Use of Quantitative Cell Transformation Assays in Risk Estimation

J. Carl Barrett and David G. Thomassen

ABSTRACT

The use of cell transformation assays to study chemical carcinogenesis allows the analysis of cellular events in neoplastic progression without interference of certain host factors such as immunological surveillance and cell–tissue interactions. An overview of cell transformation systems is presented with a discussion of the multistep nature of neoplastic progression. A hypothesis has been made that different cell transformation systems measure different stages in the transformation process and therefore their mechanisms are different. Particular emphasis is given to cell transformation systems which can quantitatively measure carcinogen-induced changes and the factors which influence this quantitation. The quantitative use of cell transformation assays in risk estimation, particularly dose–response studies, are also discussed. The use of cell culture models for the study of tumour promotion is reviewed from a mechanistic viewpoint. Systems exist to study initiation–promotion phenomena in cell culture, and the relevance of these studies to the in vivo process of tumour promotion is considered in terms of the differences in cell–cell interactions in vitro and in vivo. The use of cell transformation systems to analyse the multistage process of carcinogenesis and the relationship between cell transformation and mutation are discussed.

1 INTRODUCTION

Assessment of cancer risk associated with environmental chemicals requires experimental systems capable of measuring quantitatively their carcinogenic and tumour-promoting activity. Cell culture systems are potentially very useful experimental models for such studies. Inexpensive, short-term cell culture assays are available which have a good ability to detect known carcinogens with few false positive results (Barrett et al., 1980). In contrast to several other short-term tests, cell transformation assays are not based on a theoretical relationship between carcinogenesis and mutagenesis or other genotoxic endpoints but on the assumption that neoplastic transformation induced in cells in culture occurs by
the same mechanisms as neoplastic alteration of cells in vivo (Barrett and Elmore, 1984). This assumption can be tested by comparing results of carcinogenesis experiments in vivo and in vitro.

The difficulties that exist in the use and interpretation of cell culture assays include:

1. the more specialized technical expertise needed for these assays compared with assays using prokaryotic cells, and the variability of the assays,
2. the artificial condition of cells in an in vitro environment,
3. the many phenotypic endpoints for cell transformation that can be studied and their interrelationships, and
4. inadequate understanding of possible prealtered states of certain cells established in culture for extended times.

The first point is important in deciding whether to use these assays. It is now clear that, although they are variable, many cell transformation systems are reproducible and have been studied in numerous laboratories. The variability that occurs is generally due to differences in serum, which is a required but undefined component of most cell culture systems. Hopefully, this variable will be eliminated in the future with the advent of more defined culture conditions (Sato and Ross, 1979a,b).

There are two ways to view the artificiality of cell culture methods. The first is that cells in culture are abnormal because they are in an in vitro environment. For example, fibroblasts which are normally quiescent in vivo, proliferate extensively in culture. The second view is that cells in vitro respond normally to an abnormal environment. The ‘abnormal’ proliferation of cells in culture can be considered analogous to the stimulation of proliferation of these cells in vivo following insults such as wounding (Boone et al., 1979). The difference between these two views is more than semantic. The first view implies that placing a cell in culture makes it abnormal regardless of its environment. The second view suggests that cells in culture and in vivo are intrinsically similar but may respond differently to different environments. This would imply that better understanding of the control of the cell by its extracellular environment would allow a mimicry in vitro of the in vivo behaviour of a cell. This also suggests that cell culture techniques can be used to study the regulation of cell growth and differentiation as well as the loss of this regulation.

Recent advances in the study of factors that influence cellular responses in culture strongly support the second view. In the past, most differentiated cells failed to express differentiated functions in culture, which was cited as evidence for the abnormality of cells in culture. With changes in extracellular matrices, growth hormones and differentiation inducers, a variety of differentiated cells can now be grown and maintained in culture (Sato and Ross, 1979a,b) and these cells differentiate in culture in a manner analogous to their differentiation in vivo. When differences between the differentiation of cells in vivo and in vitro are
observed, further changes in culture conditions often result in expression of the *in vivo* function. For example, methods were developed to grow human keratinocytes in culture and these cells expressed most of the major keratin polypeptides found *in vivo*, but not one high molecular weight keratin (Fuchs and Green, 1978). However, removal of one factor (retinoid) from the medium resulted in the expression of this protein (Fuchs and Green, 1981). In certain cases where the fully differentiated state is not observed with cells in culture, the ‘normality’ of the cells can still be demonstrated. For example, rat tracheal epithelial cells in culture do not normally express mucociliary function. However, if the cells in culture are reintroduced into an *in vivo* environment (by repopulation of a trachea stripped of its epithelium and subcutaneous transplantation of this trachea), the cultured cells form normal mucociliary epithelium (Terzaghi et al., 1978). This demonstrates that cells in culture maintain the intrinsic capacity to differentiate and the environment of the cells influences the expression of these functions.

We feel that these experiments demonstrate the applicability of cell cultures for the study of normal cellular growth and differentiation. This strengthens the justification for the use of such models for the study of neoplastic alterations of cells and the influence of carcinogens on this process. The reliable response of cell transformation assays to carcinogenic agents (see section 3.1) also provides evidence that the neoplastic conversion of cells *in vivo* and *in vitro* involve similar processes. Although in these studies mainly fibroblasts have been used, recent advances in the understanding of the growth and differentiation of cells in culture have contributed to the knowledge of neoplastic transformation of epithelial cells (see section 2.4.2).

The relevance of different phenotypic endpoints associated with neoplastic transformation of cells and the differences among cell transformation systems are mechanistic considerations which are important in the interpretation of results with cell transformation assays and their use for quantitative risk assessment. These points will be addressed in the next section which also includes a synopsis of the available quantitative cell transformation assays. The final section of this review includes a discussion of the use of cell transformation assays for risk assessment, including their application in studying multistage aspects of carcinogenesis and tumour promotion in cell culture, and the relationship between cell transformation and other genetic endpoints, such as gene and chromosome mutations.

2 AN OVERVIEW OF CELL TRANSFORMATION SYSTEMS

2.1 Introduction

Before discussing experimental systems which employ cells in culture for the study of neoplastic transformation, an essential feature of cancer development *in
vivo needs to be addressed. There is now considerable evidence that neoplastic development occurs as a progressive process through qualitatively different stages (for review see Barrett et al., 1980). Experimental support for a multistage model of carcinogenesis is provided by many diverse studies including studies with cells in culture which have clearly demonstrated the progressive nature of neoplastic development. For example, if one starts with a culture of normal, diploid embryo fibroblasts, these cells will progress to a preneoplastic stage prior to conversion of the cells to a neoplastic or tumorigenic stage (Barrett et al., 1980). This process will occur either spontaneously or following treatment by a carcinogen which increases the rate and frequency of the process. A preneoplastic cell is defined as a cell which is altered in such a way that it has an increased propensity to become neoplastic as measured by tumorigenicity in vivo. In culture, preneoplastic cells often acquire some, but not all, the properties of a tumour cell. In addition, these cells acquire several other phenotypic changes which may or may not be necessary to the malignant phenotype (Barrett et al., 1979).

There are two essential points that need to be emphasized here. First, it is important to consider whether one is studying the transformation of normal cells to a preneoplastic state or the transformation of preneoplastic cells to the neoplastic or some other state. Second, the mechanisms of transformation of cells from one stage to another may vary depending on the starting point. Thus, the mechanism of transformation of normal cells to preneoplastic cells may be different than that for the change of preneoplastic cells to neoplastic cells. These two points should be considered in any discussion of the biological significance of cell transformation systems.

2.2 Neoplastic Transformation of Cells in vitro

2.2.1 Phenotypic Alterations Associated with Preneoplastic and Neoplastic Cells

The use of cell transformation assays in risk assessment depends on our ability to identify newly altered cells exhibiting characteristic preneoplastic or neoplastic phenotypes. One of the difficulties in studying neoplastic transformation is the lack of a definitive phenotypic cellular alteration which can serve as a reliable characteristic marker of malignancy. Of course, tumour formation in vivo serves to define neoplastic transformation of cells in vitro, but tumorigenicity is a multifaceted phenomenon which is difficult to analyse at the molecular or subcellular level. In addition, quantitative determination of tumorigenicity is time-consuming and costly.

Several other phenotypes have been associated with neoplastic cells in vitro and the elucidation of the significance of these cellular changes for tumorigenicity is critical to the study of neoplastic transformation. It is beyond the scope of
this review to discuss the significance of all phenotypic changes associated with neoplastic transformation (for references see Barrett and Ts'o, 1978; Franks and Wigley, 1979; McGrath et al., 1980; Yuspa et al., 1980). However, several phenotypes associated with neoplastically transformed cells do deserve special consideration. The term transformation or transformed cell indicates only an altered cell population and does not indicate how the population is altered. Its use should, therefore, be limited to instances when its meaning is clear in the context of discussion. More complete descriptions, such as neoplastic transformation, morphological transformation, transformation to anchorage independence or to an established cell line should be more frequently used.

Morphological transformation is of particular importance, as it is the basis for quantitation of most in vitro assays of carcinogen-induced transformation (see section 2.4) (Berwald and Sachs, 1965). Unfortunately, not all neoplastic cells exhibit the same alterations in morphology and some do not exhibit such changes at all. In addition, the assays are subjective and, therefore, the criteria used vary between laboratories and sometimes even between those studying the same cells. Despite these difficulties, alterations in morphology can be used to predict a preneoplastic or neoplastic alteration of cells in vitro, especially of fibroblasts. Morphological transformation alone is not sufficient for neoplastic transformation of normal diploid cells (Barrett et al., 1979). Morphological transformation is induced in these cells shortly after treatment with a carcinogen, but these cells are non-tumorigenic at the time of morphological change, and further growth in culture is required before neoplastic transformation can be demonstrated (Barrett and Ts'o, 1978; Barrett et al., 1979).

Anchorage independence of growth refers to the ability of cells to grow while suspended in a semisolid medium such as agar, agarose, or methylcellulose. Anchorage independence has correlated well with tumorigenicity in many studies (see Colburn et al., 1978b; Barrett et al., 1979) but it has also been expressed without tumorigenicity in many other studies (for example, Stanbridge and Wilkinson, 1978; Marshall and Sagar, 1981; Thomassen and DeMars, 1982). In addition, cellular growth in semisolid medium is more affected by components of the culture media (for example, serum and other supplements) than is cellular growth in liquid medium (Leavitt et al., 1977).

In the absence of readily discernible morphological alterations, carcinogen-induced transformation of epithelial cells in vitro is often scored by measuring alterations in cellular differentiation (Kulesz-Martin et al., 1980, 1981), growth potential in vitro (Marchok et al., 1977; Slaga et al., 1978; Barrett et al., 1983; Thomassen et al., 1983), or focus formation and cell line production (Terzaghi and Nettlesheim, 1979; Pai et al., 1983). Like morphologically transformed fibroblasts, cells exhibiting these early changes are not tumorigenic. The relationship between early carcinogen-induced changes in cell growth or differentiation and tumorigenicity is not known, but there is evidence which suggests that these changes are preneoplastic (Marchok et al., 1978).
Neoplastic transformation is the endpoint of *in vitro* progression as described above and it is defined as the accumulation of cellular changes resulting in the potential for the relatively autonomous growth of cells or tissue (Pitot, 1978). Neoplastic transformation is assayed by injecting cells into suitable syngeneic (see for example, Barrett and Ts'o, 1978), immunosuppressed (see for example, Marchok et al., 1978), or immunocompetent (see for example, Stanbridge and Wilkinson, 1978) hosts. Although it is important to demonstrate that carcinogen-altered cells progress to neoplastic cells to ensure that these changes are in fact preneoplastic changes, the development of neoplastic potential may not be critical for risk assessment using *in vitro* assays. In most cases (see below), carcinogen-altered cells express one of the phenotypic changes discussed above and only later do they become neoplastic as a result of secondary changes. Therefore, in most cell transformation assays neoplastic potential is only a final measure of carcinogen-induced alteration, and more frequent earlier alterations are induced by carcinogens which increase the potential for subsequent neoplastic development.

### 2.2.2 Systems for Studying Neoplastic Transformation of Cells in Culture

Several experimental systems using cells from a variety of species and tissues have been employed for studies of cell transformation by chemical carcinogens. Because fibroblasts were employed in initial studies, these systems are the best characterized and have been employed for most of the mechanistic studies to date. However, 80% of all human cancers are of epithelial origin (Harris and Cerutti, 1982); therefore, systems employing epithelial cells are very important for understanding of human malignancies and for risk assessment. At present, neoplastic transformation of epithelial cells *in vitro* by chemical carcinogens has been reported for cells derived from bladder (Hashimoto and Kitagawa, 1974; Summerhayes, 1979; Summerhayes et al., 1981), epidermis (Fusenig et al., 1973, 1978; Elias et al., 1974; Colburn et al., 1978a; Slaga et al., 1978; Kulesz-Martin et al., 1980, 1981; Ananthaswamy and Kripke, 1981), kidney (Borland and Hard, 1974), liver (Montesano et al., 1973; Williams et al., 1973; Borenfreund et al., 1975; Katsuta and Takaoka, 1975; Mondal, 1975; Schaeffer and Heintz, 1978), mammary gland (Richards and Nandi, 1978; Richards et al., 1980); salivary gland (Wigley, 1979), and trachea (Marchok et al., 1977, 1978; Steele et al., 1979; Pai et al., 1983; Thomassen et al., 1983). Neoplastic transformation of glial cells has also been reported (Laerum and Rajewsky, 1975; Roscoe and Claissé, 1978).

Neoplastic transformation of fibroplastic cells (for references see Barrett and Ts'o, 1978) have been reported with Chinese hamster lung cells, rat embryo cells, guinea pig fetal cells, Syrian hamster embryo cells, BHK cells, mouse 3T3 cell lines, a mouse ventral prostate line, and the mouse C3H/10T1/2 cell line (Table 1). The Syrian hamster embryo cells and the various mouse cell lines have
been used most often in studies employing fibroblastic cells. Neoplastic and preneoplastic transformations of normal human fibroblasts have also been reported in recent years (DeMars and Jackson, 1977; Freedman and Shin, 1977; Kakunaga, 1978; Milo and DiPaolo, 1978; Namba et al., 1978; Borek, 1980; McCormick et al., 1980; Sutherland et al., 1980; Silinskas et al., 1981).

In addition to these systems, virally infected cultures of rat, mouse and hamster cells have been employed (Hollstein et al., 1979; IARC, 1980; Heidelberger et al., 1983). Viral and chemical cocarcinogenesis appears to provide a sensitive assay for carcinogens. However, this represents enhancement of viral carcinogenesis, at least in some systems, and the significance of these observations to the mechanism of chemical carcinogenesis is unclear.

2.2.3 Qualitative Versus Quantitative Analysis of Cellular Transformation

Each of the transformation systems listed above describes the preneoplastic or neoplastic transformation of cells in vitro. However, many of these reports, most notably those involving epithelial cells, are only qualitative in their analyses of transformation events. The number of systems in which truly quantitative studies have been done on neoplasia-related changes is somewhat limited (see sections 2.3, 2.4.1, 2.4.2). Quantitation of the frequencies of carcinogen-induced changes is important in risk assessment where the relative risk imposed by any given chemical or treatment must be determined accurately. We will therefore focus on quantitative aspects of neoplastic transformation in vitro and on those cellular systems in which such quantitation is possible.

2.3 Desirable Features for Quantitative Transformation Systems

Four features are desirable in a quantitative transformation system:

(1) it should use normal, early passage cells as targets for carcinogens,
(2) the target cells employed must be capable of sufficient proliferation after treatment to allow for fixation and expression of carcinogen-induced alterations,
(3) altered cells must be readily recognizable or selectable, and
(4) the system must enable the quantitation of the number of cells exposed to the carcinogen and the number of cells surviving the treatment.

Permanent cell lines (for example C3H/10T1/2, 3T3, BHK) may play an important role in studies of neoplastic transformation and risk assessment. Such lines are useful since they can be cloned so that homogeneous cell populations can be used in some transformation studies. These lines can also be induced to express many neoplasia-associated phenotypes following exposure to a wide
variety of carcinogens. However, the use of aneuploid cell lines in cell transformation systems may result in misleading interpretations of transformation experiments with respect to transformation of normal cells. As noted above, neoplastic transformation is the result of accumulation of changes by initially normal diploid cells. Although relatively little is known about the number or nature of those changes required for neoplastic transformation (see Thomassen and DeMars, 1982), it is reasonable to assume that most, if not all neoplasms originate from diploid cells. Studies of cellular transformation, especially those in which there is concern about the biological effects of various chemicals or treatments, should therefore use normal early passage cells, if at all possible, to examine the effects of those treatments on the initial stages of neoplastic transformation. The use of early passage cells is important because changes which may affect the interpretation or outcome of transformation experiments can occur in cells as they are propagated in vitro. Cell ploidy can also affect the induction and expression of carcinogen-induced cellular changes. A given alteration could have an increased, decreased or equal chance of expression in an aneuploid cell compared with a diploid cell depending on the dominance of the expression of the change induced (Thomassen and DeMars, unpublished data). Therefore, results obtained using aneuploid cell lines must be viewed cautiously and compared with those obtained with diploid cells. Finally, both normal early passage cells and preneoplastic cell lines are important for studying the effects of carcinogens on neoplastic progression. The transitions between later stages of neoplastic transformation are also affected by carcinogens (Marshall and Sager, 1981; Bouck and di Mayorca, 1982; Thomassen and DeMars, 1982), so the effect of carcinogens on all stages of neoplastic transformation can be evaluated by using various culture systems. Cell transformation systems using either normal early passage cells or permanent cell lines are useful for studying various aspects of neoplastic transformation and for risk assessment; however, until we understand the nature of early, preneoplastic changes, it is important that both types of systems be used for in vitro transformation experiments.

Fixation and expression of carcinogen-induced alterations are essential if those changes are to be quantitated and analysed. Proliferation of altered cells allows fixation of any genetic damage present and time necessary for the expression of the effects of that damage and for the amplification of the number of cells possessing that alteration. This requirement is one factor that has limited progress in epithelial cell transformation studies because most epithelial cells grow poorly in culture, especially when compared with fibroblasts.

The need for recognizable and selectable markers for carcinogen-altered cells is self-evident if one is interested in quantitating the frequency of such cells. Identification or selection of altered cells from carcinogen-treated epithelial cell populations has been much more difficult than for similarly treated fibroblast populations.
Cellular systems in which clonal survival of carcinogen treatment and the clonal detection of altered cells can be quantitated are extremely advantageous for mechanistic and risk assessment studies. The most reliable index of cell survival is colony formation since only cells which survive and proliferate can be considered in the quantitation of carcinogenic events.

2.4 Quantitative Systems for Studying Early Carcinogen–induced Changes in Cell Culture

2.4.1 Fibroblast Systems

Table 1 provides a summary of fibroblast systems in which carcinogen-altered cells can be readily quantitated. There are many differences between the systems and several key features will be pointed out. Among the rodent cell systems, only the Syrian hamster embryo cells are early passage diploid cells while the other cells are all aneuploid cell lines and are therefore subject to the potential difficulties discussed above. Three phenotypes are scored among these various cells following carcinogen treatment:

1. individual colonies of altered cells on plastic (Syrian hamster embryo, BALB/c 3T3 and prostate cells),
2. discrete colonies in semisolid medium (BHK cells) and
3. foci of altered cells on a confluent monolayer of cells (C3H/10T1/2 and BALB/c 3T3 cells).

Carcinogen-induced changes can be easily quantitated in the first two cases while accurate quantitation of focus formation by C3H/10T1/2 cells is somewhat more complicated (see below and Barrett and Elmore, 1984). Finally, the degrees of normality of the various cells are quite different. BHK cells are clearly preneoplastic, capable of becoming neoplastically transformed in a single step (Bouck and di Mayorca, 1982) and therefore their ‘transformation’ in vitro represents a late rather than an early change in the process of neoplastic transformation. BALB/c 3T3 and C3H/10T1/2 cells also appear to be preneoplastic, although they seem to represent an earlier stage of neoplastic transformation than BHK cells. Cells from both BALB/c 3T3 and C3H/10T1/2 lines will transform neoplastically if maintained in vivo attached to a plastic or glass substrate for approximately 6 months (Boone, 1975; Boone and Jacobs, 1976). In contrast, Syrian hamster embryo cells kept in vivo under similar conditions remain non-tumorigenic. However, aneuploid, preneoplastic derivatives of these cells convert to neoplastic cells in vivo when maintained on a substrate similar to preneoplastic murine cells (Barrett, 1980). Therefore, of the
<table>
<thead>
<tr>
<th>Species of origin (cell name)</th>
<th>Source of cells</th>
<th>Cell line or strain</th>
<th>Ploidy</th>
<th>Phenotype scored</th>
<th>Time of scoring (post-treatment)</th>
<th>Units of quantitation</th>
<th>Development of tumorigenic potential</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syrian hamster (SHE cells)</td>
<td>Embryos</td>
<td>Early passage strain</td>
<td>Diploid</td>
<td>Colonies of morphologically altered cells</td>
<td>10 days</td>
<td>Foci/total colonies</td>
<td>Yes</td>
<td>Berwald and Sachs 1965</td>
<td></td>
</tr>
<tr>
<td>Balb/c mouse (3T3 mouse)</td>
<td>Embryos</td>
<td>Line</td>
<td>Aneuploid</td>
<td>Colonies of morphologically altered cells</td>
<td>10 days</td>
<td>Foci/total colonies</td>
<td>Yes</td>
<td>DiPaolo et al., 1972 Kakunaga, 1973</td>
<td></td>
</tr>
<tr>
<td>C3H mouse (10T1/2) cells</td>
<td>Embryos</td>
<td>Line</td>
<td>Subtetraploid</td>
<td>Foci of cells at high density on monolayer</td>
<td>6 weeks</td>
<td>% positive dishes</td>
<td>Yes</td>
<td>Reznikoff et al., 1973</td>
<td></td>
</tr>
<tr>
<td>C3H mouse (B1 prostate cells)</td>
<td>Ventral prostate</td>
<td>Line</td>
<td>Aneuploid</td>
<td>Colonies of morphologically altered cells</td>
<td>3–4 weeks</td>
<td>Foci/total colonies</td>
<td>Yes</td>
<td>Chen and Heidelberger, 1969</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Embryo</td>
<td>Strain</td>
<td>Diploid</td>
<td>Colony formation in semisolid medium</td>
<td>3–4 weeks</td>
<td>Colonies/total cells plated</td>
<td>Not during experiment</td>
<td>Transient nodule formation in nude mice</td>
<td>Freedman and Shin, 1977</td>
</tr>
<tr>
<td>Human</td>
<td>Embryo</td>
<td>Strain</td>
<td>n.a.</td>
<td>Colony formation in semisolid medium</td>
<td>3 weeks</td>
<td>Colonies/dye excluding cell plated</td>
<td>n.a.</td>
<td>Sutherland et al., 1980</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Foreskin</td>
<td>Strain</td>
<td>n.a.</td>
<td>Colony formation in semisolid medium</td>
<td>6–9 weeks</td>
<td>Colonies/total cells plated</td>
<td>Yes</td>
<td>Silinskas et al., 1981</td>
<td></td>
</tr>
</tbody>
</table>

Table 1  Quantitative systems for studying early, carcinogen-induced changes in cell culture: fibroblast systems
commonly used non-human fibroblast transformation systems, only the Syrian hamster embryo cell system uses early passage, diploid cells capable of detecting the early carcinogen-induced alterations in neoplastic progression. The remaining systems, though highly quantitative, employ aneuploid cell lines representing neoplastic stages.

Neoplastic and preneoplastic transformations of human fibroblasts have been reported by several laboratories (see previous section); however, only those selecting for colony formation in semisolid medium are truly quantitative (Table 1). Carcinogen-induced morphological transformation of human fibroblasts has been reported but the frequency of this transformation has not been precisely quantitated due to the fact that the experiments involved mass cell populations and numerous subcultures (DeMars and Jackson, 1977; Kakunaga, 1978; Milo and DiPaolo, 1978; Namba et al., 1978; Borek, 1980; McCormick et al., 1980). The relationship between induction of anchorage independence in human fibroblasts (Table 1) and neoplasia is not clear, although as noted previously anchorage independence does correlate with tumorigenicity in other systems. Anchorage-independent human fibroblasts have been reported to form regressing nodules in nude mice in several cases (Freedman and Shin, 1977; Silinskas et al., 1981). In contrast, Kakunaga (1978) obtained progressively growing tumours from morphologically transformed human fibroblasts following extended growth after carcinogen treatment. It is not clear whether the induction of anchorage-independent human cells represents neoplastic transformation, an early preneoplastic change, or a change not essential for malignant progression of the cells.

### 2.4.2 Epithelial Cell Systems

Table 2 is a summary of systems in which carcinogen-altered epithelial cells can be readily quantitated. As indicated in Table 2, two types of phenotypic alterations have been scored in epithelial systems: alterations in cell or colony morphology and focal proliferation of cells under conditions where normal cells do not divide. In most cases, the frequencies of carcinogen-induced alterations are scored only as the number of positive cultures or as the fraction of total cells plated. In three systems, one involving liver-derived epithelial cells (Borenfreund et al., 1975), a second, mouse epidermal cells (Kulesz-Martin et al., 1981), and the third, tracheal epithelial cells (Barrett et al., 1983; Thomassen et al., 1983), quantitation of early carcinogen-induced changes is performed at a clonal level because the cells have the potential for clonal growth in these systems. As noted in the previous section, quantitation of carcinogen-induced changes at the clonal level is important for accurate risk assessment. As culture conditions for propagating various epithelial cells are improved, our ability to quantitate the clonal induction of early, preneoplastic changes by carcinogens will improve.
Table 2  Quantitative systems for studying early, carcinogen-induced changes in cell culture: epithelial cell system

<table>
<thead>
<tr>
<th>Species of origin</th>
<th>Source of cells</th>
<th>Cell line or strain</th>
<th>Ploidy</th>
<th>Phenotype scored</th>
<th>Time of scoring (post-treatment)</th>
<th>Units of quantitation</th>
<th>Development of tumorigenic potential</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Kidney cortex</td>
<td>Primary strain</td>
<td>n.a.</td>
<td>Colonies of morphologically altered cells</td>
<td>5–7 weeks</td>
<td>Colonies/cells seeded</td>
<td>n.a.</td>
<td>Treatment in vivo</td>
<td>Borland and Hard, 1974</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Bladder</td>
<td>Primary strain</td>
<td>n.a.</td>
<td>Colonies of morphologically altered cells</td>
<td>3–6 weeks</td>
<td>% positive dishes</td>
<td>Yes</td>
<td>Explant cultures</td>
<td>Summerhayes et al., 1981</td>
</tr>
<tr>
<td>Mouse</td>
<td>Submandibular gland</td>
<td>Primary strain</td>
<td>n.a.</td>
<td>Colonies of morphologically altered cells</td>
<td>10–14 weeks</td>
<td>% positive explants</td>
<td>Yes</td>
<td>High cell density cultures; selection imposed at ≥3 weeks</td>
<td>Wigley, 1979</td>
</tr>
<tr>
<td>Mouse</td>
<td>Epidermis</td>
<td>Primary strain</td>
<td>n.a.</td>
<td>Focal proliferation in differentiation inducing medium</td>
<td>6–9 weeks</td>
<td>% positive dishes</td>
<td>Not after 8 passages</td>
<td>Kulesz-Martin et al., 1980</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Epidermis</td>
<td>Early passage strain</td>
<td>n.a.</td>
<td>Clonal proliferation in differentiation inducing medium</td>
<td>7–9 weeks</td>
<td>Frequency of altered cells</td>
<td>n.a.</td>
<td>Kulesz-Martin et al., 1981</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Epidermis</td>
<td>Primary strain</td>
<td>n.a.</td>
<td>Foci of proliferating cells (visible at 6–8 weeks)</td>
<td>3 months</td>
<td>% positive dishes</td>
<td>Yes</td>
<td>High cell density cultures</td>
<td>Ananthaswamy and Kriske, 1981</td>
</tr>
<tr>
<td>Rat</td>
<td>Liver</td>
<td>Early passage strains</td>
<td>Diploid</td>
<td>Dense colonies of morphologically altered cells</td>
<td>6–8 weeks</td>
<td>% total colonies altered</td>
<td>Yes</td>
<td>Borenfreund et al., 1975</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Trachea</td>
<td>Primary strain</td>
<td>n.a.</td>
<td>Foci of proliferating cells with high cell density</td>
<td>3–4 weeks</td>
<td>Foci/dish</td>
<td>n.a.</td>
<td>Line formation also measured</td>
<td>Pai et al., 1983</td>
</tr>
<tr>
<td>Rat</td>
<td>Trachea</td>
<td>Primary strain</td>
<td>Diploid</td>
<td>Colonies of proliferating cells under selective conditions</td>
<td>4–6 weeks</td>
<td>Colonies/colony forming cell surviving treatment</td>
<td>n.a.</td>
<td>Selection (non-permissive for normal cells) imposed 1–7 days post-treatment</td>
<td>Thomassen et al., 1984</td>
</tr>
</tbody>
</table>
2.4.3 Development of Neoplastic Potential by Cells Transformed in vitro

The use of in vitro systems for risk assessment or mechanistic studies is based on the assumptions that carcinogens induce detectable cellular alterations and that these alterations are related to neoplasia. It is important, therefore, to demonstrate that phenotypes which are scored are essential, preneoplastic changes. Such demonstrations have been made for the phenotype of morphological transformation in rodent fibroblast systems as discussed above. However, as more systems using human fibroblasts and various types of epithelial cells are developed, the preneoplastic nature of carcinogen-induced alterations will have to be proved conclusively to validate the use of those systems and those phenotypes in studies of chemically induced neoplasia.

2.4.4 In vivo --- in vitro Systems

There is a large information gap between the assessment of the carcinogenic potential of a chemical in vivo and in vitro. Several systems have been described which provide evidence that in vitro systems can reliably predict the carcinogenic potential of a chemical in vivo. In vivo exposure to carcinogens followed by selection of altered cells by growth in culture provides many of the advantages offered by both whole animal studies and in vitro assays. In vivo --- in vitro studies have included fibroblasts (DiPaolo et al., 1973), kidney (Borland and Hard, 1974), mammary glands (McGrath et al., 1980), liver (Mondal, 1975; Laishes et al., 1980), epidermis (Yuspa and Morgan, 1981), brain (Laerum and Rajewsky, 1975; Roscoe and Claisse, 1978) and trachea (Marchok et al., 1977). Although these systems are not all quantitative and have not been used for the routine bioassay of carcinogenic agents, they potentially provide a different approach to the problem of risk assessment.

Ts'o (1978) has proposed the use of a matrix system for the assessment of risk to cancer. This approach involves the use of a series of interrelated extrapolations, for example:

rodent tissues in vivo --- rodent cells in vitro
rodent cells in vitro --- human cells in vitro
human cells in vitro --- human tissues in vivo

While this approach can use a variety of endpoints for cellular effects of chemicals, cell transformation assays would be very desirable for such an approach. In addition to comparisons between rodent and human cell transformation in vitro, in vivo --- in vitro experiments of rodent or human cells transplanted to nude mice and treated in vivo could be employed for these extrapolations.
2.5 Potential Need for a Multitiered Approach to Risk Assessment Using Cell Transformation Systems: the Importance of Mechanism Studies

We have discussed differences between fibroblast systems (such as Syrian hamster embryo cells vs BALB/c 3T3 and C3H/10T1/2 cells vs BHK cells) with respect to their normality or degrees of progression toward neoplasia. As noted above, carcinogens initially will have a major effect on normal cells and the induction of early, preneoplastic changes. However, carcinogens are also likely to affect preneoplastic cells. Since we do not know the molecular nature of any of the various changes resulting in neoplasia, it is important to examine the effects of carcinogens and promoters on different preneoplastic cells. Quantitative studies of the induction of changes expressed late in the progression to neoplasia have been made (Bouck and di Mayorca, 1982; Thomassen and DeMars, 1982). This type of analysis is important to have a complete understanding of the mechanism of carcinogenesis and to fully assess cancer risk associated with exposure to chemicals.

3 QUANTITATIVE USE OF CELL TRANSFORMATION ASSAYS IN RISK ASSESSMENT

The data from cell transformation systems can be used for risk assessment in the same manner as the results from other short-term assays, a subject discussed elsewhere in this volume. Therefore, we will restrict our comments to the unique aspects of cell transformation assays.

3.1 Detection of Carcinogens with Cell Transformation Assays

Several cell transformation assays have been used as qualitative screens for detecting carcinogenic agents (see Tables 1 and 2). In particular, extensive studies with Syrian hamster embryo cells, BHK cells, C3H/10T1/2 cells and BALB/c 3T3 have been completed. In contrast, very few compounds have been analysed with epithelial cell transformation systems. The results with the fibroblast systems are very promising and indicate that cell transformation assays can detect > 90% of known carcinogens with few false negatives (Pienta, 1980). Many carcinogens, not detected in other short-term tests, are positive in cell transformation assays (Pienta, 1980; de Serres and Ashby, 1981). These results indicate the promise of these assays as screening tests for carcinogens, but also provide further evidence for a relationship between cell transformation in vitro and neoplastic development in vivo. Further detailed comparisons of the responsiveness of different cell transformation systems to a variety of chemicals can be found in a number of recent reviews of these studies (Hollstein et al., 1979; IARC, 1980; de Serres and Ashby, 1981; Heidelberger et al., 1983).
3.2 Quantitative Results of Cell Transformation Assays

Berwald and Sachs (1965) were the first to quantitate cell transformation by scoring the number of morphologically transformed Syrian hamster embryo cells following carcinogen treatment. Later studies demonstrated that the number of morphologically transformed colonies observed depends on the dose of the carcinogen used and the number of cells at risk (DiPaolo et al., 1971a). Statistical analysis of the dose–response curve of the chemically induced morphological transformation indicates that it fits very well with a 'one-hit' model for this transformation (Gart et al., 1979 and section 3.3). Data obtained with this system also demonstrate that this process results from the induction of transformed cells as opposed to the selection of pre-existing neoplastic cells (DiPaolo et al., 1971b). Dose–response analysis of induction of enhanced growth variants of rat tracheal epithelial cells also suggests a 'one-hit' mechanism for this transformation (Thomassen et al., 1983). It should be kept in mind that these results only relate to the first phenotypic change in a multistep process for the neoplastic development of Syrian hamster embryo cells and rat tracheal epithelial cells.

In contrast to the results with the above two cell types, certain aspects of morphological transformation of C3H/10T1/2 cells have recently become evident which complicate the quantitation of the frequency of transformation of these cells and suggest that this conversion is not a one-step process. Haber et al. (1977), Kennedy et al. (1980), and Fernandez et al. (1980) have followed up the original observation by Reznikoff et al. (1973) that the transformation frequency (i.e., number of transformed foci per cell treated after correcting for cell killing) depends on the initial number of cells at risk. Extensive studies by Kennedy et al. (1980) with X-ray-induced transformation have demonstrated that the absolute yield of transformed cells is constant over a wide range of initial cell number. For example, if an expression time of 12–14 population doublings after carcinogen treatment is completed and the cells are then resuspended and plated at different densities from 1 to 10,000 cells per plate, the number of transformed foci per dish is approximately 1 for all groups; hence, the apparent transformation frequency varies from 0.01 to 100% depending on the conditions employed. Also, for a given dose of carcinogen, the number of transformed foci per dish is independent of the number of cells treated. This is in contrast to the results with the Syrian hamster embryo cells (DiPaolo et al., 1971b).

According to Kennedy et al. (1980), these observations suggest that the transformed clones do not occur as the direct consequence of carcinogen treatment. Rather, these authors propose a two-step model to explain the results. The initial change induced by the carcinogen apparently occurs in a large number, perhaps in all cells. This change does not directly result in the transformation of cells, but rather, increases the probability that the transformation of these cells will occur as a rare, secondary event. Since the initial cell number and the number of population doublings do not appear to influence the
number of transformed foci, Kennedy et al. (1980) suggested that this process occurred at confluence, because the number of cells at confluence was constant in the density-inhibited C3H/10T1/2 cell line regardless of the initial cell density. The authors further considered that these observations were inconsistent with a mutational mechanism and suggested that an epigenetic process was involved in the carcinogen-induced transformation of C3H/10T1/2 cell.

Heidelberger and co-workers (Fernandez et al., 1980) made similar observations with the C3H/10T1/2 cells and proposed a 'probabilistic theory' to explain the formation of transformed foci by these cells following 3-methylcholanthrene treatment. Their theory is similar to that proposed by Kennedy et al. (1980) in that two steps must occur for cell transformation. The first step is the 'activation' of a large proportion of cells by the carcinogen which occurs with a probability \( p_1 \), and the second step is the transformation of the activated cells which occurs with a probability \( p_2 \) per cell generation. The authors have derived a mathematical equation which predicts the frequency of focus formation based on the probability of these two steps (\( p_1 \) and \( p_2 \)) and the probability of deactivation per cell generation of the carcinogen activated cells, which is termed \( p_3 \). This approach has the advantage of allowing the determination of these probabilities based on experimental results. The equation derived to describe focus formation is 

\[
\log (F/N) = \log [2p_1p_2(1-p_3)/(1-p_3) - 1] + n\log (1-p_3),
\]

where \( F \) = mean number of foci per dish, \( N \) = number of cells in a dish at confluence and \( p_1 \), \( p_2 \) and \( p_3 \) are the probabilities described above. This equation has been verified experimentally with 3-methylcholanthrene-induced C3H/10T1/2 transformation and the values of \( p_3 = 0.24 \) and \( p_1p_2 = 3.8 \times 10^{-6} \) were obtained.

Unfortunately, the values of \( p_1 \) and \( p_2 \) could not be determined separately (Fernandez et al., 1980). However, based on the results of these authors as well as on a paper by Kennedy et al. (1980), the probability of activation, \( p_1 \), must be nearly equal to 1, since transformation is commonly observed with 1–5 cells at risk. This means that following carcinogen treatment, most, if not all, cells are activated but only a few of the activated cells are subsequently transformed (\( p_2 \geq 3.8 \times 10^{-6} \) per cell generation).

Unfortunately, the probabilistic theory of Fernandez et al. (1980) for 3-methylcholanthrene-induced transformation is insufficient to describe the results of Kennedy et al. (1980) with X-ray-induced cell transformation of C3H/10T1/2 cells. The reasons for this are not clear. However, Barrett and Elmore (1984) have reanalysed the data of these two groups and have calculated a 'transformation rate' for the second step, which is comparatively constant. This calculation assumes that the 'first step' (activation of the cells) occurs with a high probability (approaching unity), while the second step is a spontaneous transformation of the activated cells which occurs randomly during the growth of the cells. The calculated spontaneous rate of this transformation is \( 1 \times 10^{-7} \) to \( 6 \times 10^{-7} \) transformants per cell per generation (Barrett and Elmore, 1984) from the results
of Fernandez et al. (1980) or Kennedy et al. (1980). In fact, good agreement is found between the results of the different groups. This spontaneous transformation rate is also similar to that reported for a subtetraploid, preneoplastic Syrian hamster embryo cell line (FOL+) (Crawford et al., 1980) which is not enhanced by mutagens, suggesting that they are equivalent to 'activated' C3H/10T1/2 cells (Barrett and Elmore, 1984).

A recent report (Mordan et al., 1983) suggests that at least part of the difficulty in quantitating focus formation of C3H/10T1/2 cells is due to the suppressive effects of normal cells on the expression of focus formation by the transformed cells. These authors suggest that a minimum colony size of approximately 128 transformed cells at confluence is required for the formation of a transformed focus. The suppression or reversion of morphological transformation of C3H/10T1/2 and BALB/c 3T3 cells has been reported previously (Sivak and Van Durren, 1967; Brouty-Boyé et al., 1979; Brouty-Boyé and Gresser, 1981). If a single transformation event in a C3H/10T1/2 cell has to be amplified 100 times to be detected, this means that quantitation of this process will be very difficult, have a low level of sensitivity and be subject to many factors inherent in the cell culture methodology. Haber and Thilly (1978) originally suggested that carcinogens affect two steps in C3H/10T1/2 transformation. The first was the induction of the potential for transformation which occurred in a large percentage of these cells. This is analogous to the activation step proposed by Fernandez et al. (1980). The induction occurs in nearly all of the cells and according to Haber and Thilly is not dose-dependent. The second step suggested by Haber and Thilly is that carcinogen influences cell—cell interactions in a dose-dependent manner to allow for the expression of the transformed potential of the cells.

It is now apparent that the expression of morphological transformation of C3H/10T1/2 cells is not a one-step process. The first step appears to be a rapid event (Backer et al., 1982) that occurs in a high fraction of cells (Haber and Thilly, 1978; Fernandez et al., 1980; Kennedy and Little, 1980). The second step could either be a second qualitative change in the cells that occurs at a low frequency during the growth of cells or at confluence (Fernandez et al., 1980; Kennedy and Little, 1980; Barrett and Elmore, 1984) or the second step could be an amplification of the transformed cells to overcome the suppressive effects of the non-transformed cells (Haber and Thilly, 1978; Mordan et al., 1983). Further experiments are needed to elucidate the mechanism of transformation with C3H/10T1/2 and the relevance of carcinogen-induced events to neoplastic progression in vivo. This system should be used in quantitative risk assessment with caution until these issues are resolved.

It is not known whether the transformation of BALB/c 3T3 cells is also a two-step process, like the other subtetraploid murine cell line, C3H/10T1/2. However, the results with a preneoplastic hamster cell line, BHK, are more consistent with a one-step process (Bouck and di Mayorca, 1976). It is intriguing to speculate
that this difference might be related to the near diploid karyotype of this cell line (Barrett and Elmore, 1984).

3.3 Dose—Response Data with Cell Transformation Assays

Few detailed dose—response curves have been published with cell transformation systems. Two carcinogens, benzo(a)pyrene and X-rays, have been studied extensively. Transformation of Syrian hamster embryo cells by polycyclic hydrocarbons does not correlate with the cytotoxicity of the carcinogen treatment (DiPaolo et al., 1971b; Umeda and Iype, 1973). Huberman and Sachs (1966) and DiPaolo et al. (1971a) reported that the logarithm of the frequency of morphological transformation of Syrian hamster embryo cells increased linearly with the logarithm of the dose. The slope of this line was approximately unity in both studies suggesting a one-hit model for this change. Gart et al. (1979) have developed statistical methods to analyse these data and have confirmed that one-hit curves fit the results from the two laboratories very well except at the highest doses. The deviation at high doses is possibly due to cytotoxicity of the chemical. Two-hit and multihit models were rejected by their analysis. The one-hit model also holds for the transformation induced by X-rays and by the joint action of X-rays and benzo(a)pyrene.

Borek and Hall (1973) determined the dose—response of morphological transformation of Syrian hamster embryo cells following X-ray treatment. The logarithm of the transformation frequency per surviving cell increased curvilinearly with the logarithm of the dose in the range from 1 to 150 rad. However, a linear response with a slope of 1 also fits the data within one standard deviation which is consistent with the one-hit model of transformation observed with benzo(a)pyrene-induced transformation (Huberman and Sachs, 1966; DiPaolo et al., 1971a). Interestingly, these authors were able to detect cell transformation with an X-ray dose of only 1 rad. The X-ray dose—response curve reached a plateau at 150-300 rads and started to decline at 600 rads. The fraction surviving at 150 and 300 rads was 0.86 and 0.75, respectively. The plateau of transformation was, therefore, not associated with a high level of cell killing. The surviving fraction at 600 rads was only 0.12—0.13, which may account for the decline in the dose—response curve (Borek and Hall, 1973).

The use of comparative dose—response studies for cancer risk analysis can be illustrated by the study of Borek et al. (1978) who compared the relative biological effectiveness of X-rays and 430-keV monoenergetic neutrons in terms of cell killing and transformation of Syrian hamster embryo cells. Parallel dose—response curves for cell transformation by neutrons and X-ray were obtained. When the data were plotted as transformed colonies/surviving cell, neutrons induced cell transformation at lower doses and to a higher maximum than X-rays. However, neutrons were also more effective in killing the cells. When the
dose curves for cell transformation per initial cell at risk were compared, neutrons and X-rays had similar maximum carcinogenic potential and dose–response curves although neutrons were effective at doses approximately 10-fold lower than X-rays. Borek et al. (1978) discussed the significance of their results in terms of the relative risk of secondary tumours associated with X-ray compared with neutron therapy. They conclude that the increased capacity of neutrons to kill cells compensates for their increased efficiency for inducing cell transformation. As a result, therapeutically effective doses of neutrons do not result in an increased risk for cell transformation relative to X-ray therapy.

The effect of split doses of X-rays on the morphological transformation of Syrian hamster embryo cells has been studied. Borek and Hall (1974) observed that if an X-ray dose was divided into two fractions, more cell transformation was observed than if the same dose was given in a single exposure. Cell killing, on the other hand, generally decreased with split doses as compared with a single dose of the same magnitude. These results are important for risk estimation because humans are generally exposed to several low doses rather than to a large, single dose of carcinogens.

The results with the Syrian hamster embryo cell transformation systems demonstrate the applicability of cell transformation systems in risk assessment. However, risk assessment requires comparative dose–response studies and such results are sometimes difficult to obtain with this system. Pienta (1980) has screened over 100 chemical carcinogens and mutagens with the Syrian hamster embryo transformation assay and a dose–response was not always observed in these experiments. The results mentioned above clearly indicate that dose–response curves can be obtained with this system; however, it should be understood that this assay is not selective like a mutation assay. Therefore, large numbers of colonies have to be screened independently to obtain sufficient numbers of transformed colonies to determine dose–response curves. For example, over $10^5$ colonies were scored in the experiments of Borek and Hall (1973) to obtain a dose–response curve for X-ray-induced transformation. This represents a tremendous amount of effort. The experiments of Pienta to screen the large number of chemicals necessary to validate the qualitative response of this system were equally extensive. His studies were not intended to be quantitative and have limited use for relative risk assessment.

This illustrates the advantage that selective assays for transformation have over non-selective assays. In the Syrian hamster embryo system microscopic examination of every colony (normal and transformed) is required. Other assay systems employ selective conditions, such as anchorage-independent growth for BHK cells (Bouck and di Mayorca, 1976; Ishii et al., 1977; Styles, 1980) and human fibroblasts (Milo et al., 1981; Silinski et al., 1981) or enhanced growth under conditions that induce the terminal differentiation of normal epithelial cells (Yuspa and Morgan, 1981; Barrett et al., 1983). These assays require one to score only the transformed cells and hence are technically much easier. Dose–
response data have been obtained for both fibroblasts and epithelial cells using selective assays.

Several detailed studies on the transformation of CH3/10T1/2 cells have been completed. In particular, the effects of ionizing radiation have been extensively examined with this transformation assay (Terzaghi and Little, 1975, 1976a,b; Miller and Hall, 1978; Elkind and Han, 1979; Han and Elkind, 1979; Miller et al., 1979). These studies had been completed before the problems associated with the quantification of transformation in this system were understood (see section 3.2). The relevance of these studies is, therefore, difficult to determine. However, it should be noted that the results with this system are very consistent with the known effects of ionizing radiation in experimental animals, including doseresponse relationships and the effects of dose rate, linear energy transfer and modifying agents (Kennedy and Little, 1980; Kennedy, 1984).

3.4 Mechanisms of Tumour Promotion

Tumour-promoting factors have been suggested to play an important role in the development of human cancers (Doll and Peto, 1981). Therefore, quantitative risk analysis of this class of cocarcinogens is very important. Tumour promoters do not induce gene mutations and, hence, are not detected by most short-term tests for carcinogens (Weinstein, 1981). Cell transformation assays may be useful in risk analysis of tumour promoters, but this requires that two-stage carcinogenesis experiments in vitro be relevant to tumour promotion in vivo. To critically evaluate this relationship one must first define the mechanism of tumour promotion in vivo, at least at the cellular level.

The mechanism of two-stage carcinogenesis, originally described nearly 40 years ago as the ability of certain treatments to promote tumour formation initiated by a non-carcinogenic dose of a chemical (see Boutwell, 1964, 1974), remains undefined today. There are two possible cellular mechanisms for tumour promotion (Farber, 1982):

1. Tumour promoters act only to amplify the number of initiated cells relative to the normal cells resulting in the appearance of a visible lesion (i.e., tumour promotion is a quantitative change only in cellular populations) (Potter, 1980; Trosko and Chang, 1980).

2. Tumour promoters modulate the initiated cell to a state which allows subsequent clonal proliferation of these cells relative to the normal cells in the tissue (i.e., tumour promotion is a qualitative plus a quantitative change in the initiated cells).

Surprisingly, little definitive work is available to distinguish whether tumour promotion is a quantitative or qualitative process. This distinction is necessary before one can relate in vitro or in vivo results.

Any model of tumour promotion in vivo has to include an amplification
mechanism since the clonal growth of the tumour requires amplification of the
initiated cell vis-à-vis the non-initiated cell. The importance of this mechanism in
tumour promotion of mouse skin is strongly supported by the observations that
(1) all known mouse skin tumour promoters are hyperplastic agents (Boutwell,
1974; Barrett and Sisskin, 1980) and
(2) regenerative hyperplasia initiated by wounding or abrasion is sufficient to

It has been stated that hyperplasia and tumour promotion are unrelated (see
Barrett and Sisskin, 1980). However, these results are often difficult to interpret
because the hyperplastic response of a single treatment of promoter is related to
tumour formation after many months of treatment. These studies assume that
the hyperplasia developing after a single treatment is related to the sustained
hyperplasia that develops after multiple treatments. This is clearly not the case
(Barrett and Sisskin, 1980; Sisskin et al., 1982). In mouse skin treated with the
potent tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), there is
a potentiation of the hyperplastic response with additional TPA treatments
(Raick et al., 1972). With other promoters (Frei, 1977) and with other species
(Sisskin and Barrett, 1981), this potentiation is not observed and sometimes the
epidermis adapts to the promoter and fails to respond hyperplastically (Sisskin
and Barrett, 1981). The differences in sustained hyperplasia with multiple TPA
treatment can also be related to different sensitivities of different strains of mice
to tumour promotion (Sisskin et al., 1982).

It is important to realize that hyperplasia and amplification of the initiated cell
are not necessarily related. In fact, stimulation of growth of the entire epidermis
would not allow expansion of the initiated cell unless the initiated cell is altered in
its differentiation potential and, therefore, is amplified relative to the non-
initiated cell which terminally differentiates after its stimulation to proliferate.
Recent data (Kulesz-Martin et al., 1980; Yuspa and Morgan, 1981; Yuspa et al.,
1981) support this hypothesis for mouse skin carcinogenesis. Amplification of
the initiated cells in other organs might occur by a specific stimulation of growth
of initiated cells or by a differential inhibition of growth of the normal cells
(Farber, 1982). Neither of these mechanisms would be associated with general
hyperplasia of the tissue.

The evidence for a modulation mechanism for tumour promotion in vivo is
limited. We assume that this is what most people are referring to when they state
that tumour promotion in mouse skin must involve something in addition to
hyperplasia. However, the nature of the additional effect is ill-defined at the
cellular or molecular level. The best evidence that tumour promoters cause a
qualitative modulation of initiated cells is the identification of different stages in
tumour promotion (Boutwell, 1974; Slaga et al., 1980a). These experiments
demonstrate that croton oil or TPA can effect changes in initiated cells which
allow them to be amplified by compounds ('second-stage promoters') that are
inactive or weak promoters by themselves (Slaga et al., 1980a). For example, mezerein, which is as potent as TPA in inducing a variety of phenotypic effects on cells, is active only as a second-stage promoter. Also, different inhibitors of promotion can be shown to be effective in inhibiting only the first or the second stage of promotion (Slaga et al., 1980b). These results suggest that potent promoters can modulate or convert (Boutwell, 1974) initiated cells qualitatively to allow them to amplify or proliferate into a clone of tumour cells. An alternate possibility (Yuspa, personal communication) is that the first stage of promotion is also a quantitative change. First-stage promoters may allow initiated cells to escape some but not all growth restraints. Second-stage promoters then enable small clones of initiated cells to continue to expand. These agents may be less effective on individual initiated cells than the first-stage promoters.

A major aspect of tumour promotion is certainly the quantitative effect of the promoter on the population dynamics in the tissue. Whether or not tumour promoters also cause a qualitative change in the initiated cells and the nature of these changes are important questions to be answered. In considering the relationship between tumour promotion in vitro and in vivo and in developing assay systems for tumour promoters, it is important to define whether the cellular changes under study are qualitative or quantitative.

### 3.5 Initiation–Promotion Experiments in Cell Culture

Based on the two-stage model of carcinogenesis (Boutwell, 1974) developed in mouse skin with the potent tumour promoter TPA, several laboratories have attempted and succeeded in demonstrating phenomena in cell culture similar to those observed in mouse skin (Kennedy, 1984), i.e., that after a low dose of carcinogen which is weak or inactive in transforming the cells, TPA causes an increase in the transformation frequency greater than the sum of the two treatments alone. TPA alone is generally weakly active or inactive as a transforming agent. Kennedy (1984) has recently reviewed the current literature in this field and the reader is referred to his paper for details on the experiments in which TPA promotes cell transformation and on other agents which have promoting activity in cell cultures.

The cellular mechanisms of cell culture models of tumour promotion have not been defined. The two models which are most widely studied are TPA-enhanced transformation of C3H/10T1/2 cells and Syrian hamster embryo cells (see Kennedy, 1984). In the latter assay, individual cells are exposed to the carcinogen, allowed to form colonies for 6–8 days, and then scored for morphological transformations. When TPA is added to the cultures during colony formation, an increase in the number of transformed colonies is observed if the cells are initiated with a low dose of carcinogen. Since the colony-forming efficiency of the cells is unaffected by the tumour promoter, amplification of initiated cells is an unlikely mechanism of this effect. (One can also consider that
Use of Quantitative Cell Transformation Assays in Risk Estimation

growth in culture allows the cellular amplification necessary for promotion.) It is possible that the tumour promoter affects cell–cell interactions in the emerging colony and allows the expression of transformed cells which are otherwise suppressed. A more likely possibility is that TPA modulates the initiated cell and allows its expression. It will be of interest to clarify if cellular modulation by TPA occurs in this system and the nature of this effect.

Although ‘tumour promotion’ has been studied more extensively in C3H/10T1/2 cells (Kennedy, 1984), the qualitative or quantitative nature of this process has not been addressed. Recent studies (Mordan et al., 1983) have suggested that TPA reduces the minimal colony size of transformed cells required to form a transformed focus. These results imply that tumour promotion in C3H/10T1/2 cells is a quantitative change which alters the population dynamics and allows the amplification of clones of initiated cells. Further confirmation of these results is necessary.

Are cell culture models of tumour promotion relevant to risk analysis? This is a critical question which is difficult to answer because of our lack of understanding of the mechanism of cellular changes during tumour promotion in vivo.

Most tumour-promoting agents, including substances in the environment which are important for human cancers, are tissue specific (Doll and Peto, 1981). For example, phorbol, the inactive control compound for tumour promotion on mouse skin, is an effective promoter of lung, liver and mammary carcinogenesis (Armuth and Berenblum, 1972, 1974, 1979). In contrast, TPA, the model tumour promoter employed in most cell culture studies, is a very active tumour promoter in vivo with a broad range of activity in a variety of tissues. Furthermore, TPA is a very potent biological substance and affects gene expression, morphology, growth, and/or differentiation of a large number of cells in culture. While the potency of TPA makes it an attractive model compound to study, it may also induce cellular phenomena unrelated to tumour promotion. Thus, the assumption that all effects induced by tumour promoters (particularly TPA) on cells in culture are related to tumour promotion is not justified. This caution also applies to ‘tumour-promotion’ studies using cell culture systems.

Cell culture promotion experiments have been somewhat predetermined, i.e., the phenomenon of tumour promotion has been defined by the in vivo experiments, and cell culture studies have been designed to mimic these results. While the anticipated results have been observed, these experiments are highly dependent on the lot of serum and sometimes even the lot of TPA used (C. Boreiko, personal communication). It should also be noted that TPA-induced promotion of cell transformation has not been observed with primary mouse epidermal cells in culture (S. Yuspa, personal communication), the target cells for classical two-stage carcinogenesis. In fact, few cell culture models exist for tumour promotion of epithelial cells, the major target cells in vivo for these substances (Kennedy, 1984). Furthermore, the effects of TPA on promotion of cell transformation in fibroblast systems may be contradictory to the effects of
TPA on fibroblasts in vivo. Bhisey and Sirsat (1976) demonstrated that if the initiation is performed by subcutaneous injection of a carcinogen which induces sarcomas, and then TPA is applied topically, inhibition rather than promotion of sarcomas is observed. Further studies of this type are needed.

Cell culture experiments are of great utility in understanding the effects of tumour promoters on cellular growth, phenotypes and differentiation and the mechanisms of these effects. The nature of modulation of initiated cells by tumour promoters may also be elucidated by cell culture studies. However, if tumour promotion in vivo is primarily an effect on population dynamics within the tissue, i.e. amplification of the initiated cell, it will be very difficult to design an experimental model with cells in culture that will reflect the cell–cell interactions that control cellular growth in vivo.

Interestingly, it has been shown that a variety of known tumour promoters can affect cell–cell interactions in a cell culture assay (Murray and Fitzgerald, 1979; Yotti et al., 1979). This assay is not a cell transformation assay but rather measures intercellular communication by interchange of small molecular weight metabolites. The potential of this assay for detecting tumour promoters should be further explored. Whether these results can be used for risk analysis remains to be determined. A further understanding of the mechanism of tumour promotion in vivo can possibly lead to future use of cell culture assays for risk analysis for promoters.

It is important to recognize that the two-stage model of epidermal carcinogenesis may involve only one step or qualitative change in the cells. The promotion stage may be a quantitative change only, i.e. the amplification of the clone of initiated cells. Of course, the identification of multiple stages in promotion suggests qualitative changes in this process as well. Regardless of the number of cellular changes during initiation and promotion, this model only describes the development of benign lesions on mouse skin. A further progression of the cells to the malignant stage has to occur for this model to describe the complete, multistep process of carcinogenesis.

3.6 Analysis of Multistage Models of Carcinogenesis in Cell Culture

As discussed in section 2.1, neoplastic transformation of diploid cells in culture is a progressive multistep process in analogy to neoplastic development in vivo. The exact number of steps involved is unknown, although at least two steps have been identified. Various multistage models have been suggested based on epidemiological data from human tumours (Armitage and Doll, 1954, 1957; Fisher, 1958; Cook et al., 1969; Moolgavkar and Knudson, 1981), but this analysis has not been extended to the cell culture models. The reason for this is that in cell culture models a single dose of carcinogen is generally used rather than a continual treatment. Therefore, the effect of the carcinogen is only on a single step in the
process. The influence of carcinogen dose on the overall process of neoplastic progression in cell culture has not been addressed.

The usefulness of merging multistage mathematical models and multistage cell transformation studies can be illustrated by considering the model of Moolgavkar and Knudson (1981), represented in Figure 1. In this model, the progression to malignancy is controlled by three factors:

1. \( \mu_1 \), the rate of transition of a normal cell to a preneoplastic cell;
2. \( \mu_2 \), the rate of transition of a preneoplastic cell to a malignant cell; and
3. the rate of growth of the preneoplastic cells, which is controlled by the rate of division (\( x_2 \)) and the rate of terminal differentiation (\( \beta_2 \)) of these cells.

The third factor in this model probably cannot be accurately determined with cell culture models because the environmental influences on these processes in vivo are difficult to mimic in cell culture. Moolgavkar and Knudson (1981) suggested that this factor was influenced by promoters, and this further points out the reasons why cell culture assays for promoters are difficult to develop. However, the transition rates, \( \mu_1 \) and \( \mu_2 \), are probably intrinsic properties of the cell which may be influenced by carcinogens. Therefore, these rates can be determined in cell culture if preneoplastic and neoplastic cells can be identified and quantitated.

### 3.7 Relationship between Cell Transformation and Mutation

Since other short-term tests for carcinogens, particularly mutagenesis assays, are used for risk analysis, it is important to determine the relationship between mutagenesis, cell transformation and carcinogenesis. Barrett and Elmore (1984) have recently reviewed the current literature and concepts on the relationship
between mutagenesis and carcinogenesis based on studies of cell culture. The following conclusions have been drawn.

Neoplastic transformation in cell culture is a multistage process and the role of mutagenesis may vary with different steps in this process. For example, aneuploid cell lines, which are non-tumorigenic, have acquired some properties of neoplastic cells and progress to neoplastic cells more readily than normal diploid cells and are, therefore, considered preneoplastic cells. The current evidence suggests that transformation of normal, diploid cells to preneoplastic cells may occur by a mechanism different from the transformation of preneoplastic cells to neoplastic cells.

Cell culture studies offer the advantage of directly comparing the processes of mutagenesis and carcinogenesis in the same target cells. Following carcino gen treatment, transformation of fibroblasts of hamster, mouse or human origin occurs with a frequency of 10–100 times that observed for gene mutation at two loci measured concomitantly. Recent studies indicate that early alterations of rat tracheal epithelial cells in culture occur at a high frequency similar to that observed with fibroblasts (Barrett et al., 1983; Thomassen et al., 1983). Induction of cell transformation can occur in the absence of measurable gene mutations (Barrett, 1981; Barrett et al., 1981; Gyi, 1982; Landolph and Jones, 1982). Aneuploid conversion and alterations of DNA methylation have been proposed as mechanisms for transformation of these cells.

These studies point out the need to better understand the role in cell transformation and cancer of changes other than gene mutations, for example rearrangements in chromosomes and alterations in DNA methylation. This understanding will be useful in evaluating the risk in humans associated with exposure to chemicals, particularly those agents which appear to act by a mechanism other than direct DNA damage.

4 CONCLUSIONS

Cell transformation assays can be used to study cellular changes involved in carcinogenesis. These studies can provide information needed for understanding the nature of these changes, the influence of environmental factors, as well as the possible identification of inhibitors of these changes. Cell transformation studies have demonstrated the multistep nature of neoplastic development and have indicated that environmental factors can act at one or more stages in this process. Furthermore, different steps in malignant progression of cells may be influenced by different environmental factors. While the complete assessment of cancer risk requires consideration of the role of host factors as well as of cellular events, cell culture assays can be used to identify possible cancer hazard to man, to define the steps in neoplastic development which are affected by these substances, and to compare the relative risk of these agents in defined steps of carcinogenesis.
5 REFERENCES


tumorigenicity of chemically transformed mouse epidermal cells. *Cancer Res.*, 38, 624–634.


Use of Quantitative Cell Transformation Assays in Risk Estimation


