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THE *src* GENE PRODUCT ($pp60^{src}$) OF AVIAN SARCOMA VIRUS RAPIDLY INDUCES DNA SYNTHESIS AND PROLIFERATION OF CALCIUM-DEPRIVED RAT CELLS

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SUMMARY: A general characteristic of neoplastic cells, but not their non-neoplastic counterparts, is the ability to proliferate in calcium-deficient medium. NRK cells infected with the transformation-defective, temperature-sensitive, ASV mutant, *ts*LA23, were unable to proliferate in calcium-deficient medium at the non-permissive 40°C, but they very rapidly initiated DNA synthesis (within 1 hour) and resumed proliferation in this medium after being shifted to 36°C, a temperature permissive for the production of active $pp60^{src}$ and for neoplastic transformation. These observations suggest that activated $pp60^{src}$ acts near the G1/S transition point in the cell cycle to bypass or stimulate a calcium-dependent mechanism required for the initiation of DNA synthesis, which enables the cells to display the neoplastic property of proliferating in calcium-deficient medium.

INTRODUCTION

The *src* gene of avian sarcoma virus (ASV) encodes for a 60,000 dalton phosphoprotein ($pp60^{src}$) possessing ATP: protein phosphotransferase activity, which initiates neoplastic transformation and maintains the neoplastic state in cultured cells (1-4). Cells infected with transformation-defective, temperature-sensitive (*ts*) ASV mutants do not produce active $pp60^{src}$ and lose their transformed phenotype at certain (usually around 40°C) non-permissive temperatures (5-8). However, active $pp60^{src}$ re-appears, and the phosphorylation of certain proteins (e.g., a cytoplasmic 36,000 dalton protein) increases, within minutes of lowering the temperature to a permissive level (9). These early events are followed by the re-emergence of the characteristic features of neoplastic transformation (10).

One of the common characteristics of neoplastic cells is the ability to proliferate indefinitely in calcium-deficient medium which does not permit the proliferation of their non-neoplastic counterparts (11-14). NRK cells trans-

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formed by ASV are identical to other neoplastic cells in this respect since they proliferate rapidly in calcium-deficient medium over the entire physiological temperature range (15). On the other hand, NRK cells infected with an ASV mutant temperature-sensitive for transformation, such as *ts*LA23, do not proliferate in calcium-deficient medium at non-permissive temperatures, but do so normally in this medium at temperatures which permit the production of active $pp60^{src}$ (15).

The available evidence indicates that calcium is required near the G1/S transition of the cell cycle in a variety of non-neoplastic cells (11,16). The fact that ASV-infected NRK cells proliferate normally and indefinitely in calcium-deficient medium while uninfected NRK cells do not (15), raised the possibility that $pp60^{src}$ acts near the G1/S boundary to bypass or stimulate a calcium-dependent control mechanism for proliferation. In this communication we will show that calcium-deprived *ts*LA23-NRK cells are blocked near the G1/S boundary of the cell cycle at a temperature (40°C) non-permissive for neoplastic transformation. However, these arrested cells *rapidly* initiated DNA synthesis (within 1 hour) and resumed proliferating in calcium-deficient medium when the temperature was lowered to a level (36°C) permissive for the production of active $pp60^{src}$ and neoplastic transformation.

MATERIALS AND METHODS

Uninfected and virally transformed NRK cells were kindly supplied by Dr. P.K. Vogt (University of Southern California, School of Medicine, Los Angeles CA). Uninfected, non-neoplastic NRK fibroblast-like cells were decendents of cells isolated from the kidney of a normal Osborne-Mendel rat (17). The transformed *wt*B77-NRK line was derived from infection of NRK cells with wild-type B77-avian sarcoma virus, and *ts*LA23-NRK cells were produced by infection with *ts*LA23 virus, a class T *ts* mutant of the Prague strain (subgroup A) of ASV. Stock cultures of all cell types were maintained at 36°C in T-75 flasks (Flow Laboratories, Rockville MD) in medium containing 90% (v/v) DEM (Grand Island Biochemical Co., Grand Island NY), 10% (v/v) calf serum (Grand Island Biochemical Co.) and the antibiotic gentamicin. Before plating experiments, cultures were subcultured into stock medium and incubated for 4 days at 40°C in order to establish an equilibrium of temperature-sensitive cellular and viral processes.

Cells were plated at a density of $3 \times 10^3/cm^2$ on 25-mm round plastic coverslips (in 8.6 cm^2 plastic dishes; Flow Laboratories, Rockville MD) in 2 ml of stock medium containing 1.8 mM $CaCl_2$ and incubated for 24 h at 40°C or 36°C in an atmosphere of 8% CO_2:92% air. They were then rinsed twice with phosphate-buffered saline and the medium replaced by 2 ml of low-calcium medium consisting of 90% (v/v) commercially prepared (Grand Island Biochemical Co.)

calcium-free DEM and 10% (v/v) calf serum, the ionic calcium concentration of which had been reduced to 0.02 mM with the specific calcium chelator EGTA (ethylene-*bis*-(oxyethylenenitrilo)-tetracetic acid) as previously described (18). The final free calcium concentration of the medium was previously adjusted to the desired level (0.02 mM - 0.06 mM) by the addition of small amounts of 10 mM $CaCl_2$. Cultures were incubated at 40°C for 42 h (unless otherwise stated) in low-calcium medium at which time experiments were initiated by placing some cultures at 36°C and by adding $CaCl_2$ (at a final concentration of 1.25 mM) to other cultures which were maintained at 40°C.

DNA synthetic activity was assessed from the proportion of cells whose nuclei were labeled during a 1 hour exposure to [^{3}H]-thymidine (5 μCi/ml of medium, specific activity 20 Ci/mmoles; New England Nuclear Corp., Boston MA). The autoradiographic procedure was that of Whitfield *et al.* (19) in which the radioactivity is found only in DNA and produces approximately 500 silver grains in the nuclear track emulsion (Eastman Kodak, Rochester NY) overlying each DNA-synthesizing cell. Between 1500 - 2000 cells were counted per sample to determine DNA-synthetic activity. The cell density of cultures was determined as previously described (15).

*ts*LA23-NRK cells were highly tumorigenic: 10^7 of them produced large (2-3 cm) fibrosarcomas within five weeks after subcutaneous injection into athymic BALB/c nude mice.

The DNA-synthetic activity in non-neoplastic, uninfected cell cultures, or in *ts*LA23-infected cell cultures maintained at a non-permissive temperature, did not fall immediately upon exposure to calcium-deficient medium (Fig. 1). The rate at which the number of DNA-synthesizing cells declined after being placed into calcium-deficient medium was a function of cell density, residual calcium concentration and the passage number of the culture. Although the *ts*LA23-NRK and *wt*B77-NRK cell lines were derived from the NRK line used in this study, differences in passage number after infection resulted in slight variations in their respective "run-down" profiles in low-calcium medium. To compensate for these variations, residual calcium levels were appropriately adjusted (final; 0.06 mM for *wt*B77-NRK and *ts*LA23-NRK, 0.02 mM for NRK) to obtain the same basal level of DNA-synthetic activity in all cultures following 42 h of incubation in calcium-deficient medium. It should be noted that regardless of the basal DNA-synthetic activity at the start of an experiment, the relative percent increase in DNA synthesis caused by a shift from 40°C to 36°C was independent of the duration of incubation (between 24-48 h) in calcium deficient medium.

RESULTS AND DISCUSSION

A progressive decline in the DNA-synthetic activity of *ts*LA23-ASV infected NRK cells was observed in calcium-deficient (0.06 mM, final) medium only at a non-permissive 40°C (Fig. 1), a temperature at which the transformed phenotype and the production of active $pp60^{src}$ *are not* expressed. In this respect, *ts*LA23-NRK cells at 40°C behaved like uninfected, non-neoplastic NRK cells maintained at either 40°C or 36°C in calcium-deficient medium (Fig. 1). On the other hand, at a temperature permissive (36°C) for transformation and the production of active $pp60^{src}$, *ts*LA23-NRK cells behaved like *wt*B77-ASV transformed NRK cells at 40°C or 36°C in that their DNA synthetic activity was

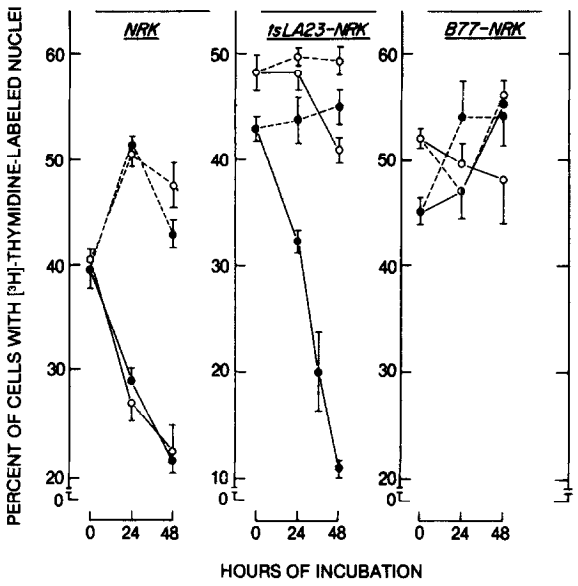


Fig. 1. DNA-synthetic activity in uninfected NRK, *ts*LA23-NRK and *wt*B77-NRK cultures maintained at 40°C (●) or 36°C (○) in complete medium (------) or in calcium-deficient medium (———). Cells were plated at 3 × 10^3/cm^2 in complete medium (90% (v/v) DEM - 10% (v/v) bovine calf serum containing 1.8 mM $CaCl_2$) and incubated for 24 hours at 40°C or 36°C. At time "0" (24 hours after plating) cultures were rinsed with phosphate buffered saline and the medium replaced with complete medium or calcium-deficient medium (see Materials and Methods). The cultures were incubated at their respective temperatures and at the indicated times the proportion of cells engaged in DNA synthesis was determined as described in Materials and Methods. The variability in DNA-synthetic activity noted with NRK cells in complete medium likely reflects partial synchronization of the cultures at plating. All points are means of ± S.E.M. of the values of 4 cultures.

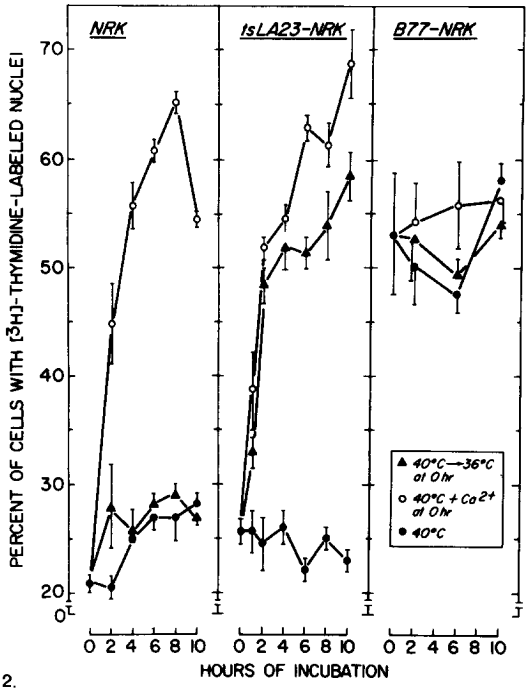
unaffected by a reduction of the extracellular calcium concentration from 1.8 mM to 0.06 mM (Fig. 1).

Reducing the calcium concentration in the medium of *ts*LA23-NRK cells actively growing at the non-permissive temperature probably suppressed DNA-synthetic activity by arresting cells at a late, calcium-requiring stage of prereplicative development, near the G1/S transition. Thus, the addition of calcium (to 1.25 mM) to *ts*LA23-NRK cells maintained at 40°C for 42 h in calcium-deficient medium caused a prompt (within 1 hour) increase in the proportion of cells engaged in DNA synthesis (Fig. 2). Predictably, a similar increase in DNA synthetic activity after calcium addition was also observed in calcium-deprived, non-infected NRK cells maintained at either 40°C or 36°C; only the restorative action of calcium at 40°C is shown for comparison (Fig. 2). The DNA-synthetic

activity of neoplastic B77-NRK cells, unaffected by a reduction in extracellular calcium levels (Fig. 1), was not further stimulated by readdition of calcium (Fig. 2). The observed responses of uninfected NRK cells and of *ts*LA23-NRK cells at non-permissive temperatures to the removal and subsequent restoration of extracellular calcium was similar to that found previously for other phenotypically non-neoplastic avian, rodent and human cells (reviewed in 16).

The appearance of active $pp60^{src}$ in calcium-deprived *ts*LA23-NRK cells also overcame the block near the G1/S transition and restored the neoplastic characteristic of being able to proliferate in calcium-deficient medium. Thus, calcium-deprived *ts*LA23-NRK were induced to rapidly (*within 1 hour*) initiate DNA synthesis while still in calcium-deficient medium by simply decreasing the temperature, from the non-permissive 40°C to 36°C, a level permissive for the production of active $pp60^{src}$ (Fig. 2). By contrast, the decrease in temperature from 40°C to 36°C *did not* stimulate DNA synthesis in calcium-deprived, uninfected NRK cells, nor did it cause a further increase in the apparently maximal DNA-synthetic activity of the continuously $pp60^{src}$-producing *wt*B77-NRK cell (Fig. 2). This rapid, temperature-induced stimulation of DNA-synthetic activity observed in calcium-deprived *ts*LA23-NRK cells was not a transient event: despite the continuous calcium deficiency, lowering the temperature to a permissive level (36°C) induced a sustained increase in proliferative activity relative to *ts*LA23-NRK cells left at 40°C (Fig. 3). By contrast, previous studies have demonstrated that calcium-deprived *wt*B77-NRK cells grow rapidly and continuously at either 40°C or 36°C, while uninfected NRK cells are unable to proliferate at either temperature in calcium-deficient medium (15).

These observations suggest that the product of the *src* transforming gene of avian sarcoma virus (having an associated protein kinase activity) acts near the G1/S transition of the cell cycle to rapidly bypass or directly stimulate a normally calcium-dependent process needed for the initiation of DNA synthesis. This action of $pp60^{src}$ enables neoplastic, ASV-infected NRK cells to proliferate in calcium-deficient medium which does not support the prolifera-



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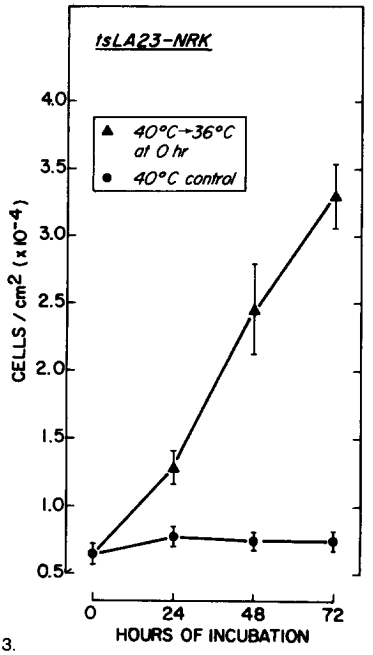

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Fig. 2. The DNA-synthetic responses of calcium-deprived uninfected NRK, *ts*LA23-NRK and *wt*B77-NRK cells to calcium addition at 40°C, or to a shift in temperature from 40°C to 36°C. All cells were maintained in calcium-deficient medium at 40°C for 42 hours prior to the initiation of experiments as described in Materials and Methods: (●), cells at 40°C; (○), cells at 40°C to which calcium chloride was added at time "0" to raise the extracellular calcium concentration to 1.25 mM; (▲), cells shifted from 40°C to 36°C at time "0". Culture conditions and the determination of DNA-synthetic activities are described in Materials and Methods. The points are means ± S.E.M. of the values in 4 cultures.

Fig. 3. The ability of *ts*LA23-NRK cells to proliferate in calcium-deficient medium at non-permissive (40°C) and permissive (36°C) temperatures. Cultures were maintained at 40°C in calcium-deficient medium for 42 hours and then: (▲), shifted to 36°C at time "0"; or, (●), left at 40°C. Culture conditions and the determination of culture density are described in Materials and Methods. All points are means ± S.E.M. of the values of 4 cultures.

tion of their non-neoplastic counterparts. These observations support the suggestion that changes in protein kinase activities are responsible for the ability of a wide variety of transformed avian, bovine, rodent and human cells to proliferate in calcium-deficient medium (11,16,20,21).

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1. Brugge, J.S., and Erikson, R.L. (1977) Nature (London) 269, 346-348.

2. Levinson, A.D., Oppermann, H., Levintow, L., Varmus, H.E., and Bishop, J.M. (1978) Cell 15, 561-572.
3. Purchio, A.F., Erikson, E., Brugge, J.S., and Erikson, R.L. (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 1567-1571.
4. Erikson, R.L., Collett, M.S., Erikson, E., and Purchio, A.F. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 6260-6264.
5. Kawai, S., and Hanafusa, H. (1971) Virology 46, 470-479.
6. Chen, Y.-C., Hayman, M.J., and Vogt, P.K. (1977) Cell 11, 513-521.
7. Baltimore, D. (1974) Cold Spring Harbor Symp. Quant. Biol. 39, 1187-1200.
8. Rohrschneider, L.R. (1980) Cold Spring Harbor Symp. Quant. Biol. 44, 1013-1022.
9. Radke, K., and Martin, G.S. (1980) Cold Spring Harbor Symp. Quant. Biol. 44, 975-982.
10. Hanafusa, H. (1977) in *Comprehensive Virology* eds. H. Fraenkel-Conrat and R.R. Wagner (Plenum Press, New York), Vol. 10, pp. 401-483.
11. Swierenga, S.H.H., Whitfield, J.F., Boynton, A.L., MacManus, J.P., Rixon, R.H., Sikorska, M., Tsang, B.K., and Walker, P.R. (1980) Ann. N.Y. Acad. Sci. 349, 294-311.
12. Lechner, J.R., and Kaighn, M.E. (1979) Exptl. Cell. Res. 121, 432-435.
13. Fisher, P.B., and Weinstein, I.B. (1980) Cancer Lett. 10, 7-17.
14. Fischer, P.B., and Weinstein, I.B. (1981) Carcinogenesis 2, 89-95.
15. Boynton, A.L., and Whitfield, J.F. (1978) Cancer Res. 38, 1237-1240.
16. Whitfield, J.F., Boynton, A.L., MacManus, J.P., Rixon, R.H., Sikorska, M., Tsang, B.K., and Walker, P.R. (1980) Ann. N.Y. Acad. Sci. 339, 216-240.
17. Duc-Nguyen, H., Rosenblum, E.N., and Ziegel, R.F. (1966) J. Bacteriol. 92, 1133-1140.
18. Boynton, A.L., Whitfield, J.F., Isaacs, R.J., and Tremblay, R.G. (1977) Cancer Res. 37, 2657-2661.
19. Whitfield, J.F., MacManus, J.P., Youdale, T., and Franks, D.J. (1971) J. Cell. Physiol. 78, 355-368.
20. Boynton, A.L., Whitfield, J.F., MacManus, J.P., Armato, U., Tsang, B.K., and Jones, A. (1981) Exptl. Cell Res. 135, 199-211.
21. Whitfield, J.F., Boynton, A.L., MacManus, J.P., Sikorska, M., and Tsang, B.K. (1979) Mol. Cell. Biochem. 27, 155-179.