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# Probabilistic view of the transformation of cultured C3H/10T1/2 mouse embryo fibroblasts by 3-methylcholanthrene

(cell transformation/chemical carcinogenesis)

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**ABSTRACT** **When C3H/10T1/2 cells are treated with a given concentration of a chemical carcinogen, the transformation frequency can vary over 4 orders of magnitude, depending primarily upon the number of cells plated. To explain this phenomenon, we have developed a probabilistic theory of the formation of transformed foci in this system. We define $p_1$ as the probability that a cell will be activated by carcinogen treatment, $p_2$ as the probability per cell generation that an activated cell will be transformed, and $p_3$ as the probability per cell generation that an activated cell will be deactivated. The equation we have derived: $\log (F/N) = \log [2p_1p_2(1 - p_3)/2(1 - p_3) - 1] + n \log (1 - p_3)$ describes focus formation; $F$ is mean number of foci per dish after carcinogen treatment, $N$ is number of cells in a dish at confluence, and $n$ is number of cell generations to confluence. This equation has been verified experimentally; $p_3 = 0.24$ and $p_1p_2 = 3.8 \times 10^{-6}$ at a single concentration of 3-methylcholanthrene. This relationship explains previously inexplicable effects of cell density on transformation frequency.**

The development of systems in which chemical carcinogens produce oncogenic transformation of cultured cells has provided powerful tools for the study of cellular and molecular mechanisms of chemical carcinogenesis as well as the means to screen environmental carcinogens at reasonable cost in time and money. These systems have been extensively reviewed (1–4), and two prototypes have been widely used. One involves primary or secondary cultures of Syrian hamster embryo cells, as first reported by Berwald and Sachs (5, 6) and used extensively by other investigators (7–10). These cells are normal and diploid, and they have a limited lifespan. Morphological transformation is scored in colonies that form in 8–10 days, and acquisition of oncogenic properties proceeds through several stages (10). The other prototype system involves the use of permanent lines such as the C3H/10T1/2 cells that were developed in our laboratory (11, 12). These cells are hypotetraploid and "immortal." Oncogenic transformation is scored after 6 weeks by focus formation on a confluent monolayer. A focus assay for transformation of Syrian hamster embryo cells has also been described (13).

The determination of the transformation frequency per surviving cell is relatively straightforward in the Syrian hamster embryo cell system in which both cloning efficiency and transformation are scored in the original colonies obtained after treatment of relatively small numbers of cells. The determination of absolute transformation frequencies in systems in which foci are scored on top of confluent monolayers is fraught with many difficulties. For example, "transformation frequencies" produced by a single concentration of 3-methylcholanthrene (MCA) in mouse fibroblast cell lines have been reported to vary over 4 orders of magnitude, depending on the experimental conditions (12, 14, 15). The most extreme example of this was reported from our laboratory (14). Single mouse prostate fibroblasts in individual dishes were treated with a noncytotoxic concentration of MCA and replated during logarithmic phase growth; all surviving dishes contained only one or two transformed foci. In a random recloning of progeny of the treated single cells, again all surviving dishes contained one or two transformed foci. Although this might be interpreted as a transformation frequency of 100%, it is difficult to explain why all the cells were not phenotypically transformed. In contrast, transformation frequencies from approximately $10^3$ C3H/10T1/2 cells per dish treated with comparable concentrations of MCA are generally reported to be ≈0.1%. We first showed that the transformation frequency obtained in C3H/10T1/2 cells varies inversely with the number of cells plated (12), and this has been confirmed in other laboratories (15–17). Because of these extreme variations in transformation frequencies, in our more recent papers we have reported transformation in terms of a directly observed quantity, the percentage of the total number of treated dishes containing type III transformed foci (12). In carefully conducted dose–response studies of the transformation of C3H/10T1/2 cells by x-irradiation, constant transformation frequencies were obtained when 100–400 viable cells survived the treatment (16, 17).

In an attempt to explain the complexities of the formation of transformed foci in MCA-treated C3H/10T1/2 cells, we have formulated a probabilistic view of this process, which we present here.

## DERIVATIONS

Our first assumption is that a carcinogen "activates" * a cell in the process of carcinogenesis. We define an activated cell as one that has a greater probability to be subsequently transformed than does a nonactivated cell. Second, we assume that activation is transmitted to daughter cells for an indefinite number of generations. Our third assumption is that an activated cell may be deactivated after removal of the activating stimulus.

Let $p_1$ be the probability that a cell is activated by a carcinogen, and let $S$ be the number of viable cells surviving treatment. Then, the number of activated cells prior to the first division will be $p_1S$. Now let $p_2$ be the probability that an activated cell will be transformed within its generation time. Then the number of transformed cells before the first division = $p_1p_2S$, and the number of activated cells remaining, $A_0$ is $p_1S(1 - p_2)$. We further assume that each transformed cell will ultimately produce a transformed focus. Hence, the number of transformed foci that originated prior to the first cell division will also be

$$F_0 = p_1p_2S.$$

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Abbreviation: MCA, 3-methylcholanthrene.

* This use of the word "activates" is purely operational and carries no implications regarding the term "metabolic activation."

Now let $p_3$ be the probability per cell generation that an activated cell becomes deactivated after removal of the activating stimulus. Because, in a typical transformation experiment with MCA, treatment is for 24 hr (which is approximately one doubling time) during which it is metabolized at a slow and constant rate, we introduce $p_3$ after the first cell division in this sequential model. Hence, after the first cell doubling has occurred, the number of activated cells will be $2p_1S(1-p_2)(1-p_3)$, from which it is evident that multiplying that number by $p_2$ will give the number of transformed foci that originated at this time,

$$F_1 = 2p_1p_2S(1-p_2)(1-p_3),$$

and the number of activated cells remaining prior to the second division will be $A_1 = 2p_1S(1-p_2)^2(1-p_3)$, and so on. Thus, the general term for the number of foci originating in the $i$th generation will be

$$F_i = p_1p_2S[2(1-p_2)(1-p_3)]^i.$$

Finally, we will obtain the mean number of transformed foci per dish, $F$, by adding all the foci that originated in each cell generation, and we will let $n$ = the total number of generations to confluence:

$$F = \left(\sum_{i=0}^{n} F_i = p_1p_2S\right) \sum_{i=0}^{n} [2(1-p_2)(1-p_3)]^i.$$

In this expression, $p_1$, $p_2$, and $p_3$ are assumed to be constant and independent of $n$ for a given experimental condition. Therefore:

$$F = p_1p_2S\,\frac{[2(1-p_2)(1-p_3)]^{n+1}-1}{2(1-p_2)(1-p_3)-1}. \qquad [1]$$

Eq. 1 is general for every case in which our assumptions are valid. However, in our case we can introduce further simplifications. Based on experimental results (see Table 3) with single cells treated with MCA at 1.0 $\mu$g/ml, we can conclude that $p_1$ is relatively high (0.05–1.0). Furthermore, because the mean number of foci per dish is invariably small compared to the total number of cells present at confluence, either the probability of deactivation is large ($p_3 \to 1$) or the probability of transformation is very small ($p_2 \ll 1$). In the former case, the terms in Eq. 1 containing the factor $(1-p_3)$ will become negligible compared to 1, and Eq. 1 will take the form

$$F = p_1p_2S.$$

This implies that the transformation frequency, $F/S$, is constant, which is contrary to experience when C3H/10T1/2 cells are treated with MCA (12, 15). Therefore, we reject the assumption that $p_3 \to 1$ and, consequently, accept that $p_2$ is a very small number. Therefore, we may simplify Eq. 1 as follows:

$$F = \frac{p_1p_2S}{2(1-p_3)-1}[2^{n+1}(1-p_3)^{n+1}-1]. \qquad [2]$$

If $p_3$ were sufficiently small, the first term of the expression in brackets in Eq. 2 would be large enough to make valid the approximation

$$F = \frac{p_1p_2S}{2(1-p_3)-1}\,2^{n+1}(1-p_3)^{n+1} \qquad [3]$$

which will be more accurate at higher values of $n$. Because the total number of cells, $N$, in a confluent dish is $2^nS$, Eq. 3 becomes

$$F = \frac{2p_1p_2N}{2(1-p_3)-1}(1-p_3)(1-p_3)^n$$

$$\text{or } \frac{F}{N} = \frac{2p_1p_2(1-p_3)}{2(1-p_3)-1}(1-p_3)^n. \qquad [4]$$

Because $p_1$, $p_2$, and $p_3$ are assumed to be constant, Eq. 4 has $n$ as the only independent variable, and we can put it in logarithmic form

$$\log\left(\frac{F}{N}\right) = \log\left[\frac{2p_1p_2(1-p_3)}{2(1-p_3)-1}\right] + n\log(1-p_3). \qquad [5]$$

Eq. 5 shows that, under the conditions of a typical transformation experiment, the logarithm of the mean number of foci per dish ($F$) divided by the mean number of cells at confluence ($N$) will be a linear function of the number of cell divisions to confluence ($n$). Thus, in a suitably designed experiment, a plot of log ($F/N$) against $n$ should give a straight line if our assumptions are valid. The slope of the line is equal to log $(1-p_3)$ and should be negative because $(1-p_3) < 1$; the intercept will be equal to log $[2p_1p_2(1-p_3)/2(1-p_3)-1]$. Once the slope is determined experimentally, the value of $p_3$ can be calculated and will show whether the approximation made in Eq. 3 is justified. From the intercept, we can calculate the value of $p_1p_2$.

The design of the experiments, therefore, involved plating different numbers of C3H/10T1/2 cells in 60-mm dishes, treating them 24 hr later with 0.5% acetone (solvent controls) or with MCA (1.0 $\mu$g/ml) for 24 hr, continuing the cultures past confluence (8 weeks for 1–400 cells or 6 weeks for 800–48,000 cells), fixing and staining, and counting the type III foci. In order to obtain consistent results, each experiment involved the use of the same batch of medium, serum, and cells and the same incubator.

## MATERIALS AND METHODS

**Cell Culture Procedures.** The C3H/10T1/2 Cl 8 cell line has been described in detail elsewhere (11, 12). Briefly, it is a cloned hypotetraploid permanent fibroblast cell line derived from C3H/He mouse embryos; it has an extremely low level of spontaneous transformation. The cells were carried in Eagle's BME plus 10% heat-inactivated fetal calf serum (GIBCO) and passaged as described (11, 12). Forty dishes were used per condition. Fluorescent lights were not used in the laminar flow hoods in which the work was done. Single cells were isolated by inspection of fragments of glass coverslips as described (14).

**Carcinogen.** MCA, obtained from Sigma, was purified by column chromatography, and its purity was determined by analytical high-performance liquid chromatography (18).

## RESULTS

Three major experiments were carried out in which different numbers (1–48,000) of C3H/10T1/2 cells were plated in 60-mm dishes. One day later, they were treated for 24 hr with 0.5% acetone (controls) or MCA at 1.0 $\mu$g/ml. The medium was then changed to medium not containing carcinogen, and the dishes were refed twice weekly until confluence and then once weekly until the experiments were terminated at 6–8 weeks by fixation in alcohol and staining with Giemsa. Only type III transformed foci were scored in these experiments, because cells from these foci are consistently tumorigenic when inoculated into immunosuppressed C3H mice (12).

$S$ is defined as the number of cells surviving treatment. In experiment 1, this was determined by plating efficiencies with 200 cells plated (higher numbers of cells gave too many colonies to count). The treatment for 24 hr with MCA did not significantly affect the plating efficiency. In experiment 2, $S$ was determined by cell counts of replicate dishes for each group trypsinized at the end of MCA treatment. Because these two types of measurements gave consistent results, in experiment

Table 1. Results obtained in experiment 3

| $c$ | $S$ | $D$ | $f$ | $F$* | $TF$, $\times 10^{-4}$ | $n$ | $-\log (F/N)$ |
|---|---|---|---|---|---|---|---|
| 10 | 2.9 | 18 | 1 | 0.056 | 192 | 18.1 | 7.16 |
| 25 | 7.2 | 28 | 2 | 0.071 | 99 | 16.8 | 7.05 |
| 50 | 15 | 20 | 2 | 0.10 | 69 | 15.8 | 6.90 |
| 100 | 29 | 20 | 3 | 0.15 | 52 | 14.8 | 6.73 |
| 200 | 58 | 25 | 13 | 0.52 | 90 | 13.8 | 6.19 |
| 400 | 116 | 29 | 4 | 0.14 | 12 | 12.8 | 6.76 |
| 800 | 231 | 25 | 8 | 0.32 | 14 | 11.8 | 6.40 |
| 1,500 | 434 | 26 | 12 | 0.46 | 11 | 10.8 | 6.24 |
| 3,000 | 868 | 31 | 25 | 0.81 | 9.3 | 9.8 | 6.00 |
| 6,000 | 1,736 | 31 | 32 | 1.03 | 5.9 | 8.8 | 5.89 |
| 12,000 | 3,472 | 33 | 42 | 1.27 | 3.7 | 7.8 | 5.80 |
| 24,000 | 6,943 | 38 | 36 | 0.95 | 1.4 | 6.8 | 5.93 |
| 48,000 | 13,886 | 36 | 53 | 1.47 | 1.1 | 5.8 | 5.74 |

All dishes were treated for 24 hr with MCA at 1.0 μg/ml. $c$, Number of cells plated; $S$, mean number of surviving cells (see text for explanation); $D$, number of dishes with cells surviving throughout the experiment (40 dishes plated); $f$, total number of type III foci in $D$ dishes; $F$, mean number of type III foci per dish; $TF$, transformation frequency ($F/S$); $n$, number of cell doublings to confluence; $N$, number of confluent cells ($8 \times 10^5$ per 60-mm dish).

* The number of foci in individual dishes followed Poisson distribution.

3 we calculated $S$ from the plating efficiency of the control cells in the batch of medium and serum used for this experiment and, because MCA exerted no cytotoxicity, used this value, 29%, to calculate all $S$ values in this experiment.

The results of the third experiment are shown in detail in Table 1 and are plotted in Fig. 1. No type III focus was observed in 38 "survivor" dishes in which 48,000 cells were plated and treated with 0.5% acetone. It is evident from Table 1 that the transformation frequency $F/S$ varied about 200-fold, inversely with the number of cells plated. This is in agreement with the earlier results from our laboratory (12) and Thilly's (15). Table 1 also shows that the total number of type III foci per dish ($F$) was small and increased with increasing cell number, although $F/S$ decreased greatly.

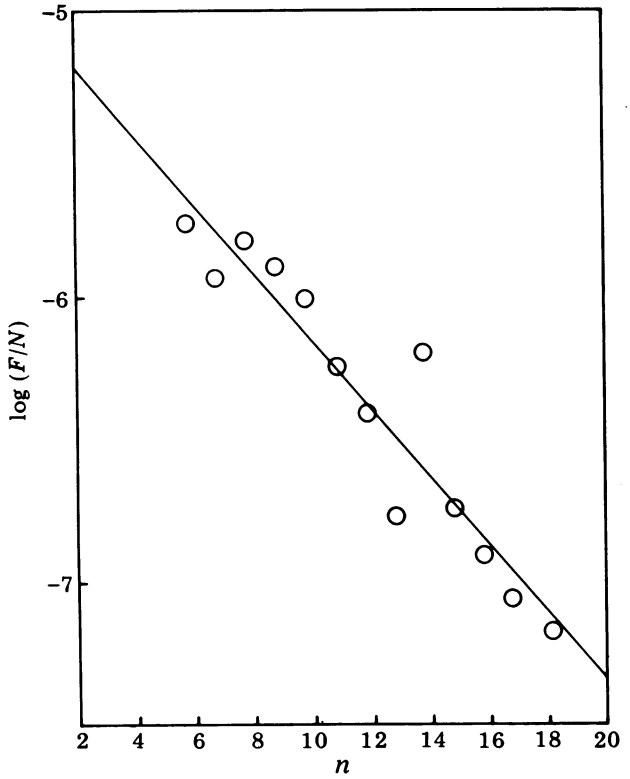


FIG. 1. Plot of log ($F/N$) against $n$; data are from Table 1.

Fig. 1 shows that, as expected from the theory, a linear relationship was obtained when log ($F/N$) was plotted against $n$. The best line obtained from Fig. 1 by a computerized least squares method is

$$\log\left(\frac{F}{N}\right) = -4.96 - 0.119\,n. \qquad [6]$$

The correlation coefficient was 0.942. As predicted from the last term in Eq. 5, a negative slope ($-0.119 \pm 0.013$) was found, from which $p_3 = 0.24$ was calculated. When this value of $p_3$ is introduced into Eq. 2, it is evident that the approximation made in Eq. 3 can be used without a significant loss of accuracy. The value of $p_3$ obtained directly from the slope of the line indicates that there is a probability of 24% that an activated cell will become deactivated during each generation. If this deactivation had not occurred, a horizontal line would have been obtained because at $p_3 = 0$, $\log(1 - p_3) = 0$. Now, when we substitute the numerical value of $p_3$ into the first term of Eq. 5, we obtain

$$\log \frac{2p_1p_2(1 - 0.24)}{2(1 - 0.24) - 1} = -4.96 \pm 0.16$$

from which we calculate that $p_1p_2 = 3.75 \times 10^{-6}$. The small value of this product is not surprising. Because we have already indicated that the probability of activation cannot be very different from 1, the probability of subsequent transformation, $p_2$, must be very small, which explains why only a small number of foci are observed in every dish. (There have been occasional reports of large numbers of foci per dish, but these are not in accord with Poisson distribution and probably result from colony splitting caused by migration of transformed cells in the dish).

Table 2 summarizes the results of three comparable experiments. Considering the experimental variations (different serum batches, cells at different passages, etc.), we believe that the results are reasonably consistent. Because experiment 3 involved the largest number of survivor dishes, we presented it in detail in Table 1 and Fig. 1.

Our main concern has been to establish the validity of our basic assumptions and derivations of this probabilistic view. We believe that we have now done so, because the results summarized in Table 2 are consistent with this view. It is now of interest to determine how many cells are activated at the end of carcinogen treatment by determining $p_1$. Unfortunately, a separate determination of $p_1$ at the present time would be extremely difficult because, even in single-cell experiments, not all activated cells give rise to foci.

We have also considered the situation, previously observed in mouse prostate fibroblasts (14), in which 100% of single cells in individual dishes treated with MCA and replated during logarithmic phase gave rise to transformed foci and all the progeny were potentially transformed. In the current series, experiments 1 and 2 contained dishes in which one attached cell (by inspection) was treated with MCA at 1.0 μg/ml, with the results shown in Table 3. When no replating was done, only one and two type III foci were obtained, respectively, in the dishes to give a mean number of foci per dish of 0.045 and 0.077. By calculation from Eq. 6, for a single cell the mean number of foci per dish in experiment 3 is 0.04. Thus, our theoretical calculations and observed results are consistent.

Table 2. Summary of three experiments conducted under comparable conditions

| Exp. | Intercept* | Slope* | $r$† | $p_1p_2$* | $p_3$ | Total of dishes, no. |
|---|---|---|---|---|---|---|
| 1 | $-5.40 \pm 0.18$† | $-0.076 \pm 0.013$† | 0.902 | $1.6 \times 10^{-6}$ | 0.16 | 292 |
| 2 | $-3.80 \pm 1.02$ | $-0.155 \pm 0.062$ | 0.897 | $4.5 \times 10^{-5}$ | 0.30 | 115 |
| 3 | $-4.96 \pm 0.16$ | $-0.119 \pm 0.013$ | 0.942 | $3.8 \times 10^{-6}$ | 0.24 | 360 |

The values were obtained from Eq. 5.
* Shown as mean ± SEM.
† Correlation coefficient.

## DISCUSSION

Our probabilistic treatment of the formation of type III transformed foci in C3H/10T1/2 cells provides a framework within which a number of previously irreconcilable facts can be explained. The experiments, particularly the one shown in Table 1 and Fig. 1, clearly demonstrate that Eq. 5 describes the formation of transformed foci in our experimental system, and hence the adequacy of our assumptions and derivations is confirmed.

The value of $p_3$ can easily be determined from the slope of the experimental line. Under the conditions of our experiments, it is 0.24 per generation. Unfortunately, although we can experimentally determine the product $p_1p_2$ from the intercept, as described above, we cannot yet determine $p_1$ and $p_2$ independently. Yet, as mentioned in the derivations, it is highly likely that, under our experimental conditions, $p_1$ lies between 0.05 and 1.0; hence, $p_2$ must have a value of about $10^{-6}$ because $p_1p_2 = 3.8 \times 10^{-6}$ as calculated from experiment 3.

Eq. 5 and its experimental verification show that when different numbers of cells are plated and treated with MCA the expected number of foci per dish ($F$) will always be small but will increase when more cells are plated per dish. The equation also shows that, even if all the original cells were activated, only a very few of its descendants would give rise to foci. Eq. 5 also predicts that, if larger dishes were used, there would be more foci per dish. It predicted correctly the small number of foci obtained when single cells were plated.

Eq. 5 shows the inadequacy of the use of the transformation frequency, $F/S$, as a general quantitative measure of transformation in focus assays because it is evident that the increment in $F$ is very small compared to the corresponding increment in the effective number of cells, $S$. Thus, $F/S$ changes very rapidly to smaller values when cells are plated at relatively high densities. Because Eq. 5 uses variables that are independent of the numbers of cells plated or the size of the dish, it can be used to characterize the carcinogen–cell system without ambiguity whereas the transformation frequency varies with the number of cells treated. Similar relationships presumably will be obtained with other carcinogens and can be used to describe their effects on the production of transformed foci. Obviously, this approach needs to be extended to study dose–response relationships, the effects of tumor promoters, etc. For example, we need to determine whether the slope of the line described by Eq. 5 is affected by the concentration of carcinogen and whether the intercept is affected by tumor promoters.

Table 3. Treatment of individual single cells with MCA (1.0 μg/ml) with no replating

| Exp. | $S$* | $D$ | $f$ | $F$ | $TF$, % | $n$ | $-\log (F/N)$ |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 22 | 1 | 0.045 | 4.5 | 19.6 | 7.25 |
| 2 | 1 | 26 | 2 | 0.077 | 7.7 | 19.6 | 7.02 |
| 3 (calculated from Eq. 6) | | | | 0.041 | 4.0 | 19.6 | 7.29 |

* Definitions as in Table 1.

Thus, apart from any theoretical considerations, the formation of transformed foci under our experimental conditions is described by the equation

$$\log (F/N) = a + bn \quad [7]$$

in which $a$ and $b$ are constants. Yet, on the basis of our theoretical speculations we were able to predict such behavior and to assign biological significance to the constants $a$ and $b$ in terms of the probabilities of activation, deactivation, and transformation. However, it is premature to assign molecular mechanisms to these probabilities.

It is unfortunate that our probabilistic theory in its present form is insufficient to describe the results of the x-ray transformation of these same cells (16, 17). In those experiments the transformation frequency was constant for 300 or less surviving cells (at a constant dose) and decreased significantly above this cell number. We did not find a constant transformation frequency at low numbers of cells (Table 1). These differences might be a consequence of one or more of the following: (*i*) the "activation" process for x-irradiation and MCA may involve different molecular mechanisms; (*ii*) the action of x-rays is practically instantaneous, whereas MCA is metabolically converted to an ultimate carcinogenic epoxide at a constant rate throughout the 24-hr treatment (19); and (*iii*) x-irradiation produces considerable cytotoxicity [5% survival at 700 rads, the dose used in an experiment in which the transformation frequency was constant with cell number (charts 1 and 3 in ref. 17)], whereas MCA was not detectably cytotoxic.

In the transformation system that uses primary or secondary cultures of Syrian hamster embryo cells (5–10), sparsely plated cells treated with chemical carcinogens give rise to primary colonies that can be scored as normal or morphologically transformed. Individual colonies are considered to arise from single cells and are usually homogeneous in appearance, although sectored colonies have been observed. If we apply our probabilistic considerations to this system, then Eq. 1 should also provide an adequate quantitative description. However, in this case we must account for the very high proportion of transformed cells that populate the transformed colonies. This implies that here the probability that an activated cell is transformed, $p_2$, is no longer small. Hence, the simplification used to obtain Eq. 3 for C3H/10T1/2 cells is not valid. Instead, if we consider that $p_2$ is approximately 1, Eq. 1 takes the form $F = p_1p_2S$. Thus, the transformation frequency $F/S = p_1p_2$. This shows that in this cell system, in which individual colonies can be scored, the transformation frequency is independent of the number of cells plated, which is in accord with experimentation (6–8). Thus, this probabilistic theory can explain the diverse behavior of the transformation both of cell lines and cell strains.

In conclusion, we have described mathematically, by Eq. 5, the formation of transformed foci produced by MCA treatment

of C3H/10T1/2 cells. This equation was derived solely from probabilistic considerations. We have experimentally verified the equation and have determined experimental values of the product of $p_1$ and $p_2$ and of $p_3$. It remains to ascribe specific biological phenomena to these probabilities.

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