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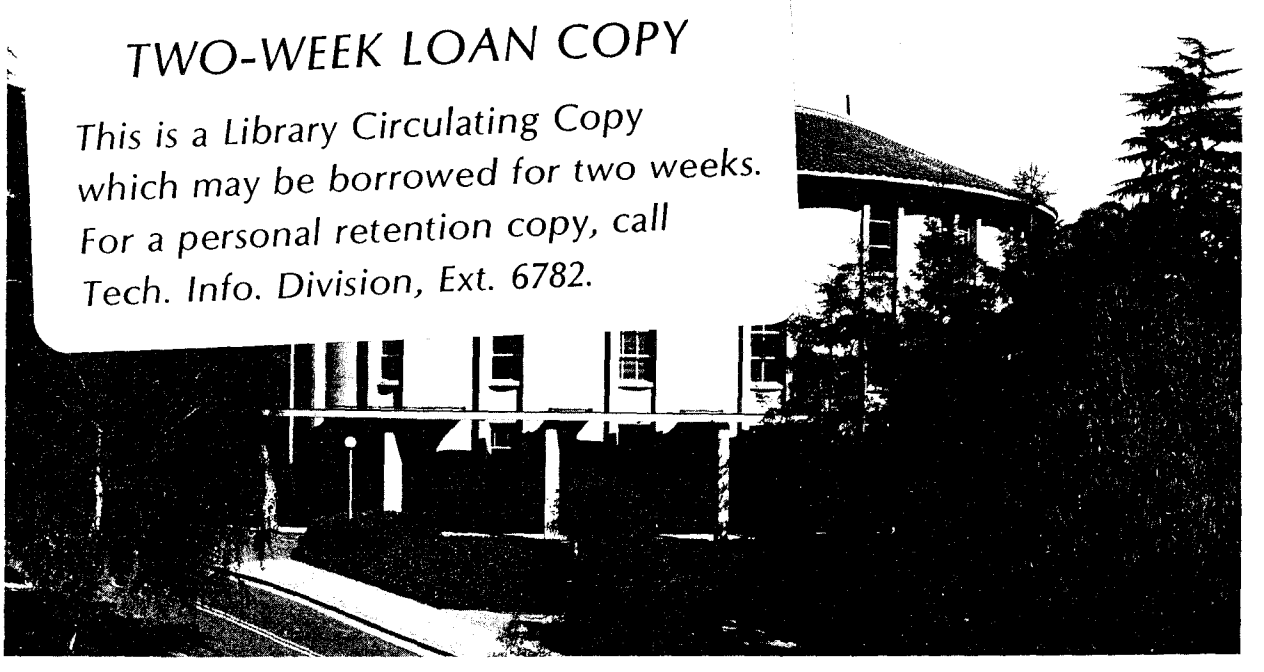

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Growth Regulation in RSV Infected Chicken Embryo Fibroblasts: the role of the src gene

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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 transformation[1-5]. 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 deprivation[2]. 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 has two distinct compartments, (for simplicity referred to as G_1 and G_0 states), (b) when rendered stationary at 41.5° by serum deprivation, normal cells enter a G_0-like state, but cells infected with the ts-mutant occupy a G_1 state, even though a known src gene product, a kinase, should be inactive at this temperature[7,8]. The possibility is discussed that viral factors other than the active src protein kinase influence growth control.

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 G_1 48 hrs later. 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, a slightly "leaky" mutant, (Schmidt Ruppin strain) (Table 1). Very few normal cells were in the S phase at either temperatures. The data thus demonstrates that at the nonpermissive temperature infected cells become blocked in G_1 phase, but that they initiate DNA synthesis upon activation of the src protein at the lower permissive temperature, confirming an earlier report[9].

When the kinetics of initiation of DNA synthesis were examined upon temperature shift from 41.5° to 35°, it was found that cells entered S approximately 8 hrs after shift (Fig. 1). This was similar to the lag period observed when LA24 infected cells at 41° were stimulated to initiate DNA synthesis by addition of serum. However, this lag period was

distinctly shorter than that observed when normal cells at 41.5° were stimulated to initiate DNA synthesis by serum addition. 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 G_1 phase.

If normal cells were initially plated at a lower density their lag period following serum stimulation could almost approach that of LA24 infected cultures. However, it was not possible to force the LA24 infected cells into the 12-14 hr lag phase state even by plating cells at much higher densities than the normal cells or by growing them at strictly 42° (data not shown). Thus, viral factors in the stationary ts-infected cells would prevent these cells from entering the stage which would necessitate the longer lag period. Experiments with cells infected with transformation defective virus showed that their lag time from serum stimulation to entrance into S was similar to normal cells (data not shown).

Two interesting conclusions can be drawn from these results. First, chicken embryo fibroblasts appear to have two distinct stationary states within the G_1 phase. One of these has the characteristics of a classical "G_0" state in that cells move into this compartment only at high density in low serum. Second, virally coded factor(s) active at the nonpermissive temperature can maintain the infected cells in the short lag stationary stage in G_1, and prevent the cells from moving into the long lag compartment. Data obtained with the transformation defective virus suggest that the <u>src</u> gene or the proximal end of the <u>env</u> gene (which is linked to <u>src</u>;[10]) code for these factors.

At present, the only known activity coded for the src gene is a protein kinase, pp 60 ^{src}[7], which has been shown to be temperature sensitive in ts mutants[8]. It is possible that a very 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 identical 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 gene[11]. Moreover, when our experiments were carried out with cells cultured at 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 any other 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 of the pp 60 ^{src} 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 of the env gene which is absent in

transformation defective mutants could code for the factors 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°[15]. Surprisingly, tumor like structures were formed at both temperatures. It is conceivable that the infected cells were "initiated" in the manner described here and that the specific environment of the chorioallantoic membrane allowed full expression of transformation parameters. These observations are also consistent with the results reported by Bissell et al.[13] 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, however, strongly supports the idea of a multi-stage model for viral oncogenesis proposed earlier on the basis of TPA action on LA24 infected cells[13].

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, equivalent to the classical G_1 and G_0 states.

Acknowledgements

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

	Fraction of the Population in Cell Cycle Phase		
	G_1	S	G_2+M[≠]
Temperature: 41.5°			
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
Temperature: 35°			
Normal Cells	0.703	0.012	0.286
NY 68	0.423	0.324	0.253
LA 29	0.407	0.478	0.114
LA 24	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 G_2+M category include some G_1 doublets which were not dissociated under our experimental conditions and hence the values in this category do not reflect true G_2 +M estimations.

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 x 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 described[17]. 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.[16]. (○) LA24 infected cells cultured at 41.5° and shifted to 35°; (△) 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 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 (2 ml medium per plate) and the proportion of cells in S at times after addition was determined as described above. (○) LA24 infected cells; and (■) Normal cells.

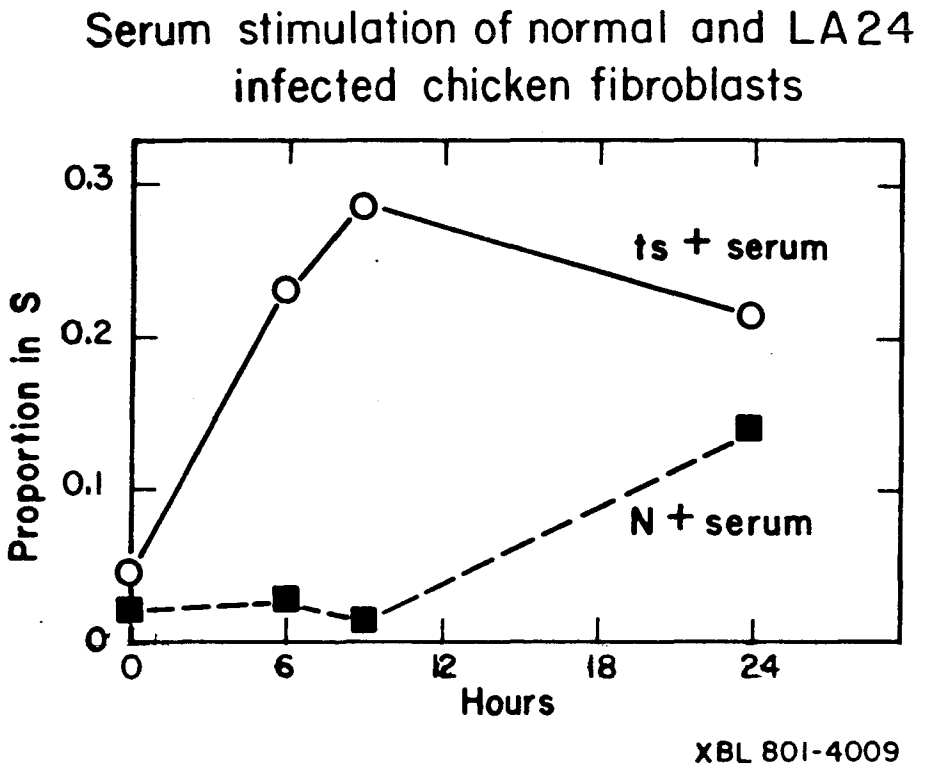

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Fig. 2

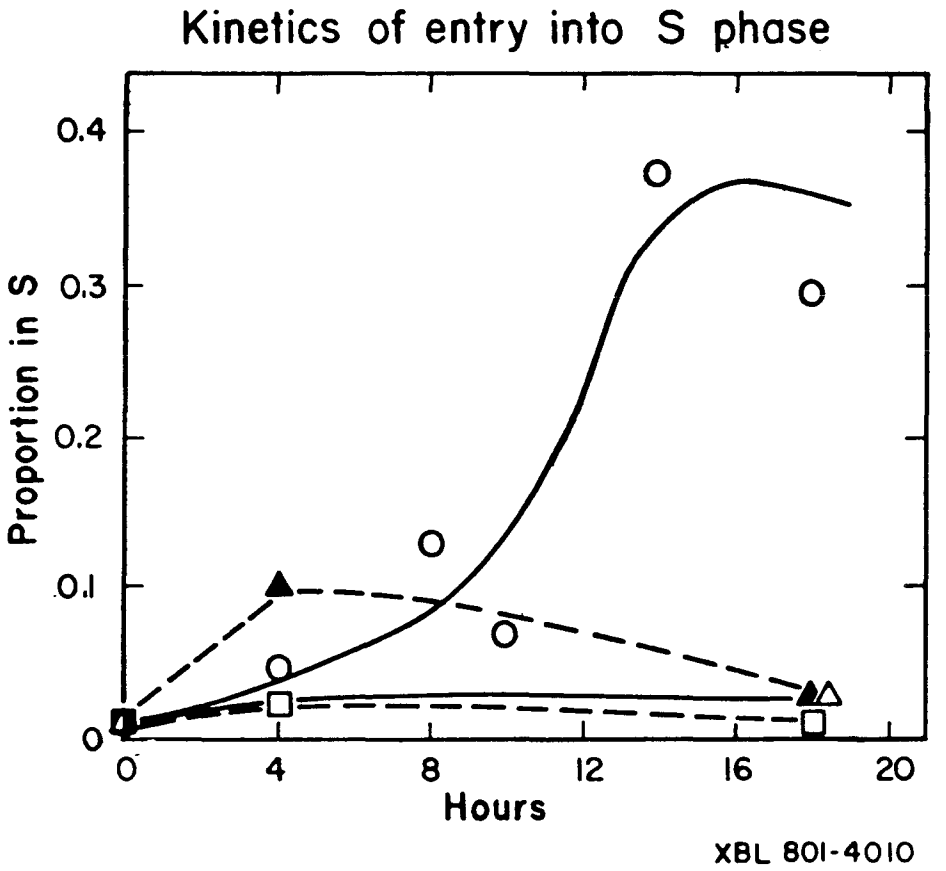

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Fig. 1