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Growth Regulation in Rous Sarcoma Virus Infected Chicken Embryo

Fibroblasts: the role of the src gene

Gordon Parry^{*}, James C. Bartholomew, and Mina J. Bissell[†]

Laboratory of Chemical Biodynamics
Lawrence Berkeley Laboratory
University of California
Berkeley California 94720

^{*}Present address:
Department of Biochemistry
St George's Hospital Medical School
Cranmer Terrace
London, SW17 0RE, United Kingdom

[†]To whom reprint requests should be addressed

The relationship between growth regulation and cell transformation has been studied in many cultured cell lines transformed by a range of oncogenic agents. The main conclusion derived from these investigations is that the nature of the growth regulatory lesion in transformed cells is a function of the agent used to induce transformation¹⁻⁵. For example, when 3T3 fibroblasts are rendered stationary by serum deprivation, normal cells accumulate in G_1 but SV40-transformed cells are arrested at all stages of the cell cycle^{1,2,6}. In contrast, 3T3 cells transformed with Rous sarcoma virus B77, accumulate in G_1 upon serum deprivation². This is also true when mouse sarcoma virus (MSV) is used as the transforming agent. MSV-transformed cells accumulate in G_1 , just as do normal cells. In this letter we report a detailed study of the mechanisms leading to loss of growth control in chicken embryo fibroblasts transformed by Rous sarcoma virus (RSV). We have been particularly concerned with the role of the src gene in the process, and have used RSV mutants temperature sensitive (ts) for transformation to investigate the nature of the growth regulatory lesion. Two principal findings have emerged: (a) the stationary phase of the cell cycle (G_1) in chick embryo fibroblasts appears to have two distinct regulatory compartments, (for simplicity we are adopting Brooks et al.'s⁷ terminology and refer to these as "Q" and "A" states). When rendered stationary at 41.5° by serum deprivation, normal cells enter a Q state, but cells infected with the ts-mutant occupy an A state. (b) While normal cells can occupy either state depending on culture conditions, the ts-infected cells do not appear to enter Q even though a known src gene product, a kinase, is reported to be inactive at this temperature^{9,10}. The

possibility is discussed that viral factors other than the active src protein kinase influence growth control in infected cultures.

The experiments involved plating cultures at high density and low serum in 35 mm plates in medium containing 0.5% chicken serum (see Legend to Fig. 1), and maintaining them at either 41.5° or 35° for 36-48 hrs before initiating an experiment. DNA synthesis was followed by staining cells with propidium iodide and analyzing cellular fluorescence by flow cytometry. When ts-infected cells were maintained at 41.5°, most cells were in G₁ 48 hrs later (Table 1). In contrast, a significant proportion of cells cultured at 35° were in S phase 48 hrs after plating. This was found to be so for mutants LA24 and LA29, (Prague A strain) and also for NY68, (Schmidt Rupp strain). Very few normal cells were in the S phase at either temperatures. When the kinetics of initiation of DNA synthesis were examined upon temperature shift from 41.5° to 35° (without a medium change), it was found that cells entered S approximately 8 hrs after shift (Fig. 1). The data thus demonstrates that at the nonpermissive temperature infected cells become blocked in G₁ phase, but that they initiate DNA synthesis upon activation of the src protein at the lower permissive temperature, confirming an earlier work of Bell *et al.*⁸ This lag period was distinctly longer when normal cells seeded at high density and maintained in low serum were stimulated to initiate DNA synthesis by serum addition at 41.5°. In six separate experiments, normal cells at 41.5° were found to enter S phase 12-14 hrs after stimulation while LA24 infected cells entered S phase 6-8 hrs after stimulation (Fig. 2). These data implied that in the stationary state normal and LA24-infected cultures were in distinctly different stages of the G₁ phase.

If normal cells were initially plated at a lower density or serum

stimulated 24 hrs after plating instead of 48 hrs, then their lag period before entry into S approached that of LA24 infected cultures (Fig. 3). This clearly demonstrated that there are two compartments in the stationary phase of the chick embryo fibroblasts and that, by manipulating culture conditions, normal cells can be made to occupy either compartment. In contrast, it was not possible to force the LA24- infected cells into the compartment with a 12-14 hr lag even by plating the cells at much higher densities than the normal cells or by growing them strictly at 42°. Thus, viral factors in the stationary ts-infected cells prevent these cells from entering the state which would necessitate the longer lag period.

Experiments with cells infected with transformation defective virus showed that their lag period from serum stimulation to entry into S was similar to that of normal cells (data not shown).

That chicken embryo fibroblasts appear to have two distinct stationary states within the G_1 phase is consistent with the recent work of Brooks et al.⁷ These investigators discuss the evidence for two indeterminate states (Q and A) in mammalian cell cycle models (while chick cells are not mammalian, they would, presumably, fall within the same category). Exit from each of these states occurs at random. In the chicken embryo fibroblast one of these states has the characteristics of a Q state in that the rate constant (K_Q) for exit from this state at high density and low serum is very low. It is possible to argue that the data in Fig. 2 could also be explained if there were only one state from which normal and ts-infected cells would exit, but that their rates of progression toward S could be different. The data in Fig. 3, however, argue against this interpretation in that normal cells can be stimulated into S with similar kinetics as ts-infected cells if the stimulation occurs soon after cultures

reach saturation density and the cells are seeded at low cell density. This indicates that the two cell types do not have inherently different rates of progression.

Our data also indicate that virally coded factor(s) active at the non-permissive temperature must be responsible for maintaining the infected cells in the short lag stationary stage in G_1 or preventing the cells from moving into the long lag compartment. At present, the only known activity coded for by the src gene is a protein kinase, pp 60^{src}, which has been shown to be temperature sensitive in ts mutants¹⁰. It is possible to argue that a low level of kinase is expressed at 41.5° and that this is sufficient to advance the cells further along the cycle. Such levels, however, would have to be very small in LA24 and LA29 infected cells since the "in vivo" (cellular) activity of the kinase has been shown to be similar to uninfected controls even at 41°. This activity can be measured by the level of phosphotyrosine, which is reported to be a specific product of the protein kinase associated with the src gene¹¹. Moreover, when our experiments were carried out with cells cultured at strictly 42° when such "leakiness" would be minimized, the same differences in cycle position was observed. It is also important to note that in earlier studies examining the expression of many transformation parameters at permissive and nonpermissive temperatures using LA24, we have failed to detect "leakiness" of other usual transformation parameters at the nonpermissive temperature^{12,13}.

An alternative possibility that seems more likely is that an activity other than the kinase is responsible for these observations. This could be another activity associated with pp 60^{src} at the non-permissive temperature or possibly a different protein coded for by a different reading frame

of the src gene. Support for this possibility comes from other observations which suggest that growth regulating activity of src and its other transformation related functions may be dissociable. Calothy et al.¹⁴ have identified an RSV mutant, PA-101, which is defective for "transformation", but causes proliferation of neuroretinal cells. The 60 K protein produced by these cells is shown to be inactive as a kinase in the immune complex assay (J.M. Bishop, personal communications). It is also possible that the adjacent region(s)¹⁵ to the gene coding for pp 60^{src} (which then has to be absent in the transformation defective mutants we used in our experiments) could code for the factor(s) causing this activity.

Recently, Poste and Flood have examined the ability of cells infected with temperature sensitive mutant viruses to grow on chick chorioallantoic membranes at 35° and 41°¹⁶. Surprisingly, tumors were formed at both temperatures. It is conceivable that the infected cells were "initiated"¹³ in the manner described here (or in the terminology of Stiles et al.¹⁸ were "committed") and that the specific environment of the chorioallantoic membrane allowed full expression of transformation parameters ("promotion"¹³ or "progression"¹⁷). The observation by Harley and Goldfine¹⁸ that cells infected with LA24 need not synthesize lipids before initiating DNA synthesis upon shift from 41° to 35° may be also interpreted as another manifestation of this initiation at the non-permissive temperature. While these investigators did not examine the requirement for lipid synthesis by the uninfected chick cells, such a requirement exists for other uninfected cell types, (for example, mouse lymphocytes, human fibroblasts and rat myogenic cell lines (see 18 for references). These latter cell types are shown to be reversibly blocked in G after addition of inhibitors of lipid synthesis. These observations provide further support for the model

proposed by Bissell et al.¹³ based on the enhanced effects of tumor promoters on cells infected with temperature sensitive mutant viruses of RSV at the nonpermissive temperature as compared with normal cells. In preliminary experiments we have examined whether the well-known tumor promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA) has the same effect as serum in differentially stimulating the initiation of DNA synthesis in LA24 infected cells. Early data suggests that the cell's response to TPA is complicated in that there is an initial inhibition of DNA synthesis by TPA. The information presented here, nevertheless, strongly supports the idea of a multi-stage model for viral oncogenesis proposed earlier on the basis of TPA action on LA24-infected cells¹³.

Aside from this, and the implication of these results in postulating a hitherto unknown activity at the nonpermissive temperature, the results are also interesting in terms of current models of cell cycle regulation, providing clear evidence for two stationary states in chicken fibroblasts, analogous to those postulated by Brooks, et al.⁷.

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Table 1. Growth Regulation in ts Mutants LA29
 LA24 Prague A
 NY68 Schmidt Ruppin A

	Fraction of the Population in Cell Cycle Phase		
	G ₁	S	G ₂ +M [†]
<u>Temperature: 41.5°</u>			
Normal Cells	0.694	0.027	0.279
NY 68	0.688	0.028	0.304
LA 29	0.688	0.042	0.271
LA 24	0.688	0.028	0.304
<u>Temperature: 35°</u>			
Normal Cells	0.703	0.012	0.286
NY 68	0.423	0.324	0.253
LA 29	0.407	0.478	0.114
LA24	0.498	0.424	0.078

[†]doublet population significant

Legend to Table 1

Chicken embryo fibroblasts were cultured as described in the legend to Fig. 1 except that 4 hrs after plating some cultures were shifted to 35° while others were maintained at 41.5°. Cultures were maintained at these two temperatures for 48 hrs and the distribution of cells throughout the cycle analyzed after this time period, using the procedures described in the legend to Fig. 1. The cells in the G₂+M category include some G₁ doublets which were not dissociated under our experimental conditions and hence the values in this category do not reflect true G₂ +M estimations.

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Figure Legends

Figure 1. Kinetics of entry of chicken embryo fibroblasts into S phase after shift from 41.5° to 35°. Tertiary cells were plated in medium 199 supplemented with 2% tryptose broth, 0.5% chicken serum and 0.1% glucose, at a density of 2×10^6 /35 mm plate. They were kept at 41.5° for 36-38 hrs and either maintained at this temperature for a further 18 hrs or shifted to 35°. At given times, plates were removed, the cells harvested, and stained with propidium iodide as previously described²⁰. The stained cells were subsequently analyzed in a flow cytometer and histograms of the distribution of cells throughout the cell cycle were obtained. The proportion of cells in S was determined using a fitting procedure, and data was analyzed using a program described by Bartholomew, *et al.*¹⁹. (○) LA24 infected cells cultured at 41.5° and shifted to 35°; (Δ) normal cells cultured at 41.5° and shifted to 35°; (□) LA24-infected cells cultured at 41.5° and held at that temperature; and (▲) normal cells cultured at 41.5° and held at that temperature.

Figure 2. Stimulation of DNA synthesis in normal and LA24-infected cells maintained at 41.5°. Cells were seeded at $2.0-2.5 \times 10^6$ /35 mm dish, cultured as in Fig. 1 and maintained at 41.5° for 48 hrs. DNA synthesis was initiated by the addition of 200 ul of calf serum to the cultures (no medium change; 2 ml medium per plate) and the proportion of cells in S at times after addition was determined as described in Fig. 1. (○) LA24-infected cells; and (■) normal cells.

Fig. 3. Stimulation of DNA synthesis in normal and LA24-infected cells maintained at lower density at 41.5°. Cells were seeded at $1.2 \times 10^6/35$ mm plate under the conditions described in Fig. 1. They were kept at 41.5° for 18-24 hrs. DNA synthesis was initiated by changing the medium with fresh medium containing 2-5% chick or calf serum. Proportion of cells in S was determined as described in Fig. 1. Closed symbols: control cultures. Open symbols: serum stimulated cultures.

Serum stimulation of normal and LA24
infected chicken fibroblasts

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Figure 3.
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