Effect of pH on the induction of competence and progression to the S-phase in mouse fibroblasts

Rodrigo Bravo and Heather Macdonald-Bravo

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, FRG

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The pH dependence of competence induction and progression to the S-phase in quiescent stimulated cells has been studied. The results show that: (i) induction of competence by fibroblast growth factor in these cells is relatively independent of the external pH between pH 5.6–7.6; (ii) progression of cells to the S-phase is highly sensitive to pH and shows a dramatic increase between pH 6.8–7.2. These observations suggest that the intracellular alkalinization triggered by growth factors is fundamental for progression but not for competence induction.

DNA synthesis *Quiescent cell* *Fibroblast growth factor* *Cell cycle* *Platelet-poor plasma*

1. INTRODUCTION

A variety of cultured animal cells express on their surface a Na^+/H^+ antiporter system which can extrude protons from the cell using the inwardly directed Na^+ gradient as a driving force [1–7]. This system is rapidly activated by serum and growth factors in quiescent cells [2,5,6,8–10], resulting in an intracellular alkalinization [6,11–13] which has been postulated as a possible 'messenger' of growth factor action [8,10–14]. Recently, it has been demonstrated that the induced alkalinization by growth factors is a determinant step in the events required by stimulated cells to reach the S-phase [12,15,16]. This internal increase in pH can be efficiently counteracted by a slightly acidic medium [16].

Quiescent mouse fibroblasts briefly exposed to fibroblast growth factor (FGF) become 'competent' to synthesize DNA [17]. The addition of 'progression factors', which are present in platelet poor plasma (PPP) allows competent cells to progress through G_1 and enter the S-phase [18,19].

Here we have studied the effect of pH in the induction of competence and progression of stimulated quiescent mouse fibroblasts. The results indicate that progression but not competence induction is highly dependent on the external pH.

2. MATERIALS AND METHODS

2.1. *Cells*

Mouse NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and antibiotics (penicillin, 10 units/ml; streptomycin 50 μg/ml). To obtain quiescent cells, cultures of 70% confluency were kept for 2–3 days in DMEM supplemented with 0.5% FCS. Stimulation of cells by growth factors was done in HCO_3^--free medium at 0% CO_2 atmosphere. The buffers used in the different HCO_3^--free media were: Mes for pH 5.6, 6.0 and 6.4; Mops for pH 6.8 and 7.2; Hepes for pH values above 7.2. In all cases a final concentration of 30 mM was added.

2.2. *Autoradiography*

Cells were grown on glass coverslips (9×9 cm) and made quiescent as described above. Stimulated cultures were labelled for 24 h with 1 μCi/ml [^{3}H]thymidine in the indicated media. Then cells were washed twice in Hank's and fixed 15 min in

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2.5% glutaraldehyde at room temperature. The fixed cells were rinsed thorougly with Hank's and incubated for 5 min in 2% $HClO_4$ on ice. Coverslips were then washed with distilled water, mounted on slides and covered with stripping film (Kodak, AR10). Autoradiographs were developed after 2 days of exposure at −20°C.

PPP was prepared from human outdated platelets as described [18]. FGF was purchased from BRL.

3. RESULTS AND DISCUSSION

Previous studies have demonstrated in fibroblast cells that in addition to the Na^+/H^+ antiporter there is a Cl^-/HCO_3^- system which is also very active in the regulation of the intracellular pH [20]. To eliminate the possible effect of the latter antiporter system we have carried out all our experiments in DMEM without HCO_3^- and 0% CO_2 atmosphere. The buffer systems used for the different pH values required are described in section 2.

Initially we studied the effect of the external pH on the capacity of quiescent NIH 3T3 cells to reinitiate DNA synthesis. For this, quiescent cells were incubated for 3 h at the corresponding pH before adding 100 ng/ml FGF and 2% PPP. Then, following stimulation, cells were incubated for a further 24 h in the presence of the growth factors and 1 μCi/ml [^{3}H]thymidine. DNA synthesis was determined by autoradiography. Fig.1 clearly shows that reinitiation of DNA synthesis is dependent on external pH. Interestingly the capacity of quiescent cells to reach the S-phase after growth factor stimulation, is completely inhibited at external pH values lower than pH 6.8. Thereafter a dramatic increase in the ability of stimulated cells to reinitiate DNA synthesis is found between pH 6.8 and 7.2. No significant changes on the thymidine labelling index were observed between pH 7.2 and 8.2 (not shown). These results agree with previous observations in Chinese hamster lung fibroblasts stimulated with α-thrombin in which these cells showed a rapid increase in [^{3}H]thymidine incorporation between pH 7.0–7.4 [16].

To investigate further the effect of the pH during the transition of cells from G_0 to the S-phase we decided to study the induction of competence in

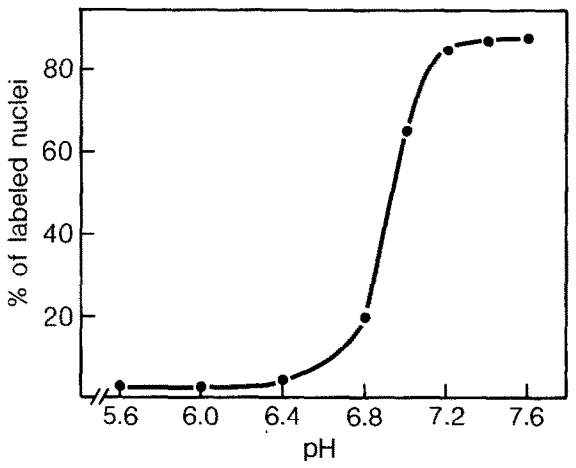

Fig.1. Effect of pH on the reinitiation of DNA synthesis. Quiescent NIH 3T3 cells were stimulated for 24 h with 100 ng/ml FGF in the presence of 2% PPP and 1 μCi/ml of [^{3}H]thymidine at the indicated pH values. About 500 nuclei were counted in each case.

quiescent cells and their progression to S-phase at different pH values.

To study the effect of external pH on competence induction, quiescent cells kept at different pH values, were incubated with 100 ng/ml FGF for 3 h. Then the growth factor was washed out and cells were incubated for another 6 h in serum-free medium at the indicated pH to eliminate possible traces of the growth factor. During this period the competent state slightly declines in these cells [21]. The medium was replaced by 2% PPP at pH 7.6 in all cases. Cells were further incubated for 24 h in the presence of 1 μCi/ml [^{3}H]thymidine.

The results in table 1 illustrate that a significant induction of competence is found at all the pH values tested. Thymidine labelling is 49% of maximal at pH 5.6 and increases slowly thereafter till it reaches a maximum of 85% at pH 7.6. These observations contrast with those described in fig.1, where no reinitiation of DNA synthesis is detected between pH 5.6 and 6.8, while at these pH values competence induction is 49–78% of maximal. The difference observed in competence induction between pH 5.6 and 7.6 could be due to an effect in the stability of the competent state. To study this, we stimulated quiescent cells with FGF for 3 h at pH 7.6, then cells were washed and kept in serum-free medium at different pH values for another 6

Table 1

Effect of pH on the induction of the competent state

Medium[a] pH	FGF treatment[b] (h)	Incubation in serum-free medium		Platelet poor plasma[c]		Thymidine labelling (%)[d]
		pH	h	pH	h	
5.6	3	5.6	6	7.6	24	42
6.0	3	6.0	6	7.6	24	49
6.4	3	6.4	6	7.6	24	55
6.8	3	6.8	6	7.6	24	66
7.2	3	7.2	6	7.6	24	70
7.6	3	7.6	6	7.6	24	85

[a] Cells grown on coverslips were made quiescent as described in section 2. Cultures were preincubated for 3 h at the respective pH before adding growth factor

[b] Cultures were treated with 100 ng/ml FGF

[c] Cells were incubated with 2% PPP

[d] Autoradiography was performed as described in section 2. About 500 nuclei were counted in each case. Thymidine labelling values are expressed as percentage of [^{3}H]thymidine labelled nuclei, relative to the total number of nuclei counted

h. The medium was replaced and cells were incubated for 24 h in 2% PPP at pH 7.6 in the presence of 1 μCi/ml [^{3}H]thymidine. The results demonstrate that there is no significant variation in the stability of the competent state at the different pH values studied at least during a 6 h period (not shown). It is possible that the difference observed is due to an effect on the growth factor-receptor binding.

As the effect of the pH observed in the induction of competence could not explain the complete inhibition found in reinitiation of DNA synthesis at those pH values, we decided to study the pH dependence of the progression period. For this, quiescent cells were induced with FGF for 3 h at pH 7.6, washed and kept at the same pH for another 6 h. Then 2% PPP was added at the indicated pH values and cells left for 24 h in the presence of [^{3}H]thymidine. As shown in table 2 progression of cells to the S-phase is highly sen-

Table 2

Effect of pH on the progression towards the S-phase

Medium[a] pH	FGF treatment[b] (h)	Incubation in serum-free medium		Platelet poor plasma[c]		Thymidine labelling (%)[d]
		pH	h	pH	h	
7.6	3	7.6	6	5.6	24	2
7.6	3	7.6	6	6.0	24	3
7.6	3	7.6	6	6.4	24	2
7.6	3	7.6	6	6.8	24	25
7.6	3	7.6	6	7.2	24	62
7.6	3	7.6	6	7.6	24	80

[a,b,d] As table 1

[c] Cultures were washed 3 times at the respective pH before adding 2% PPP

sitive to the external pH. The ability of competent cells to progress to the S-phase is completely inhibited at pH values lower than pH 6.8. The thymidine labelling index increases dramatically thereafter, reaching a maximum at pH 7.6. These observations are similar to those illustrated in fig.1.

The comparison of the results shown in tables 1 and 2 clearly indicates that progression of cells to the S-phase is far more dependent on pH than induction of competence. At pH 6.4, for example, no reinitiation of DNA synthesis occurs, however induction of competence is 70% of the maximum. These are strong evidence that the alkalinization of the intracellular pH is more important for the progression period than for competence induction. Previous reports have demonstrated that an increase in intracellular pH is required for phosphorylation of the ribosomal protein S6 [8], an event that is possibly necessary for the stimulation of protein synthesis observed by growth factors. These and our observations would suggest that induction of competence is therefore less dependent in increased protein synthesis than progression.

The levels of c-*myc* and c-*fos* mRNAs are stimulated several-fold after growth factor treatment of quiescent cells [21–25] suggesting that both genes could be involved in the events that trigger the competent state. Recently it has been demonstrated that induction of c-*myc* ([16]; Bravo et al., submitted) and c-*fos* (Bravo et al., submitted) by growth factors is independent of the external pH. This observation would support the evidence presented in this report that the cellular events required for the induction of competence are relatively independent of intracellular alkalinization.

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