Modelling the Birth of Transformation Foci in Cell Cultures

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Abstract

In vitro tests are very useful tools to assess the carcinogenic effects of chemicals and the effectiveness of drugs and chemopreventive agents. A cellular automata model of the birth of so-called transformation foci is presented here, based upon biological knowledge about the process of cell transformation, which is the *in vitro* analogue of tumor formation. The model describes the dynamics of the process, and it provides indications concerning the dependence of the number of foci upon the number of cells that have been initially seeded.

It is shown that the model can account for known experimental data; moreover, different model versions lead to different scaling behaviours, which seem compatible with the results obtained by using different experimental protocols.

This line of research appears therefore able to provide useful information to interpret the results of *in vitro* tests, and to suggest further experiments.

Keywords: transformation foci, cell cultures, dynamical models, cellular automata, cancer growth

1 Introduction

While many studies exist concerning *in vivo* cancer growth (see e.g. Adam and Bellomo, 1997, for a review), the mathematical analysis of *in vitro* assays is less developed. However, in order to study the main features of tumor formation, *in vitro* tests provide very useful information, reducing the need for animal experimentation (Kakunaga and Yamasaki, 1985; Yamasaki et al, 1996; Vaccari et al, 1999). Moreover, the recent developments of molecular biology allow for a careful comparison, at the level of patterns of gene expression, between *in vivo* and *in vitro* systems, and it is therefore expected that the importance of these latter methods will further increase in the near future.

These tests are based upon the use of well defined cell clones (Kakunaga and Yamasaki, 1985): some cells are plated on a Petri dish and are exposed to a chemical (e.g. a suspect carcinogen) for a short period of time. After that, the chemical is washed away and the

cells are cultured for a longer period. They reach confluence (i.e., they cover the bottom of the plate) in some days, but the test continues for some more weeks. While the growth of the number of normal cells is inhibited, transformed cells, which are not affected by "contact inhibition", experience further growth giving rise to macroscopic structures (transformation malignant foci), each one composed by many transformed cells.

Macroscopic foci are counted at the end of the experiment; in this way it is possible to evaluate the carcinogenic effect of the chemical under study by comparison with the results of other known substances; indeed, some of these tests, e.g. those using Balb/c 3T3 clones, show good correlation with *in vivo* tests (Matthews et al, 1993). Moreover, the use of *in vitro* systems with well defined cell clones makes the molecular characterization of the steps leading to transformation easier than their *in vivo* analogues.

Anyway, even these *in vitro* systems are complex biological systems and are subject to a high level of variability among different tests. The variables that are usually determined are

- the number of transformation foci per dish which are found at the end of the experiment, F_{fin}, , in different experimental conditions (e.g. by using different concentrations of a suspect carcinogen)
- the transformation frequency $T_f \equiv F_{fin}/M_0$, where M_0 is the number of cells which survive after initial plating and exposure to the carcinogen (which may well have a citotoxic effect).

However, by focusing only upon the number of foci at different carcinogen concentrations one is likely to ignore some useful information that may be provided by these methods, perhaps introducing some further tests. Mathematical modelling might therefore improve our capability to extract meaningful information from *in vitro* tests and to suggest further experiments, by providing a framework to interpret the time development of cell cultures.

Existing dynamical models of the birth of transformation foci are usually of the population dynamics type, and they treat cell growth as spatially homogeneous (Fernadez et al, 1980). This were appropriate if the cells were free to wonder in the plate, but they actually are bound to the bottom of the dish, so they cannot move and interact freely with each other. A simple difference which should be noticed concerns the dependence of the growth rate upon the number of cells: if cells were free to wonder, than this dependence would be linear (leading to exponential growth) until the effects of crowding ("contact inhibition") were felt; however, if the growth takes place in a 2D way, around an initially seeded cell, one expects (and actually observes) the development of roughly circular clusters. Cells within the cluster start to feel crowding much earlier than in the previous case, and the only cells which actually contribute to growth are those close to the boundary (therefore providing a growth rate roughly proportional to $N^{1/2}$).

A different model was proposed by Mordan et al (1983), which takes into account the local features related to the development of cell clusters; however, this model is phenomenological, as it assumes that the number of final foci is a logistic function of

the average size of the cell clusters (microcolonies) at the time of confluence, and no attempt is made to follow the dynamics of growth and transformation.
Therefore, in order to properly describe the phenomena which take place during cell growth, it is important to develop a truly dynamical model which takes into due account the locality of the processes involved.
We have developed such a model using the framework of cellular automata (shortly, CA) which are well suited for this task (Burks, 1970; Serra and Zanarini, 1990). The available space, i.e. the bottom of the Petri dish, is divided into pieces of equal size, which are called cells, in CA jargon; each of these cells may be either occupied by a biological cell or not. In order to avoid confusion related to the two different meanings of the term "cell", we will limit the use of this latter term to biological cells, and we will explicitly refer to the "CA cell", "lattice site" or simply "site" in the other case.
The flexibility in defining the transition function of a CA allows one to introduce the modelling assumptions (detailed below) concerning the way how different types of cells interact.
While a preliminary version of the model has been outlined elsewhere (Serra et al., 2000a), we present here novel results concerning an important variable which can be experimentally determined, i.e. the dependence, *ceteris paribus*, of the number F_{fin} of final transformation foci upon the number M_0 of cells which are initially seeded and which survive the initial treatment. In order to study this variable, a slightly improved version of the original model has been developed, which is also described here. Moreover, a comparison of some robust features of the model with published experimental data is discussed.

2 The model and its dynamics

As several phenomena take place during cell growth, it is appropriate to try to develop a minimal model, which aims at taking into account the relevant aspects, leaving aside many details (unless comparison with experimental data does not compel us to take them into account).
The model which is proposed here is based upon the well known fact that the cell transformation process involves more than one step (Fernandez et al, 1980; Kennedy et al., 1980, 1984); a minimal model therefore requires only two steps, so we will suppose that normal cells (called "B" cells) can become active ("A" type) under the effect of the carcinogen. As the cell lines which are used for *in vitro* tests have already undergone some of the mutations which finally lead to the "transformed" state, they are not really "normal" cells like those found in vivo, but are "normal" from the viewpoint of the test. When no activation event occurs, B cells grow from their initial number to a full monolayer, which covers the bottom of the culture plate.
A-type cells can then spontaneously undergo a further change, leading them to transformed (i.e., "T") cells. A-type cells can possibly be detected by the cell defense system, and induced to death, so their dying probability may exceed that of B-type cells. Activation represents the intermediate step between the original cell and the

transformed one: we will suppose that it is a property which is inherited by daughter cells, like e.g. a mutation.
A and B-type cells, which are supposed to be indistinguishable at the microscope, feel contact inhibition, but T cells don't and continue to grow. A single T cell can give rise to a full macroscopic transformation focus.
The growth of T cells could be described by a further CA model (which can be built on top of the previous one) which should describe how a single transformed cell may give rise to a focus; this process is not modelled in the present work where we will assume that each newborn T cell gives rise to a full focus (unless it is too close to another T seed; in this case, as coalescence between nearby foci may occur, we will count the two as a single focus, in comparing the model with experimental data).
Following Fernandez et al (1980) we will assume that during the initial exposure to the carcinogen some B cells may become activated (A cells). The model presented here does not describe the events which take place during this initial phase (which lasts typically 1 or 2 days), but it focuses upon what happens after the carcinogen has been washed away. During this 3-5 weeks period, the culture medium is periodically changed, so that cells are provided with fresh nutrients and metabolic wastes are removed. It is assumed that some A cells may spontaneously become transformed during this period and, as it has already been stressed, that each newborn T cell develops into a transformation focus.

2.1 The cellular automata model

We will divide the 2-D space (which physically corresponds to the bottom of the culture plate) in a fixed number N of CA cells, whose size is chosen to match the average size of a biological cell. A-type cells are supposed to be macroscopically indistinguishable from B cells, so their average size is the same. T cells are smaller than B cells, but we will neglect this aspect here.
Therefore, in any lattice site there may be either a B cell, an A cell, a T cell or no cell at all. The state of site i will be called X_i; $X_i \in \{B,A,T,E\}$, where the first three symbols refer to the kind of (biological) cell which may occupy the site, and the latter refers to the case where the site is empty.
The topology is square, with the 9-membered Moore neighbourhood (Toffoli and Margolus, 1987). Updating is synchronous, as is typical in cellular automata.
At each time step, a cell may either do nothing, or duplicate itself, or give birth to a different cell, or die. Note that it is supposed that the major events in a cell life, like the transition from A to T type, take place only when the cell enters its reproductive cycle and its DNA double strand is open.
Therefore, at each time step, a lattice site which is occupied by a biological cell of type A or B may either try to reproduce or not: this can be described by a stochastic variable r_i which can assume either the value 1 (try) or 0 (stay idle) according to a given probability. Let us concentrate upon the case when reproduction is "on" at time t, i.e. $r_i(t)=1$. For every site which is either in state A or B, a check will be performed, to test

whether there are some empty neighbours; let $e_i(t)=1$ if there is at least an empty neighbour of lattice point i (i=1 ...N), $e_i(t)=0$ otherwise.
For every time step t, for every CA cell i such that $r_i(t)e_i(t)=1$, some state change will then take place; the frequencies associated to the different alternatives are $p_{B->A}$ (probability that a B cell gives birth to an A cell), $p_{A->T}$ (probability that an A cell gives birth to a T cell) and p_{RB} , p_{RA} (probability that a B or an A cell duplicates itself respectively).
A reproducing cell will place its daughter cell in one of the empty neighbours, at random. Actually, the algorithm for updating the state of the lattice sites proceeds in several steps: first, the CA sites occupied by B or A-type cells are considered, and each reproducing cell identifies the empty site which is to be occupied by its daughter cell; in a second step, all the empty CA cells are considered, and those which have been selected by a neighbour for reproduction become occupied. If there are conflicts (i.e. two or more neighbours of different kinds all trying to occupy the same empty space) then a stochastic choice is performed.
The search for empty neighbours is iterated: indeed, without iterations, an unrealistically high slow down of growth would be observed, due to the fact that some empty space would be left unoccupied even if it were available: for example, two biological cells might try to reproduce in the same site, intermediate between them, even if they are both surrounded by other empty sites. In this case the algorithm described above would allow only one biological cell to reproduce, while, when iteration is used, both can reproduce. It has been experimentally verified that three iterations suffice to make the artificial slow down negligible.
A cell may also die: let p_{DA} and p_{DB} denote the probability that, at each time step, an A or B cell dies off (note that cells may die at every time, not only when trying to reproduce). Due to the presence of cell surveillance systems which try to eliminate abnormal cells, it will be assumed that $p_{DA} > p_{DB}$. The possibility that a T cell dies off will be neglected here, as the dynamics of T cells will not be described in detail.
In the following simulations, we will also assume that $p_{B->A}$, the probability of spontaneous activation, is negligible during cell growth. Cells become activated in the initial phase of exposure to the carcinogen. The time origin is set at the end of this exposure period.
We will also assume that there is no nutrient limitation during all the culture period: the growth of the cell population is bounded only by the crowding of other cells, and no lack of essential nutrients is experienced.
A key variable, in order to compare simulations and experiments, is the number of transformation foci. Note that, if two activated cells which are close to each other become transformed, each of them generates a focus; however, from the viewpoint of the experimenter, a single focus will be observed. Therefore, in foci counting, we will consider that two transformed cells which are very close to each other give actually rise to a single focus.
Let us briefly summarize the model which has been developed according to the above hypotheses.

The CA is formally defined as a quadruple A=<**G**,**V**,**Q**,f>, where

- **G** is the cellular space, in our case $\mathbf{G} \subset \mathbf{Z}^2$ is a finite subset of the set of points with integer coordinates in 2-D euclidean space
- **V** defines the neighbourhood; using relative coordinates **V**={(0,0),(0,1),(1,1),(1,0),(1,-1),(0,-1),(-1,-1),(-1,0),(-1,1)}
- **Q** is the state space: it is the cartesian product of the state space of i) a variable describing the kind of biological cell, ii) a variable describing the reproductive state and iii) a variable describing the relative direction in which reproduction could take place: **Q**=**X**x**Y**x**D**, where **X**={B,A,T,E} ; **Y**={0,1} ; **D** = **V**-{0,0}.
- f is the transition function which describes how the state of a CA cell is determined form the knowledge of the previous states of its neighbours. The basic steps are the following

for every t

{

for every site i such that X_i#E

- determine whether reproduction will be attempted (by comparing a stochastic variable with a threshold which determines the reproduction rate); let $\mathbf{G}_1$ be this set of sites
- for every site in $\mathbf{G}_1$, verify whether there is at least an empty site in the neighbourhood; let $\mathbf{G}_2$ be the set of CA cells with at least an empty neighbour
- repeat
- {
- for every site in $\mathbf{G}_2$, determine the direction of reproduction; if there are more available sites, choose at random among them
- for every site i such that X_i=E
  - determine whether at least one of the neighbours has a direction of reproduction pointing to i; let $\mathbf{G}_i$ be the set of these CA cells
  - if all the sites in $\mathbf{G}_i$ have a common state, then set $X_i(t+1)$ equal to that state; otherwise, choose at random among the states
  - assign at random the newborn biological cell in $\mathbf{G}_i$ to one of its parents (let it be P_i)
  - set $X_i(t+1)$ equal to the state of P_i
  - if the new state is A, then change it to T according to a fixed probability
- }
- remove P_i from $\mathbf{G}_2$

}

2.2 Modifications of the basic model

The model described above introduces, besides others, a simplification which might *a priori* appear dangerous; indeed, it has been assumed that an A cell can become fully transformed in a single generation. Looking at the phenomenon at a more microscopic level, it may be supposed that "transformation" corresponds to a further mutation, which is likely to take initially place only on one of the two DNA strands of one of the two daughter cells of the original "A" parent. If "transformation" were dominant, it would show up immediately, otherwise it would need another generation to appear at a phenotipic level (in one out of four offsprings of the initial parent).
In this latter case, competition for available space from other cells and contact inhibition might actually change the transformation frequency, and it is not obvious a priori that the model features remain unaltered. Therefore, we have also tested a modified version of the model, where the final transformation from A to T takes actually place in two generations: A -> A' -> T.
However, the main features of the model which are described in Section 3 are not deeply affected by this modification (which might be important to describe what happens in the initial period of exposure to the carcinogen).
A further modification which has been tested is motivated by the fact that, in general, some form of taxis (cell movement) in the dish cannot be a priori excluded. In this case, cells could migrate far from their parents, so that the effects of contact inhibition would be initially mitigated. We have therefore also tested a model where newborn biological cells are allowed to move one CA cell away from their parents, therefore limiting the crowding effects in the initial growth phases (later, when confluence is close, moving away does not lead to significant benefits).
Also in this case, the modification did not affect the major features of the model behaviour which have been explored.

3 Simulations

A series of experiments has been performed, to study the dynamical properties of the CA model described in section 2, on a grid of 400*400=160.000 CA cells. The graphs and picture shown in this section have been obtained with the set of parameters of table 1 (where N_p is the number of simulations run for each set of parameter values and initial conditions). In the following, A(t), B(t) and T(t) will denote the number of cells of A, B and T type at time step t; M(t)=A(t)+B(t) is the total number of non transformed cells; y(t) = A(t)/M(t); subscript "0" denotes initial values, e.g. M_0=M(0). As it has already been observed, the origin of time is set at the end of the exposure to the carcinogen (which lasts 1-2 days)
Note that, in order to use this model to simulate actual in vitro carcinogenesis tests, one must stop the replication after a certain number of generations (t_{end}), instead of studying the limit $t->\infty$.

Table 1 (meaning of symbols is given in main text)

parameter	value	Parameter	Value	parameter	Value
N_p	10	M_0	160-32000	$p_{B->A}$	0
p_{RB}	1	y_0	0.1	p_{DB}	0
p_{RA}	0.1-0.7	$p_{A->T}$	0.001	p_{DA}	0

The cells which have survived seeding and treatment may then have already undergone some reproduction, so we will investigate two classes of cases, differing for the configuration of initial cells ("seeds"):

a) sparse cells, where at t=0 there are M_0 cells, placed at random in the spatial grid
b) grouped cells, where the initial clusters are composed by two cells; in this case, whenever there is an A cell in the initial seed, there is also a B cell in the same seed (due to the fact that A cells originate from B cells, and to the hypothesis that "activation" initially affects only one strand of DNA). In some cases initial clusters formed by 4 cells (with at most one A cell) have been tested.

In order to specify in a concise way, for each figure shown below, which model has been used, we will use the following shorthand notation: BM: base model, described in section 2.1; 2GM: two-generation model, as described in section 2.2; MCM: moving cell models, as also described in section 2.2

A minuscule letter ("s" or "g"), added to the previous notation, indicates whether sparse or grouped cells are considered, so e.g. BMs is "base model with sparse seed".

The expected growth of the clusters of cells is observed, until confluence (fig. 1). The growth of the number of cells in time follows a familiar S-shaped curve, as shown in fig. 2. Note that the MCM has a higher initial growth rate than that of the BM, as it should be expected. Moreover, in a log-log plot of M vs. time, one easily verifies that, in the first part of the curve, M grows with t^2, as it is to be expected if the growth takes place on the borders of a 2D cluster ($dM/dt \propto M^{1/2}$)

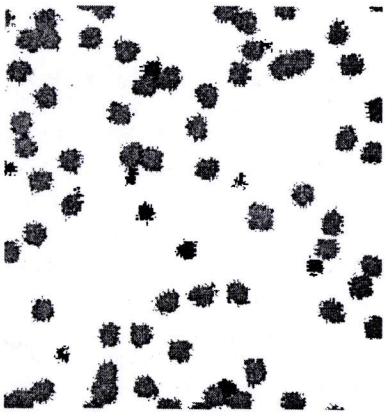

Fig.1 : Cluster development after 9 generations
(model MCMs, M_0=160, p_{RA}=0.7, p_{DA}=0)

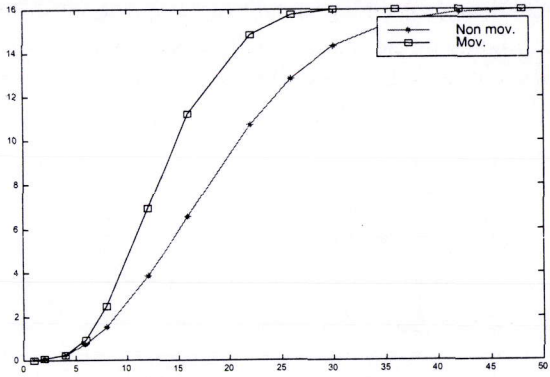

Fig. 2: time evolution of M(t): x-axis: generations; y-axis: number of cells $*10^{-4}$ (Models MBs and MCMs, M_0=160, p_{RA}=0.7, p_{DA}=0)

Let us consider the dependence of the final number of transformation foci upon the number of initially seeded cells. As coalescence is a rare event, we can actually consider T_{fin}, the number of cells of T type which are found, instead of F_{fin} - note that T cell reproduction is not considered, so all the T cells which are found have been produced by an A cell.
A typical diagram, showing how the number of T cells scales with M_0, is given in fig. 3

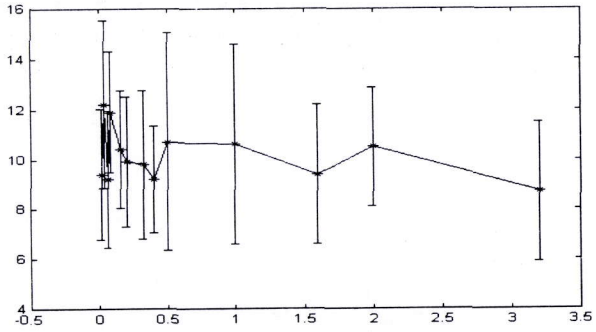

Fig. 3: number of transformed cells found at the end of the experiment (T_{fin}) vs the number of initially seeded cells M_0*10^{-4} (Model MCMs, p_{RA}=0.7, p_{DA}=0)

It can be seen that, as the transformation frequency is low, the variance of the data is high. Since in this version of the model transformation occurs with a fixed probability every time a new A cell is generated, we can study directly the number of newly generated A cells during the whole experiment (let it be N_A) which is much larger and

less affected by noise, and which should be proportional, in the limit of a very large number of simulations, to the number of T cells. Indeed, it can be seen that error bars are considerably reduced in this case (fig. 4).

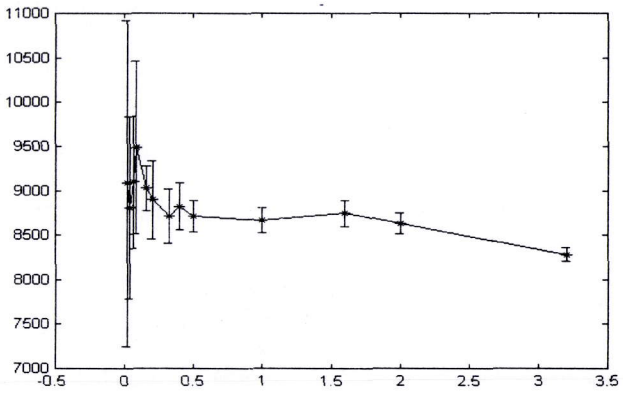

Fig. 4: number of A cells which have been generated during the experiment (N_A) vs the number of initially seeded cells M_0*10^{-4} (Model MCMs, $p_{RA}=0.7$, $p_{DA}=0$)

The dependence of N_A upon M_0 is rather flat; for reasons given above, we expect the same also of the dependence of the number of transformation foci - and it is actually the case, although of course with large error bars. Let us however observe that, if A cells are considerably less prone to reproduction than B cells, there is a different scaling behaviour, which leads to an increasing number of foci as M_0 increases (see fig.5)

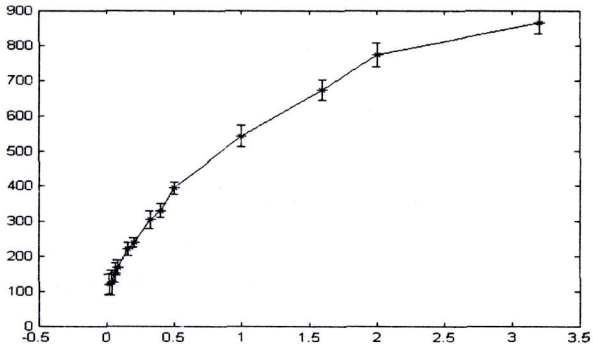

Fig. 5: number of A cells which have been generated during the experiment (N_A) vs the number of initially seeded cells M_0*10^{-4} (Model MCMs, $p_{RA}=0.1$, $p_{DA}=0$)

These results can be understood by considering that, under the hypotheses which have been done, there is no spontaneous death of A and B cells, so every new A, and every new T, is formed before confluence, in the phase of rapid growth. If A's reproduction is

highly unfavoured with respect to that of the B's, then we can expect that higher M_0, which implies a higher A_0 value, would lead also to more A reproduction and therefore to more T's (fig. 5). But if A's reproduction is only slightly depressed, then this disadvantage in rate is compensated by the effects of crowding, and no appreciable overall trend is observed.

But let us now consider the case where the seeds are formed by pairs of cells, recalling that the initial clusters are composed either by two B cells or by one A and one B cell. In this case, each initial A cell immediately faces competition with the faster growing B's, and the overall behaviour is such that the number of A's reproductions, and therefore the number of foci, is a growing function of M_0 (figs. 6,7).

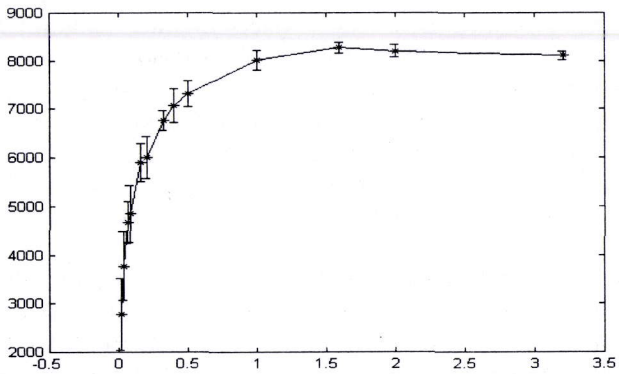

Fig. 6: number of A cells which have been generated during the experiment N_A vs the number of initially seeded cells M_0*10^{-4} (Model MCMg, $p_{RA}=0.7$, $p_{DA}=0$)

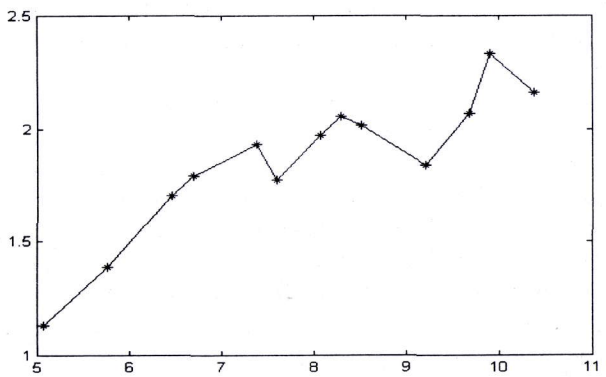

Fig. 7: number of transformed cells found at the end of the experiment T_{fin} vs M_0*10^{-4} (data on a log-log scale, Model MCMg, $p_{RA}=0.7$, $p_{DA}=0$)

Behaviours similar to those shown above have been found also for the different models which have been tested, i.e. with or without cell movement. Also the introduction of the

two-generation procedure described in section 2.2 does not modify the main conclusions given above.

4 Comparison with experimental results

We have seen that the CA model can lead to two different scaling behaviours. Unless the reproduction of the A's is highly unfavoured with respect to that of the B's, in the case where the clusters develop from isolated cells the dependence of the number of final foci (F_{fin}) upon the initial number of cells (M_0) is rather flat, for a wide range of parameters, and for the different model versions which we have tested (including moving and non moving cells, as well as one-generation and two-generation models).
On the other hand, if the initial seeds are formed by groups of cells, F_{fin} is a growing function of M_0. This is again a robust result which continues to hold, for example, if we suppose that the initial seeds are composed by quadruples of cells, instead of couples.
It should be remarked that this difference in behaviour is not due to the actual number of initial cells (as can easily be checked) but to the fact that in the second case each A cell is born close to at least one B cell, and it is therefore subject to competition pressure from the very beginning - while in the former case nuclei composed by all A's have some generations available for reproduction, before colliding with other nuclei composed by B cells.
So, what about the experimental results? It is interesting to observe that most experimental data which have been reported, concerning the growth of cell cultures exposed to a chemical carcinogen, show a power-law increase of F_{fin} wrt M_0. The data by Fernandez et al (1980), concerning C3H10T1/2 cells exposed to methylcholanthrene, show an initial slope of about 0.4, in impressive agreement with those of fig.7. Also the data by Haber et al., (1977) concerning C3H10T1/2 cells exposed to benzo(a)pyrene, and those of Reznikoff et al (1973b), concerning C3H10T1/2 cells exposed to 3-methylcholanthrene show an increase in the number of foci per dish, with increasing seeding density, with a similar slope.
Note that no adjustable parameters have been used, although we have chosen a value for the ratio of the parameters $p_{RA}/p_{RB} = 0.7$, which agrees with the estimate of Fernandez et al. of the analogous parameter in their model (which, however, was supposed to describe a possible repair of activated cells, leading them back to their normal state). As B>>A, the fact that the A's which are detected by the cell surveillance system are repaired or killed does not significantly influence the overall outcome.
This is the kind of behaviour which we would expect in our model, if the initial event of activation would affect only one of the two strands of the DNA of a replicating cell - so that A's always appear close to their parent B's.
There are however other experiments where some cells are taken from a dish after reaching confluence and then replated to a new dish; in this case, replating would lead to initially sparse cells, so we would expect that the dependence of F_{fin} wrt M_0 be rather low; actually, interesting data by Kennedy et al (1980, 1984) and Mordan et al (1983) show that in replating experiments there exist large intervals of M_0 values where no

appreciable difference in foci per dish is observed (but note that data by Haber et al. (1977) seem to show an increase of F_{fin} with growing M_0 in their replating experiments). Note also that some interesting experiments have been performed using x-ray irradiation, instead of a chemical carcinogen (Kennedy et al; 1980, 1984; Little, 1985, 1998). It has been suggested that in this case the intermediate, activated state of the cell corresponds to a large, genome-wide damage to the DNA (Little, 1998), which is supposed to be inheritable, and prone to develop later in a fully transformed state. Therefore, our model should be applicable also to this case. One could speculate that in such a case the wide damage could affect both DNA strands, and therefore there would exist initial clusters formed by activated cells only.
We would therefore guess that F_{fin} be approximately independent of M_0, and this is in agreement with experimental data (Little, 1985, fig. 2).

5 Discussion

The interpretation of experimental data which has been suggested is speculative at this stage, and needs further testing, which might in turn lead to a better understanding of the phenomena which are involved. However, it already shows the usefulness of models like the one which has been studied here in providing a clearer picture of the complex processes which take place in *in vitro* tests, and in identifying different action patterns of different carcinogenic agents.
Moreover, as far as the dependence of the number of foci upon the number of initial cells is concerned, the very hypothesis that the seeding procedure may deeply affect the overall outcome of the process, leading to qualitatively different behaviours in the case of re-seeding with respect to the standard tests, had never been considered in the previous literature on the subject, to the best of our knowledge.
Further modifications to the model, which are under investigation, concern

- The explicit introduction of chemicals, in order to accurately describe the actual cell growth under the influence of nutrients as well as the use of chemicals which may either enhance or inhibit the transformation frequency (e.g. tumor promoters or chemopreventive agents)
- The description of the initial phases of exposure to the carcinogen
- The use of a more sophisticated cell model, coupling the genetic/metabolic pathways of the cell to its growth and to the chemicals which are available in the culture plate (see Serra et al., 1997, 2000b)
- the modelling of the growth of transformation foci, in order to extract further information from their geometrical features

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