRNA would remain constant during differentiation of EC cells. We have studied by Northern analysis the level of β-actin mRNA in Nulli-SCC1 cells treated with 10^{-6} M RA or 3 mM HMBA for increasing periods of time (Fig. 1) (unpublished observations). We have observed with both agents a slight reduction in the level of β-actin mRNA 30 min after addition of the drugs. Expression of β-actin mRNA then returns to uninduced levels only to decrease once again beyond 12 hr of treatment and to remain below control values thereafter. Quantitatively, the response to HMBA vs. RA was found to differ when results from several experiments were averaged (the effect of HMBA treatment was generally more profound than that of RA). This might reflect the different phenotypes to which Nulli-SCC1 cells progress when treated with the two agents (*66*).

Insofar as other cytoskeletal proteins are concerned, the network of tubulin does not show any change in relation to cell differentiation (*65*). When PCC3 cells are induced to differentiate with HMBA, there is a large increase in the synthesis of tropomyosin followed by its association with actin bundles (*67*).

The lamina is a structural component of the nuclear envelope that interacts with peripheral chromatin and the nucleoplasmic face of the nuclear membrane. It plays an important role in nuclear envelope breakdown and formation during cell division. The lamina is formed by three

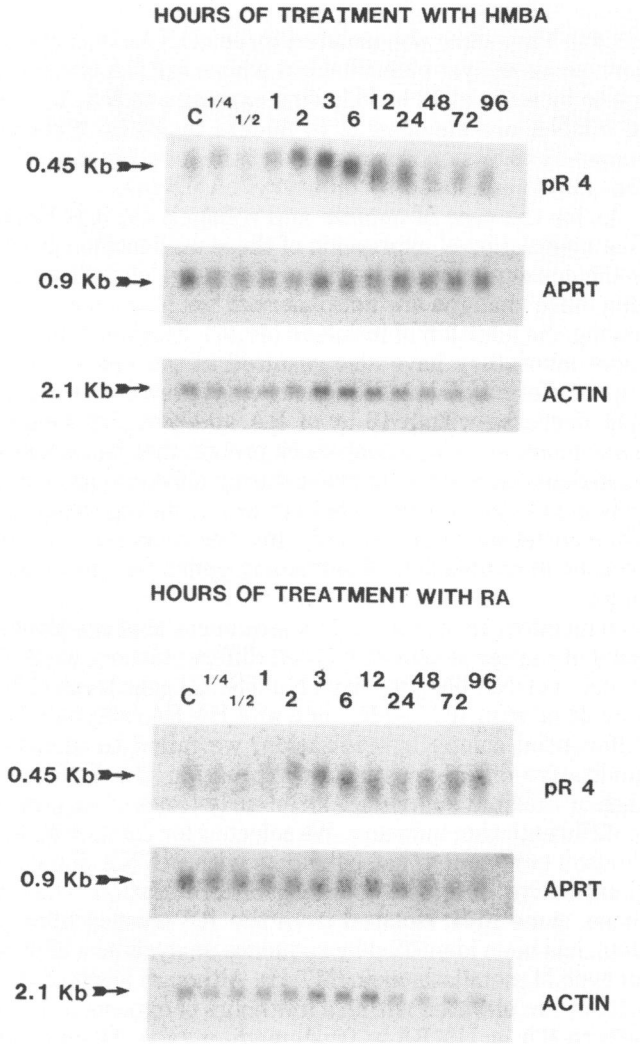

FIGURE 1. Northern blot analysis of MT II (pR4), β-actin, and APRT levels in Nulli-SCC1 EC cells. Inducers (10^{-6} M RA or 3 mM HMBA) were added to fresh medium, cultures were collected at the indicated times from addition of inducers, and RNA was isolated. Total RNA (5 μg) were fractionated by electrophoresis on 1.4% agarose-formaldehyde gels, blotted onto nylon membranes, and hybridized with [^{32}P]-labeled nick-translated plasmid pR4. After autoradiography the labeled probe was removed and the filters were reprobed in the same way with plasmid DNA containing β-actin or APRT sequences.

lamins, A, B, and C. Differentiation of F9 cells into visceral endoderm is accompanied by the appearance of lamin A and C; lamin B is present both in undifferentiated and differentiated cells (*68*).

Cell Surface Components

The SSEA-1 antigen has been very useful as a marker for EC cell differentiation because it is no longer detectable regardless of the resulting differentiated phenotype (*69,70*). It has been claimed that the SSEA-1 antigen is involved in cellular interactions during differentiation to endoderm (*71*). However, Rosentraus (*72*) isolated a mutant cell line from F9 cells (SOTF9-11) that is deficient in SSEA-1 binding activity and found that it exhibits the same tumorigenic, developmental, and cell adhesion properties as parental cells, suggesting that the SSEA-1 haptenic site is not required for those functions.

F9 EC cells express the blood group antigen I, but not i, on their surface. After 4 days of culture under conditions promoting formation of F9 embryoid bodies, the i antigen appears, and changes are observed in the distribution of the I antigen in the different cell layers (*73*).

Epidermal growth factor (EGF) has little or no effect on the proliferation or differentiation of mouse EC cells (e.g., PC13, OC15S1). These cells bind very small amounts of EGF. However, when they are induced to differentiate into endodermlike derivatives, the number of EGF surface receptors increases. The differentiated cells are induced to proliferate by the addition of the hormone. Treatment of the endodermal cells with RA causes a further increase in the number of EGF receptors (*74*). Similar effects have been observed with transforming growth factor beta (TGF-β) on PC13 and F9 cells. Differentiation of these two cell lines leads to a 16- to 40-fold increase in the binding of TGF-β, corresponding to an increase in the number of high affinity receptors. The differentiated cells are growth inhibited by TGF-β (*75*).

Secreted Proteins

The production of plasminogen activator is one of the more widely used markers for EC cell differentiation. This enzyme, which converts plasminogen into plasmin, is often secreted when EC cells are induced to differentiate (*76,77*). α-Fetoprotein is synthesized and secreted when EC cells differentiate into visceral endoderm, but not parietal endoderm, cells (*66,73,78*). Apolipoprotein E (apoE), a protein that plays an important role in cholesterol metabolism, is secreted in peripheral adult tissue and visceral yolk sac endoderm of midgestation embryos. The synthesis and secretion of apoE by F9 cells is correlated with the differentiation of these cells (*79*).

The study of EC cells under different *in vitro* conditions has established that EC cells condition their culture medium. It appears that multiple factors are produced, as medium conditioned by EC cells can support the growth of pluripotent cell lines from mouse embryos or promote the anchorage-independent growth of nontransformed cells (*80–82*). It has been shown that F9 and PC13 EC cells can produce a factor that is able to compete with human platelet growth factor (PDGF) for binding to membrane receptors. Differentiation results in a reduction in the secretion of this PDGF-like factor (*83*).

Protooncogenes

Protooncogene expression in undifferentiated and differentiated EC cells is detailed elsewhere in this volume (*84*), and so the topic will be dealt with only briefly here.

The cellular homolog of the transforming gene of the FBJ osteosarcoma virus, c-*fos*, encodes a nuclear protein with unknown functions. c-*fos* expression can be induced in a number of cell types by agents known to affect cell growth and differentiation (e.g., TPA, growth factors) but

its role in EC cell differentiation is not clear (*85,86*). Introduction of the c-*fos* protooncogene into F9 cells results in the appearance of differentiation markers in some of the cells. The fraction of morphologically altered cells remains the same even after many passages and is not increased by induction of c-*fos* expression. This suggests that other factor(s) as well are required for the induction of the differentiated phenotype (*87,88*). In fact, when F9 cells are induced to differentiate into parietal endoderm cells by RA and dibutyryl cAMP, only a small increase (3-fold) in c-*fos* mRNA is observed in 30 min, followed by a rapid return to uninduced levels (*89*). In the cell line 01A1, an increase in c-*fos* transcripts is evidenced during aggregation-induced differentiation regardless of the nature of the differentiated phenotype. The increase is first observed at 5 days after plating the aggregates, and a peak is reached on day 7, the same day on which maximal α-fetoprotein transcription can be detected (*90*).

Another protooncogene encoding a nuclear protein whose expression is induced when fibroblasts and lymphocytes are stimulated with growth factors is c-*myc*. Treatment of F9 cells with RA results in a 50% reduction in the level of c-*myc* mRNA after 3 hr of treatment and a 90% reduction after 12 hr. This early decrease has led some authors to propose a causal relationship to cell differentiation (*91,92*). However, the studies of Dean et al. (*93*) suggest a correlation with the rate of proliferation rather than differentiation. The p53 oncogene, like c-*myc*, decreases during differentiation of F9 cells in 2 to 3 days. Both c-*myc* and p53 appear to be posttranscriptionally regulated in EC cells (*94*).

Expression of N-*myc*, c-*src*, and c-*myc* was studied in four different EC cell lines (PCC7, PCC4, PCC3 and F9). N-*myc* mRNA is detectable in all cell lines and its expression decreases dramatically when PCC7 cells differentiate into neuronlike cells or when F9 cells differentiate into parietal endoderm. In PCC7 cells, the decrease in N-*myc* mRNA is paralleled by an increase in c-*src* expression. No c-*src* transcripts are detected in F9 cells, and PCC7 cells do not express c-*myc*. Serum starvation did not affect the expression of any of the protooncogenes analyzed (*95*).

Changes in Expression of Other Genes

With the exception of protooncogenes, whose role in differentiation of EC cells is still unclear, all of the alterations in gene expression described occur relatively late during the differentiation process, and they appear to be characteristic of the differentiated phenotype rather than to be involved in the initiation of the differentiation process *per se*. By two-dimensional gel electrophoresis, changes in protein synthetic profiles can be detected in Nulli-SCC1 and F9 cells as early as 3 and 6 hr, respectively, after the addition of RA to the cultures (*96,97*). Gudas and her colleagues (*60,61,98*) have analyzed cDNA libraries from PSA-G cells (derived from the PSA1 EC line) treated for several days with RA + dibutyryl cAMP. As mentioned above, they were able to identify cDNA clones for collagen IV and laminin, proteins whose levels were already known to increase during differentiation (*60,61*). They have also isolated three cDNAs that represent genes not yet identified but whose mRNA levels begin to increase at 12 hr following exposure to RA. Levels of mRNA are elevated 5- to 30-fold by 24 hr. This increased expression is controlled at the level of transcription and is enhanced by dibutyryl cAMP (*61*).

As for the case of laminin and collagen IV, it is likely that modulation of expression of these unidentified genes is the consequence, rather than the cause, of differentiation since changes are not observed for many hours following the addition of inducers (*60,61*). Studies from the same laboratory have also resulted in the discovery of eight different cDNA sequences whose levels of expression decrease within 12 hr of RA addition (*98*). One of these genes encodes a heat-shock protein that had already been demonstrated to decrease during differentiation (*99*). It is important to stress that critical initiating events in differentiation might equally involve increases or decreases in expression of particular genes (see next section).

In an effort to identify DNA sequences that are modulated at earlier stages of EC cell differentiation, we constructed cDNA libraries from Nulli-SCC1 cells treated for only 24 hr with 10^{-6} M RA or 3 mM HMBA (*100,101*). By differential colony hybridization, we failed to identify qualitative differences in the expression of mRNAs of high or medium abundance after early times of exposure to differentiation inducers. We selected for further investigation two clones that hybridize with mRNA showing quantitative differences during differentiation. One of these, clone pR4, isolated from the RA-treated library (*100*), has been identified by sequence analysis as a cDNA for type II metallothionein (MT II). Although levels of this mRNA are elevated within a few hours of exposure of the cells to RA or HMBA in fresh medium (Fig. 1), much of these early alterations are presumably a response to serum factors, since the differentiation-inducing agents alone have minimal effects on MT II mRNA levels (not shown). The steady state levels of MT II mRNA appear to increase in some EC cells with culture age (unpublished observations).

The second clone (pH34) that we analyzed in detail was isolated by U. Barkai from the HMBA-treated library and hybridizes to a 650-nucleotide mRNA (*101* and unpublished results). Expression of pH34 mRNA in Nulli-SCC1 cells increases modestly and transiently within minutes of addition of fresh medium. However, in the presence of HMBA, the level of this mRNA decreases in 8 hr, reaching barely detectable levels by 24 hr. Addition of RA produces a smaller decrease in the level of pH34 mRNA (2.5-fold at 48 hr) (*101*). The decrease of pH34 mRNA levels appears to be due to posttranscriptional regulation. The largest open reading frame predicted from the pH34 cDNA sequence would result in a 14 kd protein, which we have, in fact, observed in *in vitro* translation experiments. The predicted amino acid sequence of the protein shows no significant homology with other known proteins. pH34 mRNA is abundant in Nulli-SCC1 and F9 cells, which have a restricted pattern of differentiation, but is present at much lower levels in P19 and PCC4 Aza1R, EC cell

lines that differentiate more extensively. To date we have failed to detect pH34 mRNA in non-EC cell lines or adult and late gestation embryonic tissues. It remains to be determined whether this gene plays any role in EC cell differentiation (*101*; unpublished results).

Exogenous Gene Expression and Differentiation of Embryonal Carcinoma Cells

It is well documented that regulation of gene expression in mammalian cells can be transcriptional, posttranscriptional, translational, or posttranslational. The degree to which each of these mechanisms contributes to the establishment of the differentiated state of a cell and the maintenance of the differentiated phenotype remains to be elucidated. The use of small viral genomes as probes to study cellular regulatory mechanisms has provided an approach to the problem of gene regulation in animal cells since these viruses rely heavily on the host transcriptional and translational machinery to express their genetic program. EC cells are restricted to infection by papovaviruses and ecotropic type-C retroviruses, whereas they become permissive upon differentiation. There have been extensive and detailed studies with these viruses and EC cells because this system could shed light on mechanisms involved in regulation of viral gene expression, and because important information might be gained which could promote, by analogy, an understanding of how differentiation-specific genes are controlled.

Simian virus 40 (SV40) produces an abortive infection in differentiated mouse cells, resulting in expression of early viral proteins but no viral DNA synthesis or late viral protein expression. In some cases a small fraction of infected cells undergo malignant transformation (*102*). By contrast, EC cells are refractory to SV40 and early viral proteins cannot be detected following infection (*103–105*). It was shown initially that the block to infection of EC cells was not at the level of virus adsorption, penetration or transport to the nucleus (*103,104*). Moreover, SV40 DNA extracted from infected EC cells was infectious to monkey kidney cells, ruling out DNA modification as the blocking mechanism (*106*). Segal et al. (*105*) reported that only a small amount of nonspliced early viral RNA was present in SV40-infected EC cells, suggesting both transcriptional and posttranscriptional regulation. Induction of differentiation by RA and cAMP removed the block to viral gene expression (*107*).

The identification and dissection of the SV40 regulatory sequences that act to control viral gene expression have revealed the existence of a transcriptional enhancer region located upstream of the early promoter. This enhancer is typical in that it is *cis* acting, regulates both homologous and heterologous promoter elements, acts over long distances and is orientation independent (*108–110*). Knowledge of this enhancer region made possible a more detailed molecular analysis of the restrictive response of EC cells to SV40 early gene expression. By using DNA-mediated gene transfer techniques, Gorman et al. (*111*) showed that following calcium phosphate transfection of F9 cells with pTSV3, a plasmid containing the entire SV40 genome, 30 to 60% of the cells expressed both large T-antigen (T-ag) and small t-antigen (t-ag) as detected by indirect immunofluorescence at 40 hr posttransfection. The level of T-ag expression was shown to depend on the amount of viral DNA transfected. This dependence on DNA concentration occurred only with viral DNA and was not seen in differentiated cells. These data showed that the SV40 early promoter is functional when introduced into EC cells by calcium phosphate-mediated tranfection and that there is no absolute block to its expression. Since large amounts of viral DNA are introduced into individual cells by gene transfer, as opposed to viral infection, the authors suggested that most likely a negative regulatory factor(s) was being titrated out. However, alternative interpretations are possible. Unfortunately, in these studies, early viral RNA was not analyzed for efficiency of splicing.

Calcium phosphate transfection studies with homologous and heterologous promoter-enhancer constructs has shown that transcription from the SV40 early promoter is enhancer-independent in undifferentiated EC cells; i.e., the enhancerless promoter works as efficiently in transient expression assays as the enhancer-containing promoter. Moreover, Sassone-Corsi et al. (*112*) demonstrated that the only sequences upstream from the SV40 early promoter required to activate transcription from a β-globin promoter (otherwise silent in EC cells) was the GC-rich, 21 base-pair repeat region, the TATA box and sequences in between these two elements. In contrast, differentiation restores the requirement for the SV40 enhancer as shown by the lack of transcription from the enhancerless promoter in F9 cells treated with RA for 4 days (*111*).

If there is a repressor factor(s) in EC cells as suggested by Gorman et al. (*111*), it would be likely to act on the enhancer element since the enhancerless promoter does not seem to be subject to repression. This would appear contradictory to the results obtained with the enhancer-containing promoter, which is as active as the enhancerless promoter in EC cells. However, this apparent discrepancy could be explained by the presence in these cells of a *trans*-acting factor that renders the SV40 early promoter enhancer-independent. There is evidence to support the existence in undifferentiated (but not differentiated) F9 cells of a *trans*-acting regulatory protein that resembles the adenovirus (Ad) E1a proteins (*113*); experiments with other cell types suggest that E1a can repress the action of the SV40 enhancer (*114,116,117*). In fact, it has now been well documented that E1a can both activate and repress transcription of several genes. Therefore, it is not unreasonable to suggest that the SV40 early promoter activity in EC cells could be subject to both negative and positive regulatory mechanisms. This E1a-like activity could neutralize transcriptional activation from the SV40 enhancer but at the same time could render the promoter enhancer-independent by *trans*-activation at either the 21 bp repeat, the TATA box, or both. This possibility will be considered in more detail in the following discussion.

Further experimental evidence to support the concept that enhancers can be targets for repression in EC cells comes from studies with retrovirus long terminal repeat (LTR) promoters. Retroviruses do not replicate in EC cells. Moloney murine leukemia virus (Mo-MLV) and murine sarcoma virus (MSV) LTRs do not function as promoters in transient expression assays when transfected into F9 EC cells (*111,118*). In contrast, differentiated mouse cells use both viral LTRs very efficiently. Deletion of the enhancer region (a 72 bp tandem repeat) from the MSV LTR to some extent removed this repression, allowing appreciable levels of transcription to occur in undifferentiated F9 cells (*111*). As with the SV40 early promoter, transcription seemed to require at least the GC-rich region located upstream of the TATA box, as further deletion of this element rendered the LTR transcriptionally silent. Transcriptional activity of the Mo-MLV LTR was shown to be restored by replacing the 72 bp tandem repeat by a heterologous enhancer sequence known to be active in EC cells (*118*). The LTRs seem to be more effectively repressed than the SV40 early promoter in EC cells since enhancer-independent *trans*activation does not overcome the transcriptional block imposed by the presence of the MSV enhancer region. Furthermore, replacement of the SV40 enhancer by the MSV enhancer completely abolished transcription from the SV40 early promoter in EC cells (*111,112*). This repressor activity present in EC cells appears to be saturable since cotransfection with large amounts of either homologous or heterologous enhancer-containing constructs releases the inhibition of the MSV LTR normally seen in undifferentiated EC cells (*111*).

Studies on polyomavirus (Py) replication have added a genetic dimension to viral gene expression in EC cells. Undifferentiated EC cells are refractory to infection by Py: both viral transcription and DNA replication are blocked (*103,104*). However, the virus undergoes a full lytic cycle in differentiated mouse cells. This has provided a system to screen for Py mutants adapted to grow in EC cells following chronic infection of two cell lines, PCC4-azal (PCC4) and F9 (*119–124*). Host-range mutants capable of overcoming the expression block in EC cells were isolated and found to have mutations and/or sequence rearrangements in the enhancer region (also involved in viral DNA replication). Initially, Fujimura and Linney (*124*) showed that the F9-Py mutants affected two processes in F9 cells, one involving expression of Py early genes and a second involving viral DNA replication. Moreover, in transient expression assays the wild-type Py early region was minimally effective in promoting transcription from heterologous genes in EC cells, whereas DNA fragments containing Py enhancer mutants were severalfold more efficient in stimulating transcription of the same genes (*125*).

The Py enhancer region does not contain a tandemly repeated sequence like the SV40 or LTR enhancers. This region has been shown to include two distinct contiguous enhancer elements (*126*). The A element, located next to the late transcription unit (nucleotide 5021 to nucleotide 5128 of the Py sequence), contains a region of homology to the AdE1a enhancer (E1a-core), whereas the B element, extending from nucleotide 5128 to 5265, contains a region of homology to the SV40 enhancer (SV40-core). Both elements work independently in promoting transcription from heterologous genes in transfection experiments, although with different cell specificities. For instance, element A provided a 3-fold enhancement of the α-2-collagen promoter than element B in 3T6 cells. By contrast, in PCC3 EC cells, the B element showed the same efficiency as in fibroblasts, whereas element A was several-fold less efficient (*126*). In summary, host-range mutants adapted to grow in F9 cells always exhibit modifications in the B element, usually a single base pair change (AT to GC at position 5233), often followed by a tandem duplication also containing the point mutation. On the other hand, PCC4-adapted mutants lack the B element and contain a duplicated A element.

Two groups have recently demonstrated that the E1a products of Ad5 and Ad12 repressed expression from the Py early promoter in 293 (a human transformed cell line that constitutively expresses low levels of the E1a proteins) or HeLa cells (*114,127,128*). The target of repression was again shown to be the enhancer region. Unexpectedly, E1a proteins could even repress enhancer mutants which have overcome block to expression in F9 and PCC4 EC cells (*128*), although other studies suggest that the dosage of E1a must be high for repression of enhancer mutants to occur (*127*). Taken together these results and studies with other cell lines (*127*) suggest that: a) the Py early promoter is more sensitive to repression by E1a than is the SV40 promoter; b) Py enhancer mutants adapted to growth in EC cells require elevated levels of the E1a proteins to be repressed; and c) E1a proteins more efficiently *trans*-activate the enhancerless SV40 promoter than the Py promoter.

Whether the *trans*-activator and repressor activities present in EC cells reside in the same or different protein molecule(s) remains to be determined. However, it is possible to construct a scenario that could explain how a cellular E1a-like activity, specific to undifferentiated cells, could result in the observed transcriptional regulation of the SV40 and Py early promoters and retroviral LTRs in EC cells. It is likely that the SV40 enhancer is either not repressed or only modestly repressed by the low levels of E1a-like proteins existing in EC cells. Transcription from the early SV40 promoter would thus be expected to be weakly *trans*-activated by this E1a-like factor(s) in an enhancer-independent manner by interaction, either directly or indirectly, with sequences located between the enhancer and the start site for the early mRNA.

Upon differentiation, the E1a-like activity declines to unmeasurable levels and could be replaced by a new factor(s) that *trans*-activate(s) the SV40 promoter in an enhancer-dependent fashion. This model seems to be contradictory to the DNA concentration dependency for large T-ag expression observed by Gorman et al. (*111*). These authors suggested that a negative regulatory factor(s) was being titrated out. However, other interpretations are possible. For instance, a positively acting transcription factor could be present at limiting levels and/or

be of low affinity such that no transcription would take place until a threshold level is reached. Alternatively, as shown earlier, splicing could be playing a major role in the block to SV40 early gene expression in EC cells, and no mature, correctly spliced T-ag mRNA would be observed until the rate of transcription reached a threshold. Two observations support the latter explanation. First, concentration dependency was only observed when Gorman et al. used viral DNA and measured expression of T-ag. Second, in transient expression assays where splicing was not required for production of mature functional mRNA, the enhancerless SV40 early promoter was transcriptionally indistinguishable from the enhancer-containing promoter. Thus, further experiments that address this question of splicing in SV40 early expression in EC cells are required before more definitive conclusions can be drawn.

In contrast to SV40, retrovirus LTRs and the Py early promoter seem, overall, to be subject to negative regulation in EC cells by repression targeted to the enhancer. The repressor factor(s) is likely to be an E1a-like activity. Repression overrides enhancer-independant *trans*-activation of these promoters. This is consistent with the observation that the Py early promoter is more sensitive to E1a repression than the SV40 promoter and that the MSV LTR enhancer suppresses enhancer-independent *trans*-activation of the SV40 early transcription unit.

In the case of the Py promoter there is evidence to suggest that the B enhancer element is the target of repression. For example, Py mutants able to grow in PCC4 EC cells always lack the B enhancer but possess a duplicated A enhancer. On the other hand, F9 Py mutants always contain an altered and, in many cases, duplicated B enhancer. By contrast, the A element is never deleted in F9 or PCC4 EC mutants; thus, it is unlikely to be the target of repression. Rather, once strong repression of the early promoter is released by deletion of the B element in PCC4-adapted mutants, a second site for a weak and/or low abundance *trans*-activating factor (acting on the A element) is created that can now synergize with enhancer-independent *trans*-activation, allowing more efficient expression of the Py promoter.

F9-adapted Py mutants seem to follow a different strategy to overcome the block to expression, perhaps reflecting subtle differences in gene expression when compared to other EC cells. Whereas negative regulation may be abolished by the point mutation, it is unlikely that such a change would be so specific (always at the same position) and so often duplicated only to release suppression. Thus, it is plausible to propose that such an alteration creates a new sequence motif that can now facilitate the binding of a positively acting factor present in F9 cells (but probably inactive or absent in PCC4 cells).

A nuclear factor has recently been described to be present in F9 cells that can discriminate between the wild-type and a F9 Py mutant enhancer. This protein factor, which can bind to the mutant sequence but very poorly to the wild-type, is present in a variety of cells, including F9 cells and differentiated derivatives (*129*). This is in good agreement with the observation that F9 Py enhancer mutants are always more efficient than the wild-type Py enhancer in promoting transcription from heterologous promoters, not only in F9 cells but also in a variety of differentiated cells, including RA-treated F9 cells (*125,126*). In addition, F9 Py mutants with tandem duplications containing the point mutation are at least twice as efficient as mutants without it (*125*). Since F9 Py mutants are still restricted in PCC4 cells, this lends support to the idea that the single base pair change in element B does not only release repression. Instead, it is possible that the new sequence motif created allows the positively acting factor described above to compete out the repressor, overcoming the block to expression. In contrast, PCC4-adapted Py mutants can still be expressed in F9 cells, although at low efficiency.

That the Py early promoter is subject to negative regulation in EC cells has also been suggested by experiments with protein synthesis inhibitors. Cremisi and Babinet (*130*) showed that treatment of Py infected-PCC4 cells with low doses of cycloheximide for 24 hr allowed substantial expression of T-ag (25- to 60-fold increase), suggesting the presence of a short-lived repressor protein(s). Moreover, cycloheximide treatment of PCC4 cells infected with a PCC4-adapted mutant showed only a marginal effect (3- to 10-fold increase) on T-ag expression, again suggesting that the main target of repression is the B enhancer.

Transcriptional regulation appears to be the major mechanism used by EC cells to restrict expression of papovaviruses and ecotropic type-C retroviruses; however, it is increasingly evident that in many cases viral transcription units are not completely silent in these cells, strongly suggesting additional control mechanisms in virus suppression. For example, early studies with retrovirus-infected EC cells showed that following infection and integration, the provirus became heavily methylated (*131*). This led to the suggestion that DNA methylation was responsible for the block to expression. However, it was later demonstrated that methylation was a late event, suggesting an effect of, rather than the cause for, lack of expression (*132,133*). In addition, methylated provirus genomes could not be activated even after induction of differentiation, a situation known to restore virus permissiveness.

Recent studies on retrovirus restriction in EC cells have uncovered some new information that could shed some light on other control mechanisms. Infection of EC cells with retroviruses leads to integration of proviral DNA into the genome at normal levels, but viral-specific RNA is detected at less than 1% the level of infected NIH/3T3 cells (*131,132*). However, infection of EC cells with recombinant retroviruses containing the selectable neomycin phosphotransferase gene results in rare colonies that are resistant to the antimetabolite G418 (*134–136*). Studies of these host-range variants have indicated that proviruses can overcome suppression by two different mechanisms: integration at or near *cis*-acting host DNA sequences and mutations in the viral control elements. Several proviruses whose expression is mediated by 5′-flanking host sequences have been isolated. These studies have

shown that there are a limited number of chromosomal positions in the cellular genome of EC cells that allows virus expression (*136*). Not surprisingly, one study led to the isolation of one of these flanking sequences which was shown to behave as an enhancer element in transfection assays (*137*).

Using the same approach, Barklis et al. (*136*) isolated a Mo-MLV host-range variant with a single point mutation in the 5′ untranslated region of the virus (within, the tRNA primer binding region). In contrast to Py, no host-range variants with single base pair changes within the LTR enhancer have been isolated. However, a mutant of MSV, the myeloproliferative sarcoma virus (MPSV), which has been shown to be efficiently expressed in F9 EC cells (but not in PCC4 cells), contains several specific point mutations within the U3 region of the LTR when compared to the MSV and Mo-MLV LTRs (*138–141*). Most of these mutations are clustered in or around the enhancer region (*142*). Additional changes and rearrangements are required for expression in PCC4 cells (*143*). Thus, more than a single base pair change within the LTR seems to be required to alter the host-range specificity of retroviruses in EC cells, and this explains the difficulty in isolating such mutants by this procedure.

The host-range mutant with the single base pair change within the tRNA primer-binding site has been shown to be expressed in both F9 and PCC4 cells (*144*). This mutation enhances the level of stable RNA present in F9 cells by about 10-fold, whereas it has no effect in NIH/3T3 cells. How this mutation actually increases the levels of RNA is presently unknown. Because it maps outside the LTR, it is tempting to speculate that a posttranscriptional mechanism is involved. For instance, the 10-fold enhancement of stable neomycin phosphotransferase mRNA (transcribed from the gene driven by the viral mutant LTR) contrasts with a 1000-fold stimulation in the formation of G418-resistant colonies in F9 and PCC4 cells (*144*).

Whether the extent of selectable gene expression is now close to threshold levels or whether the point mutation affects, for example, the efficiency of translation, are issues that require resolution before further speculation is warranted. Nevertheless, it should be pointed out that there are precedents for the involvement of 5′-untranslated sequences in the regulation of retrovirus gene expression (*145,146*). Whatever the mechanism of regulation might be, it does not seem to involve $tRNA_{pro}$ primer binding because the MPSV 5′ noncoding sequence does not differ from the Mo-MLV sequence within the tRNA binding region itself, but in sequences close to it. However, MPSV is as active as the B2 mutant in transducing neomycin resistance in EC cells (*144*). The corresponding Mo-MLV and MSV 5′-untranslated regions are inactive. Since the recombinant retroviruses used in these studies lack most, but not all, of the viral functions, it is not clear whether a viral or a cellular factor mediates control at this site.

From all of the above studies it is apparent that EC cells are unusual in that they can actively suppress expression from otherwise strong viral transcription units. In some cases (e.g., Py, LTRs), transcriptional enhancers in differentiated cells actually become the target of repression in EC cells. In other instances (e.g., the SV40 early promoter), suppression of transcription in EC cells is not absolute, although viral proteins are not produced, suggesting posttranscriptional regulation as yet another mechanism responsible for virus suppression. Upon differentiation of EC cells, the block to expression of these viral genomes is removed and their enhancers become fully functional. Since no viral proteins are required in the early stages of virus expression, repression must be mediated by cellular factor(s) present in EC cells but either absent (or at low abundance) or inactive in differentiated cells. In addition, it is likely that new factor(s) required for enhancer function are induced during differentiation (*147*).

Endogenous differential gene expression could be similarly regulated by EC cells. In other words, differentiation-specific genes could be maintained in a state of repression in undifferentiated EC cells by specific negative regulatory factor(s), perhaps the same ones that suppress viral gene expression. If this is the case, how, then, is the cascade of events leading to differentiation triggered? One can imagine that either repression of negative factor(s) or induction of new activator(s) would serve to overcome or reverse the effect of the repressor molecule(s). Recent evidence obtained by Montano and Lane (*148*) strongly suggests that it is the repression of certain genes that triggers irreversible differentiation of EC cells. These authors showed originally that transfection of EC cells with plasmids encoding Ad5 E1a gene products underwent radical morphological and biochemical changes characteristic of the differentiated state induced by treatment with RA. However, they then demonstrated that a plasmid encoding the E1a enhancer-dependent repressor activity, but lacking the *trans*-activating activity, induced the same changes as the complete E1a coding region.

Further support for the repressor hypothesis derives from our observation that exposure of EC cells to low concentrations of cycloheximide [a treatment which allows substantial expression of Py T-ag in Py-infected PCC4 cells (*130*)], results in differentiation of a significant proportion of cells in the cultures (P. Abarzúa, unpublished observations). We propose, therefore, that EC cells are maintained in the undifferentiated state by the presence of one or more short-lived repressor proteins. We also feel it likely that viral genes are regulated in a negative fashion in undifferentiated EC cells because their regulatory sequences share elements in common with genes selectively expressed in differentiated cells.

In view of the close relationship between EC cells and germ cells (*3*), it is possible that refractoriness of EC cells to viral gene expression reflects an evolutionary mechanism for minimizing virus infection of germ cells (which in turn would serve to regulate the introduction into the species of new genetic material).

It seems reasonable to assume from the studies reviewed in this section that in both viral and cellular systems, repressors and *trans*-activating factors will compete and interact with differing affinities and specificities for any genetic control region, allowing a delicate differential

control of gene expression. In this context, the stem cell phenotype of EC cells can be thought of as a dynamic equilibrium maintained by the interaction (and relative abundance) of both positive and negative regulatory molecules with specific sequence motifs within regulatory elements of critical genes. Inducers of differentiation could then readily be envisaged to act by disturbing this equilibrium as a consequence of either direct or indirect changes in gene expression.

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