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High efficiency DNA transfection in murine embryonal carcinoma cells: expression of pSV3neo in wild type and retinoid-resistant cell lines

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ABSTRACT **Embryonal carcinoma cells provide a convenient and manipulable model for early embryogenesis. Like their counterparts in the inner cell mass, they are refractory to infection by several viruses. In their undifferentiated state, EC cells are resistant to calcium-phosphate DNA transfection. This resistance is compounded by the inefficient and/or actively inhibited expression of transfected genes driven by certain viral promoters. Conversely, the differentiated derivatives do not share this resistance and readily express virally promoted genes. We have developed a protocol for liposome-mediated gene transfer in EC cells and compared its efficiency in wild-type and retinoid-resistant variants. Dose response experiments with the EC cell line PCC4.aza1R showed a linear progression of colony formation when transfected with the vector pSV3neo and selected in medium containing the antibiotic G418. DNA concentrations of 10 μg per plate resulted in over 600 colonies per 10^6 cells. This represents a 20-30 fold greater efficiency over reported values for calcium-phosphate methods even though the neomycin resistance gene in this plasmid is driven by the SV40 viral promoter. The retinoid-resistant line PCC4(RA)$^{-2}$ also showed enhanced transformation by lipofection, but despite the relatively high efficiency, colony formation rate for the differentiation-defective cells was less than 25% of the parental line. Our data indicates that there is no absolute block of genes driven by the SV40 early region promoter in murine EC cells if enough DNA is introduced to titrate out negative regulatory factors.**

KEY WORDS: *embryonal carcinoma, virus, gene expression, transfection*

Introduction and review

The ability to study mechanisms of early events in mammalian development is often hampered by the technical limitations of the embryo. Murine embryonal carcinoma (EC) cells provide an effective model for studying these events as they are similar to inner mass cells of the blastocyst and can be manipulated to undergo analogous differentiation (Pierce, 1967; Lehman and Speers, 1974). As with early mouse embryos, murine EC cells show a host range restriction for virus-cell interaction and are resistant to infection by Moloney murine leukemia virus (MMuLV), SV40 and polyoma (Py) virus (Swartzendruber and Lehman, 1975; Speers and Lehman, 1976). Conversely, post-blastocyst embryonic cells and the differentiated derivatives of EC cells do support permissive Py and MMuLV infections and exhibit a nonpermissive infection by SV40 with T antigen expression (Segal and Khoury, 1981). These findings suggest that the mechanisms controlling the expression of viral genes in differentiating EC cells parallel the cellular controls of gene expression in early development and that the EC cell system may be useful for identifying some of these mechanisms.

The Moloney murine leukemia virus (MMuLV) is a class C retrovirus, the expression of which has been widely studied in EC cells. Expression and replication of this virus is restricted in undifferentiated EC cells. However, differentiated EC cells are permissive for productive MMuLV infection (Peries *et al.*, 1977). Teich *et al.* (1977) showed that the time of differentiation was an important determinant as to whether or not the cells would be permissive. EC cells infected with MMuLV and then induced to differentiate did not produce virus. If EC cells are first induced to differentiate and then infected, virus is produced. MMuLV is able to penetrate the cell membrane, reverse transcribe itself to double-

Abbreviations used in this paper: RA, retinoic acid; EC, embryonal carcinoma; MMuLV, Maloney murine leukemia virus; Py, polyoma; LTR, long terminal repeat; PBS primer binding site; NRE, negative regulatory element.

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stranded DNA and migrate to the nucleus where it integrates into the host genome. The block to expression and replication of progeny virus presumably occurs here, after integration. No viral transcripts could be detected in undifferentiated EC cells. It was shown that the block to viral growth could be complemented for by fusion of undifferentiated EC-A EC cells (a derivative of PCC4.aza1) with the permissive mouse embryo fibroblast line SC-1 (Gautsch, 1980). The best results were obtained when fusion was performed 8-12 h after viral infection. These results suggested that MMuLV requires some host cell factor at a discreet time during its life cycle. To determine whether this was a cytoplasmic or nuclear factor, fusions with either cytoplasm or nuclei from permissive cells were performed (Gautsch, 1982). Complementation occurred only after fusion of EC-A cells with nuclei from the permissive SC-1 line. MMuLV DNA can be detected in EC cells 10 h after infection and it can be detected in the nucleus 10-12 h post-infection. The DNA is not inactivated in the EC cytoplasm. If a differentiated nucleus is introduced into the cell 12 h after infection, the viral DNA could enter it as well as the native EC nucleus. The DNA entering the differentiated nucleus may be integrated and transcribed resulting in the production of progeny virus by the heterokaryon while DNA entering the native nucleus is still being functionally repressed. Two possible explanations were offered: some function required for viral growth is present in the differentiated nuclei that is either not present or is non-functional in the undifferentiated nuclei. This could be a factor or enzyme needed for RNA transcription or processing. The other possibility is that the EC nucleus may interrupt the progression of proviral replication by an inhibitor acting to repress transcription.

Methylation of MMuLV DNA was proposed as a possible mechanism of repression when it was shown that the integrated sequences of the MMuLV genome were highly methylated at GCGC sequences (Stuhlman, 1981). Several studies had previously shown an inverse correlation between methylation levels and eukaryotic gene expression (Razin and Riggs, 1980; Sutter and Doerfler, 1980; Wigler, 1981). MMuLV DNA was shown to become methylated upon integration into undifferentiated EC cells but not upon integration into the differentiated cell genome. This methylation correlated with the loss of infectivity and lack of viral gene expression in undifferentiated EC cells (Stewart *et al.*, 1982).

Further studies to determine the role of DNA methylation in the repression of MMuLV expression were undertaken using 5-azacytidine, an inhibitor of methylation (Niwa *et al.*, 1983). 5-azacytidine is able to activate endogenous viral genomes in both avian and mouse embryo fibroblasts. Treatment of infected, undifferentiated EC-A1 cells did not result in the activation of the MMuLV genome suggesting that methylation does not cause repression in these cells. It was also shown that methylation of MMuLV sequences occurs very slowly (10-15 days) and may be a consequence of methylation of inactive genes. It would appear that by the time methylation occurs, the viral genome has already been inactivated. Additionally, comparison of the methylation patterns between undifferentiated and differentiated cells showed no differences.

Other evidence that methylation was not repressing MMuLV expression was that the viral genome can be activated by treating EC cells with bromodeoxyuridine. This thymidine analog becomes incorporated into the DNA and is thought to interfere with DNA:protein interactions. This evidence supported the possibility of a cellular factor binding to the DNA to either repress or activate MMuLV.

The restriction of expression of polyoma and MMuLV in EC cells led to the speculation that a similar mechanism of action functioned for both viruses (Linney *et al.*, 1984). Polyoma mutants, altered in their enhancer regions and able to replicate in EC cells had been isolated. Deletion mutants of the MMuLV enhancer region were made to determine whether this was also true for MMuLV. Removal of the MMuLV long-terminal repeat (LTR) resulted in a complete loss of production of infectious viruses upon transfection into NIH-3T3 cells. Chloramphenicol acetyltransferase (CAT) constructs with the MMuLV LTR replaced by the mutant polyoma enhancer were made and transfected into NIH-3T3 cells and the EC line F9. Induction of CAT activity was observed in both lines. No CAT activity was detected in the F9 cells when the construct was driven by the MMuLV LTR. These data suggested that the MMuLV LTR functioned as an enhancer and that the primary block in MMuLV infection was at the level of the LTR.

Several laboratories began to focus their efforts on the MMuLV LTR. It was postulated that like the polyoma virus, there might be a defect in early viral replication and that EC cells may contain a factor that negatively regulates viral enhancers, thus inhibiting transcription from the viral promoters. Transfection studies utilizing the neomycin resistance gene driven by deletion mutants of either the MMuLV enhancer, promoter, or both were performed (Loh *et al.*, 1987). Evidence for a negative repressor was not found. Rather, it appeared that a lack of a positive regulatory factor in undifferentiated EC cells resulted in restricted expression. Additionally, an element within the provirus appeared to play a role in the EC-specific down-regulation. This element was within the RNA leader sequence of the genomic transcript. It was localized to a 29 base pair region that included the tRNA primer binding site (PBS).

Other laboratories used gel retardation experiments to determine DNA:protein interactions at the level of the MMuLV LTR. Using crude nuclear extracts, in one instance, four sequence-specific binding sites were described, one of which was specific for undifferentiated EC cells (Flamant *et al.*, 1987). It was named the EPBF-binding site and was located between nucleotides -87 and -59, which includes the CCAAT box portion of the promoter. They showed that deletion of the CCAAT box reduced viral infectivity, but had a limited effect in transient expression experiments. This suggested that transient expression and infection experiments were not directly comparable and may have accounted for some of the conflicting data. The implication was that the effect of EPBF may not be detectable in a transient assay.

Conflicting data were reported by other laboratories (Speck and Baltimore, 1987; Tsukiyama *et al.*, 1989). Using gel retardation assays, Speck and Baltimore (1987) found binding sites for six distinct nuclear factors within the MMuLV LTR, one of which was not present in EC cells. This was a binding site for NF-1, a conserved SV-40 core-like motif found in several other genes. Tsukiyama *et al.* (1990) also found several proteins that bound to the LTR, one of which was specific for undifferentiated EC cells. This protein, called ELP, bound upstream of the enhancer region and caused specific repression in EC cells. Proteins binding in the region of the CCAAT box were identified in both differentiated and undifferentiated cells. This conflicted with Flamant's data (1987), possibly due to differences in the techniques used for collecting the nuclear extracts. Additionally, differences in the numbers and amounts of proteins that bound to the enhancer and GC-rich regions were detected when the cells were induced to differentiate. These proteins were thought to be transcriptional activators and their shortage may cause

repression of the LTR in undifferentiated EC cells. Two mechanisms of LTR repression were proposed: a lack of activator proteins and the presence of a negative factor such as ELP.

The negative regulatory element (NRE) within the tRNA primer binding site (PBS) was further characterized by Loh *et al.* (1990). This intragenic domain restricted expression by 20-50 fold in EC cells, but not in permissive cell types. A single point mutation at nucleotide position +160 (G to A) completely abolished the repression. Also, competition with the leader fragment allowed rescue of expression in EC cells, suggesting that the EC-specific restriction was caused by recognition of sequences at or near the tRNA PBS domain by a negative *trans*-acting factor. The NRE inhibited expression in an orientation- and position- (relative to the promoter) independent manner. It was proposed that this domain acted as a silencer element, analogous to those seen in yeasts.

Specific DNA-factor interactions were detected at the NRE in both undifferentiated and differentiated EC cells. However, significantly less complex was detected in the differentiated cells. Again, this argued for a *trans*-acting cellular repressor that restricts MMuLV expression in EC cells through interaction with the negative regulatory element at the tRNA primer binding site.

Several mechanisms are probably acting in tandem to repress MMuLV expression in undifferentiated EC cells. Specific factors remain to be isolated and characterized. Additionally, with the exception of methylation, primary and secondary effects have not been well characterized. Currently, it appears that repression occurs at the enhancer as well as the tRNA PBS. The enhancer is inactive, possibly due to a negative repressor or to a lack of positive activators. At the tRNA PBS, inhibition may be mediated by a *trans*-acting factor, probably at the level of transcription.

As with MMuLV, early studies utilizing SV40 and Py showed a consistent lack of susceptibility to infection in undifferentiated cells. The mechanisms governing this phenomenon were not readily apparent. Possible explanations included simple failure of the virus to absorb or penetrate the host cell. Alternatively, blocks in uncoating or rapid degradation of the viral genome were also proposed. Using isotopically labelled virus and electron microscopy, Swartzendruber *et al.* (1977) found that SV40 and polyoma virus are capable of penetrating EC cells and can travel to the nucleus. Later, Friedrich and Lehman (1981a,b) showed that intracellular SV40 DNA, introduced either by infection or transfection, was gradually lost from the cell, was not irreversibly modified, and recoverable SV40 DNA could be infectious in permissive cells. Evidence of faulty splicing of SV40 RNA was presented by Segal *et al.* (1979) which was compatible with RNA degradation and a nonproductive infection, lack of viral genome integration, and a subsequent loss or dilution of viral DNA from infected EC cells. Using somatic cell hybrids, Balint *et al.* (1980) proposed that the block in viral RNA processing might be the lack of appropriate cellular functions which would be induced upon differentiation. Post-transcriptional events were also invoked by Linnenbach *et al.* (1980) in their studies utilizing a construct containing the SV40 genome in tandem with the herpes thymidine kinase gene. Transfection into thymidine kinase-deficient EC cells resulted in early viral transcripts but no T antigen expression. However, others noted that if splicing was the only block, T antigen expression might be expected following differentiation of putatively infected EC stem cells (Knowles *et al.*, 1980). As this did not routinely occur except in cases of viral genome integration, Friedrich and Lehman (1981b) postulated that the presence of a repressor might be a key factor in viral gene expression. Furthermore, they concluded that if a repressor was operant, their transfection experiments suggested it could be removed by phenol extraction.

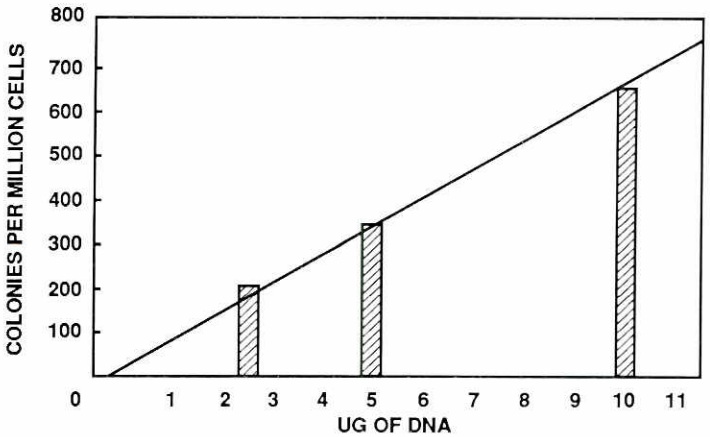

Fig. 1. Dose-response curve for lipid-mediated DNA transfection of PCC4.aza1R cells. *EC cells were treated with 40 μg of DOTMA and increasing amounts plasmid DNA. A near linear progression of colony formation was obtained in the range tested.*

Investigations involving the interactions of polyoma virus and EC cells resulted in the isolation of viral mutants capable of productive infections in undifferentiated cells (Katinka *et al.*, 1980; Vasseur *et al.*, 1980). Analysis of these mutants showed alterations in the noncoding region near the origin of replication. Fujimura *et al.* (1981) demonstrated that a single point mutation in this region was sufficient to confer infectivity. Constructs utilizing mutated Py enhancer regions showed active expression in undifferentiated EC cells (Hen *et al.*, 1986). Extensive deletion mapping of similar regions in SV40 clearly defined the regulatory sequences governing RNA transcription (Fromm and Berg, 1982). Subsequent construction of positive selection vectors with genes of interest driven by the SV40 early-region promoter allowed further exploration of the regulation of viral gene expression in EC cells (Southern and Berg, 1982). Nicolas and Berg (1983) found that transcription dependent on this promoter was very inefficient in EC stem cells but was markedly increased in differentiated derivatives. Additional studies with genes driven by a composite SV40-herpes thymidine kinase promoter demonstrated efficient expression in both differentiated and undifferentiated cells (Nicolas and Berg, 1983; Rubenstein *et al.*, 1984). Using a transient expression system and variable plasmid constructs, Sleigh and Lockett (1985) found that functional enhancer sequences were necessary for increased expression in differentiated F9 cells when transfected with genes driven by the SV40 early region promoter. Coincident transfection experiments by Gorman *et al.* (1985) stressed that negative regulation of viral enhancers in undifferentiated EC cells was dominant and supported the proposal of a titratable trans acting repressor element. Taken in sum, these investigations and many others indicated the presence of both negative and positive regulators in the control of viral RNA transcription in stem cells. Complicating issues governing which factor(s) dominates included cell type, state of differentiation

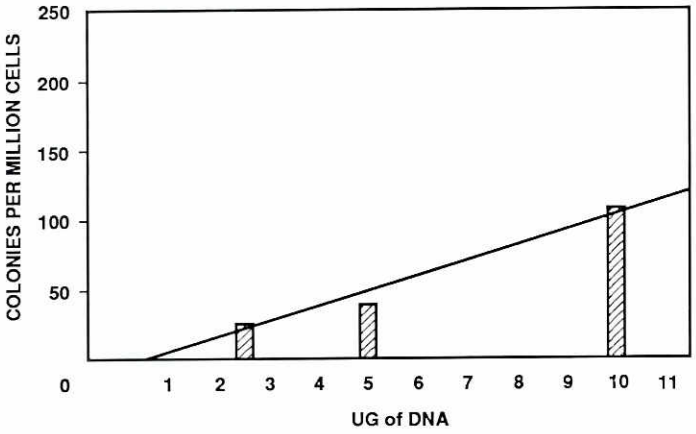

Fig. 2. Dose-response curve for lipofection of PCC4(RA)$^{-2}$ cells. *As with the parental line, the differentiation-defective mutant cells showed a linear response to increasing plasmid concentrations, albeit at a lower level.*

and stage of development (Kellermann and Kelly, 1986; Hales and Lehman, 1987; Nomiyama *et al.*, 1987; Bronson *et al.*, 1990).

Negative regulation of viral gene expression in EC cells appears to be competitively mediated by a *trans*-acting, labile protein factor. The resistance of PCC4 cells to polyoma virus infection was shown by Cremisi and Babinet (1986) to be partially blocked by coinfection with SV40. Additionally, they were able to remove the resistance by temporarily inhibiting protein synthesis with cycloheximide. Transcription of the intermediate filament endo A gene was also enhanced upon cycloheximide treatment of undifferentiated stem cells, supporting the analogy for similar regulation of early gene expression in embryogenesis (Cremisi and Duprey, 1987). Later experiments supported these results and showed that the shared negative regulation of polyoma virus, SV40 and the endo A gene could be derepressed by the products of the proto-oncogene c-*myc* (Onclercq *et al.*, 1989). Along similar lines, Sleigh *et al.* (1987) performed competition experiments to investigate repressor function in F9 cells. Utilizing an excess of polyoma virus enhancer region sequences co-transfected with a chloramphenicol acetyl transferase marker driven by the SV40 early region promoter, a small but measurable increase in CAT activity was found. By varying constructs, repressor binding was localized to one half of the polyoma enhancer and was lost upon further fragmentation. Assuming genes driven by the SV40 early region promoter were under net negative regulation, Sleigh (1987) reasoned that appropriately transfected cells treated with cycloheximide should show an increase in expression due to the removal of repressor molecules. This in fact was found but, interestingly, an increase was seen in undifferentiated and differentiated cells. These results indicated repressor molecules were present in both developmental states. The c-*myc* experiments by Onclercq *et al.* (1989) also showed increased viral gene expression in differentiated states, suggesting gene repression and activation were operating in tandem and that the net regulation of early developmental gene expression involved more than simple derepression. La Thangue and Rigby (1988) have argued convincingly that the concentration of positive regulating factors represented the limiting step in the control viral gene expression. They developed an *in vitro* transcription system to investigate the regulation of SV40 early gene expression in EC cells utilizing whole cell extracts from differentiated and undifferentiated F9 cells. Mixing experiments showed the differentiated cell phenotype was dominant and also confirmed that a transacting repressor influenced transcription in both cell types. However, in their system, the activity of SV40 driven genes in stem cell extracts remained low relative to differentiated cell extracts despite high template conditions.

A direct way to test for the influence of repressor elements in undifferentiated EC cells might be to put increasing amounts of SV40 driven genes into the stem cells and quantitate expressing colonies. This strategy should titrate out any negative regulatory factors, resulting in an increase in transformed cells. Depending upon the presence of rate-limiting positive activators, the rate of increase may or may not be linear. Unfortunately, murine EC cells are somewhat refractory to calcium-phosphate DNA transfection (Pellicer *et al.*, 1980). Transformation frequencies (expressed as the fraction of plated cells producing expressing colonies) vary from $1x10^{-6}$ to $3x10^{-5}$. Compounding the technical aspects of transfection is the fact that various promoters governing gene expression in exogenous constructs do not function with the same efficiency in EC cells (Hasegawa *et al.*, 1990). We have undertaken studies to optimize DNA transfer into undifferentiated EC cells utilizing an SV40 driven reporter gene for neomycin resistance (pSV3neo). Methods and dose response curves were analyzed.

Additionally, we examined the expression of the pSV3neo under optimum conditions in differentiation-defective mutant cells. Through liposome mediated DNA transfer, we have achieved transfection frequencies of $6x10^{-4}$ in wild type cells.

Results

All experiments were performed in triplicate. Lipofections were repeated three times. Values in the Figures represent averages of all experiments. Fig. 1 shows the dose response curve for lipid

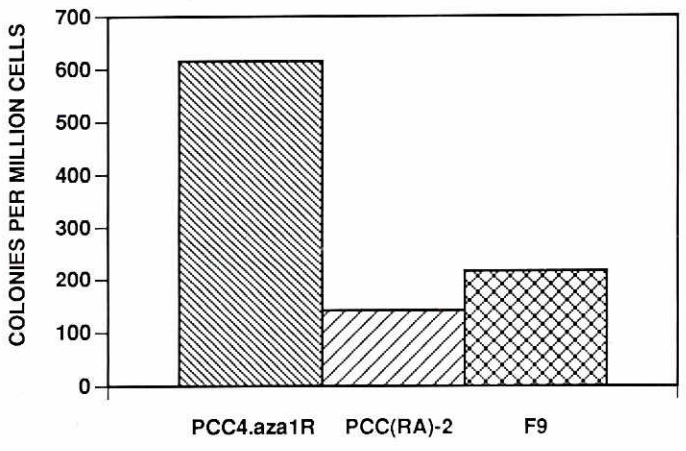

Fig. 3. DNA transfection frequencies of murine EC cell lines using cationic liposome-mediated transfer. *Three different cell lines were transformed with 10 μg of plasmid vector and 40 μg of DOTMA. Under optimized conditions, PCC4.aza1R cells yielded 616 colonies per million cells. F9 cells gave 216 colonies and the differentiation-defective line PCC4(RA)$^{-2}$ produced 142.*

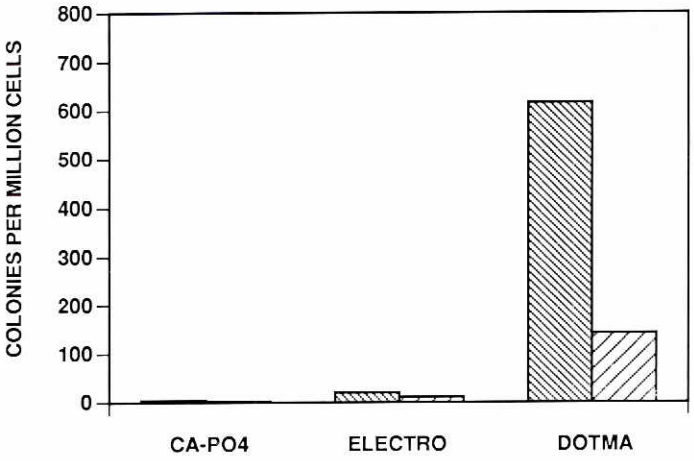

Fig. 4. Colony yields for transfection techniques using PCC4.aza1R and PCC4(RA)$^{-2}$ cells. *With the murine EC cell line PCC4.aza1R, average counts of 4, 20 and 616 were obtained via calcium-phosphate precipitation, electroporation and lipofection respectively. PCC4(RA)$^{-2}$ cells gave counts of 2, 11 and 142 for the three methods, respectively.*

mediated DNA transfection in PCC4.aza1R cells. At a concentration of 10 μg of plasmid, over 600 colonies per 10^6 cells were obtained. The dose response was linear in the range tested. This range was somewhat limited by the toxicity of the DOTMA and the recommended ratio of DNA to lipid. PCC4(RA)$^{-2}$ cells also showed a linear response, albeit at lower frequencies (Fig. 2). A maximum of approximately 10^{-4} was obtained for the differentiation-defective cells.

Additional lipofections were performed comparing the three cell lines PCC4.aza1R, PCC4(RA)$^{-2}$ and F9 using an optimized concentration of 10 μg of pSV3neo and 40 μg of DOTMA. These results are depicted in Fig. 3. Frequencies of 6.16×10^{-4}, 1.42×10^{-4} and 2.16×10^{-4} were obtained, respectively. The majority of colonies from all three cell lines retained their EC morphology after transfection. Individual cells were small, rounded, and contained one or more prominent nucleoli. In the presence of 10^{-7}M retinoic acid(RA), PCC4.aza1R and F9 cells responded as usual. The cells enlarged, flattened, and assumed a polygonal shape. Many cells sent out processes. The PCC4(RA)$^{-2}$ line retained its undifferentiated phenotype upon exposure to RA. Transformed colonies of PCC4.aza1R cells did show some spontaneous differentiation, accounting for approximately 5% of the transformants. Rare differentiated colonies were observed in transfected F9 and PCC4(RA)$^{-2}$ cells, occurring at a rate of less than 0.5 in 10^7 cells. Genomic DNA from long-term cultures is currently being analyzed for neomycin phosphotransferase sequences. The retention of neomycin resistance over several passages (20-40) suggests that the plasmid has stably integrated into the host genome.

Concurrent experiments were performed with PCC4.aza1R and PCC4(RA)$^{-2}$ cells comparing calcium phosphate transfection and electroporation to lipofection. In each set, 10 μg of pSV3neo and 40 μg of DOTMA was used. The results are shown in Fig. 4. In our hands, PCC4.aza1R cells averaged 4 colonies per 10^6 cells for the calcium phosphate method and 20 colonies for electroporation. Others have reported frequencies of 2×10^{-5} for wild type murine EC cells using calcium phosphate precipitation (Gorman *et al.*, Wagner and Mintz, 1982; De Groot *et al.*, 1990).

Total RNA was isolated and used for slot blot and northern blot analysis. Slot blots showed that the neomycin resistance gene is expressed in all transfected lines tested (see Fig. 5).

Northern blot analysis of PCC4aza.1R plus pSV3neo (clone A), PCC4(RA)$^{-2}$ plus pSV3neo (clone B), and untransformed controls were performed. An approximately 2.8 kb band representing the neomycin phosphotransferase mRNA was detected in the two transfected cell lines tested. This band was not present in the untransfected cell lines (Fig. 6).

Discussion

Although the murine EC cell has been shown to be a convenient model for gene expression in early development, DNA transfection studies are often hampered by relatively low colony yields. Deciding whether this is a problem of method or a result secondary to *cis*- or *trans*-acting regulatory elements is often problematic. Our experiments show high transformation rates can be obtained in undifferentiated EC cells utilizing cationic liposomes (Felgner *et al.*, 1987). Frequency rates of over 6×10^{-4} have been achieved as compared to 2×10^{-5} via calcium phosphate techniques (Wagner and Mintz, 1982; De Groot, 1990). Lipofection also appears to have a yield advantage over electroporation in our hands, showing a 20-fold

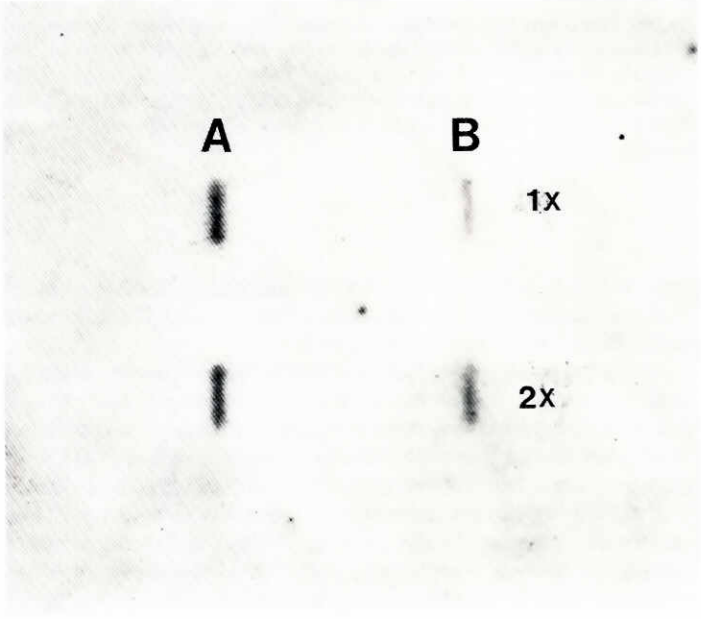

Fig. 5. RNA slot blot analysis of PCC4.aza1R and PCC4(RA)$^{-2}$ cells transfected with pSV3neo and probed with the Bam HI/Hind III fragment. *Total RNA was harvested from representative colonies transformed with pSV3neo and selected in the antibiotic G418. RNA from PCC4.aza1R, clone 1A is shown in column A at 1X and 2X concentrations; column B contains RNA from PCC(RA)-2, clone 1B.*

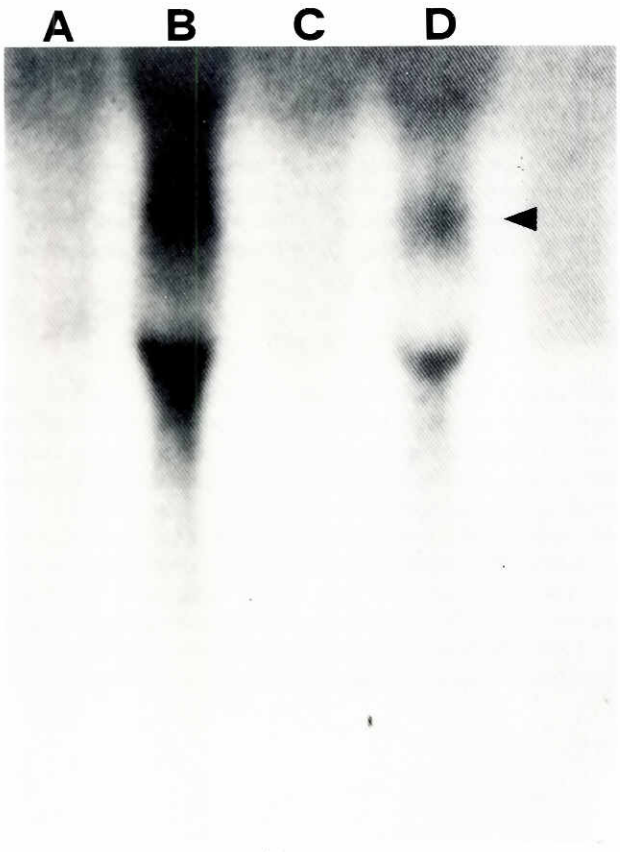

Fig. 6. Northern blot analysis of transfected and mock transfected PCC4.aza1R and PCC4(RA)$^{-2}$ cells. *The filter was hybridized with the Bam HI/Hind III fragment of the neo gene. The lanes are as follows:* **(A)** *PCC4.aza1R;* **(B)** *PCC4.aza1R plus pSVSneo;* **(C)** *PCC4(RA)$^{-2}$;* **(D)** *PCC4(RA)$^{-2}$ plus pSV3neo. Note 2.8 kb band in lanes B and D. Ribosomal marker present at 2.1 kb.*

increase. This enhanced frequency is comparable to other efficient cell lines transformed by conventional techniques (Southern and Berg, 1982; Rubenstein *et al.*, 1984).

Using the positive selection vector pSV3neo, all undifferentiated lines had frequencies on the order of 10^{-4}. The pluripotent cell line PCC4.aza1R showed the highest transfection rates via lipofection of all lines tested. The differentiation-defective variant PCC4(RA)$^{-2}$ gave the lowest and the restricted EC cell line F9 was intermediate. The majority of transformed colonies maintained the usual EC cell phenotype, although in the case of PCC4.aza1R cells, approximately 5% showed morphologic evidence of spontaneous differentiation. This phenomenon may in part account for the higher transfection rates. Interestingly, PCC4(RA)$^{-2}$ cells, representing the other end of the differentiation spectrum, gave the lowest rates. The resistance to transformation held true for all methods tested, including past experiments with microinjection (Taketo *et al.*, 1983). Perhaps the induced mutation(s) in these cells involves the regulation of repressor elements preventing the induction of early developmental genes and subsequent differentiation.

Genes driven by the SV40 early region promoter have long been known to be inefficiently expressed in murine EC cells. Analogies have been drawn to the resistance of the blastocyst to certain viral infections and to the way early gene expression is regulated in the developing embryo (Speers and Lehman, 1976). This regulation is most likely multifactorial, but in part depends on the presence of *trans*-acting repressor elements. The importance of repressor elements in the EC system has been postulated by several authors (Friedrich and Lehman, 1981b; Gorman *et al.*, 1985; Sleigh *et al.*, 1987). Experiments with metabolic inhibitors pointed to a labile protein factor (Cremisi and Babinet, 1986; Cremisi and Duprey, 1987; Sleigh *et al.*, 1987). *In vitro* mixing experiments and *in vivo* co-transfection studies have indicated limiting amounts of repressor elements were present in EC cells and could be titrated out (Sleigh *et al.*, 1987; La Thangue and Rigby, 1988). Studies by Gorman *et al.* (1985) have shown that high levels of transforming DNA resulted in relatively efficient functioning of the SV40 early promoter in EC cells and that there was no absolute block to its utilization. *In vitro* transcription systems have given equivalent results under conditions of competing templates for repressor binding (La Thangue and Rigby, 1987). Similarly, Hales and Lehman (1987) have demonstrated that the block to SV40 expression in EC cells can be overcome in a transient manner at high template concentrations.

Our data supports the presence of a titratable repressor element in undifferentiated murine EC cells. We agree with Gorman that there is no absolute block to expression of genes driven by the early region of SV40 and, if enough transforming DNA can be introduced into the cell, efficient expression occurs in a linear, dose-response manner (Gorman *et al.*, 1985). A similar result has been reported with the standard calcium phosphate technique using the SV40 early region cloned in front of a promoterless rabbit beta-globin gene (Nomiyama *et al.*, 1987). In this instance, it was proposed that high transfection efficiencies might have titrated out putative repressor factors. Additionally, the linearity of our results (in the DNA concentrations tested) argues for the predominance of negative regulation over limiting amounts of positive activators. The use of lipofection appears to overcome the inherent restricted capacity of EC cells to take up exogenous DNA and consistently give high transfection efficiencies. Whether this is also true for inner mass cells has yet to be determined.

Materials and Methods

Cells and tissue culture

PCC4.aza1R EC cells and their differentiation-defective mutant derivatives PCC4(RA)$^{-2}$ were obtained as previously described. (McCue *et al.*, 1983) Cells were plated into 60 mm culture dishes at a density of $5x10^5$ and grown until 85% confluent (approximately 2 days) in Dulbecco's Modified Eagle Medium (Mediatech, Herndon,VA) supplemented with 10% fetal calf serum (HyClone, Logan, UT). F9 cells were grown on gelatin-coated flasks (0.1%, Sigma, St. Louis, MO.). Retinoic acid was also obtained from Sigma.

Plasmid DNA

pSV3neo was obtained from the American Type Culture Collection (Rockville, MD). The plasmid contains the neomycin resistance gene from the TN5 transposon as well as an SV40 transcription unit in the EcoRI/PvuII fragment of pBR322, and the entire SV40 early region inserted into the BamHI site of pBR322.

Lipofection

10 µg pSV3neo was mixed with 1.5 ml Optimem (Gibco BRL, Gaithersburg, MD). DOTMA lipofectin reagent (BRL Life Technologies, Gaithersburg, MD) was mixed with 1.5 ml Optimem at a concentration of 40 µg per plate. The

aliquots were then carefully mixed to avoid precipitation of the DNA/DOTMA complexes and incubated for 1 h at 37°C. The 3 ml mixtures were then added to the plates.

Prior to transfection, the cells were washed twice with Optimem and incubated with the DNA-Liposome mixtures overnight at 37°C and 5% CO_2. After 18 h in the DNA/DOTMA/Optimem medium, the cells were washed twice with DME and then allowed to recover 6 h in cDME. The cells were then harvested with trypsin and plated into 100 mm dishes at a density of $5x10^6$ cells per plate and incubated overnight. The next morning, the medium was replaced with 15 ml selection medium consisting of cDME plus 500 µg/ml G418. Selection medium was changed as needed to keep the cultures healthy. Colonies were counted after 10 days in selection medium. Several colonies were subcloned and expanded for further study. Controls included untransfected plates as well as plates treated with DOTMA alone.

Electroporations

Cells were harvested with PBS and washed twice in electroporation buffer containing 30 mM potassium acetate, 220 mM sucrose, 0.3 mM dibasic potassium phosphate and 0.85 mM monobasic potassium phosphate. The pH was adjusted to 7.2 with acetic acid. The cells were resuspended at $1x10^8$ cells per millimeter and incubated on ice for 10 min with 10 µg plasmid DNA. The mixture was placed into 6-well tissue culture plates and pulsed for 10 milliseconds at 300 volts using a Hoefer Pro-Genetor (Hoefer Scientific Instruments, San Francisco, CA). Cells were then incubated 10 min at 37° C and 5% CO_2 with sealing buffer (120 mM NaCl, 3.5 mM KCl, 8 mM K_2HPO_4, 0.5 mM MgAc, 0.1 mM CaAc, 10 mM glucose). Cells were then plated into cDME. G418 at 500 µg/ml was added to the medium 24 h later.

Calcium phosphate transfections

Cells were plated into 100 mm dishes at a density of $5x10^5$ and allowed to grow 24 h before transfection. The culture medium was changed 2 to 4 h before transfection. A solution containing 10 µg pSV3neo in 250 mM $CaCl_2$ was incubated 30 min at room temperature. This solution was mixed slowly with an equal volume of Hepes buffered saline solution (280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4, pH 7.05). The precipitate was applied directly to the cell cultures at a final concentration of 5 µg DNA per dish. Glycerol shock (25% in DME) was performed for 2 min after having incubated the cells plus DNA for 4 h. Cells were then washed with PBS and cDME was added. Two days later the cells were trypsinized and split into 100 mm dishes. Medium containing 500 µg/ml G418 was added one day later.

RNA extraction

Total RNA was obtained from cultured cells using the guanidine thiocyanate/phenol/chloroform procedure outlined by Chomczynski (Chomczynski and Sacchi, 1987).

Northern blot analysis

Total RNA (40 µg per lane) was size fractionated on a 0.9% formaldehyde agarose gel. RNA was transferred to Nytran filters (Schleicher and Schuell, Keene, NH) according to manufacturer's instructions. The Bam HI/Hind III neomycin cDNA fragment was labeled with the random prime method to a specific activity of $1x10^8$ cpm/µg DNA. Hybridizations were performed overnight at 42°C in buffer containing the radiolabeled probe, 50% formamide, 1X SSPE, 0.1% SDS and 1X Denhardt's solution. The filters were washed twice at room temperature for 15 min each in 6X SSPE/0.1% SDS followed by two washes at 37°C for 15 min each in 1X SSPE/0.1% SDS. A final wash was performed for 15 min at 65°C in 0.1X SSPE/0.1% SDS. Autoradiographs were developed after 4 days at -70°C.

Slot blot analysis

Slot blot analysis was performed as described by Maniatis (Maniatis *et al.*, 1989). The Bam HI/Hind III neomycin cDNA fragment was radiolabeled to a specific activity of $1x10^8$ cpm per µg DNA using the random prime method. Blots were hybridized overnight in buffer containing the labeled probe, 50 mM PIPES, 100 mM NaCl, 1M Na_2HPO_4, 400 mM EDTA and 5% SDS. Washes were performed in 5% SDS and 1X SSC at 65°C for 30 min. Autoradiographs were developed after 24 h.

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