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Staurosporine suppresses replicon initiation in mammalian cells

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Replication in cellular replicons of mouse Ehrlich ascites, human CCRF-CEM and hamster BHK-21 cells was analyzed, after exposition of the cells to staurosporine, by measuring the overall DNA synthesis rate, by alkaline sedimentation analysis of length distributions of growing daughter strand DNA and by DNA fibre autoradiography The results consistently indicated that micromolar concentrations of staurosporine caused, in all three cell lines, a fast suppression of replicon initiation which was reversible if the drug treatment did not exceed about 2 h. The inhibition of initiation was accompanied by a slight reduction of rates of propagation of replication forks. The data are interpreted in terms of the existence of a so far unknown factor which seems to be involved relatively directly in the initiation process of cellular replicons and has to be activated, like the large T antigen of SV 40 for the replication initiation in the viral genome, by a specific phosphorylation event. Unlike several other protein phosphorylations of cellular regulation, the kinase concerned here seems to be inhibited only by relatively high staurosporine concentrations.

DNA replication; Replicon initiation; Protein kinase; Staurosporine; Mammalian cell

1. INTRODUCTION

There is now convincing evidence that protein phosphorylation is a common cellular mechanism of fundamental importance in biological regulation. This type of control seems to be acting at almost all levels of molecular cell physiology. Recent results acquired by SV 40 model systems indicate that so far unknown key proteins of mammalian DNA replication may also be regulated by a change of their state of phosphorylation (reviewed in [1–3]). In the present study, we asked whether an involvement of protein phosphorylation in the control of cellular replication can be demonstrated in intact mammalian cells. For this purpose, we studied the influence of staurosporine, which is a potent general inhibitor of protein kinases [4], on firing of cellular replicons in 3 mammalian cell lines: mouse Ehrlich ascites cells, human lymphoblastoid CCRF-CEM cells, and baby hamster kidney (BHK-21) cells. We used a combination of analysis of length distributions of growing DNA daughter chains by alkaline sedimentation [5] with DNA fibre autoradiography [6] to analyze the responses of the mammalian replication machinery. This strategy has proved successful in analyzing the O_2 dependent regulation of replication in mammalian cells [7–9]. Micromolar concentrations of staurosporine were found to cause fast suppression of replicon initiations whereas the rate of DNA chain growth at already active replication forks was only slightly reduced.

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Dedicated to Prof. Dr. D. Mecke on the occasion of his 60th birthday

2. MATERIALS AND METHODS

2.1. *Materials*

Staurosporine, obtained from Boehringer-Mannheim was used from an 10 mM stock solution in DMSO which was stored at −20°C in the dark. [*methyl*-^{3}H]Thymidine (82 Ci/mmol) and [2-^{14}C]thymidine (70 mCi/mmol) were purchased from Amersham. Non-radioactive thymidine (Boehringer-Mannheim) was used from a 40 mM aqueous stock solution stored at −20°C.

2.2. *Cells and cell culture*

The handling of suspension cultures of the mouse Ehrlich ascites cells used, designated ELT-Bonn according to [11], has been described in detail earlier [12]. The human T lymphoblastoid cell line CCRF-CEM (American Type Culture Collection CCL 119) was grown as a suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml). The fibroblast-like hamster cell line BHK-21(C13) (American Type Culture Collection CCL 10) was cultured as a monolayer in DMEM medium supplemented with fetal calf serum and penicillin/ streptomycin as above.

2.3 *Radioactive labeling*

Cells to be used for measuring [^{3}H]dThd incorporation rates were prelabeled for 16–18 h with 0 002–0.005 μCi/ml [^{14}C]dThd and supplemented about 2 h before starting staurosporine treatments with 30–50% fresh medium. Aliquots of about 10^6 prelabeled cells were exposed at the end of the staurosporine treatment to 5 μCi/ml [^{3}H]dThd (diluted to 2 μM with non-radioactive thymidine) for 10 min and then processed for LSC counting of acid insoluble radioactivity as described [14]. DNA labeling for alkaline sedimentation analysis of length distributions of growing daughter strand DNA was performed

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with 15 μCi/ml [^{3}H]dThd. 'Hot' pulses (20 min) for DNA fibre autoradiography were performed with 80 μCi/ml [^{3}H]dThd, the subsequent 20 min period of 'warm' labeling was initiated by addition of 10 μM non-radioactive thymidine.

2.4. *Analytic procedures*

For recording dose response curves of cell growth 0.5% of appropriate dilutions of staurosporine in DMSO were added to sets of identically prepared 2 ml cell cultures (5×10^5 Ehrlich ascites cells or 10^6 CCRF-CEM cells in 12 ml tubes with a beveled flat bottom or 5×10^4 BHK-21 cells in 35 mm petri dishes) at time 0. After 24 and 48 h, 50% of the medium were replaced by fresh medium containing staurosporine at respective concentrations. The total DNA of the cultures attained after 72 h was estimated by the bisbenzimidazole method of Labarca and Paigen [13]. Details of the analysis of length distributions of daughter strand DNA by alkaline sedimentation after cell lysis on the top of sucrose gradients are given in [14]. The DNA fibre autoradiography procedure used and the evaluation of the autoradiographs were performed as described earlier [15].

3. RESULTS AND DISCUSSION

Fig. 1 compares the long-term effects of different staurosporine concentrations on cell growth during an 72 h interval (Fig. 1a) with the short-term effects on the rate of DNA synthesis after a 2 h incubation (Fig. 1b). Separate analyses (unpublished) revealed that a 2 h exposition to 5 μM staurosporine had no significant influence on cellular deoxynucleotide pools. Under this precondition, relative rates of [^{3}H]dThd incorporation reflect relative rates of DNA synthesis.

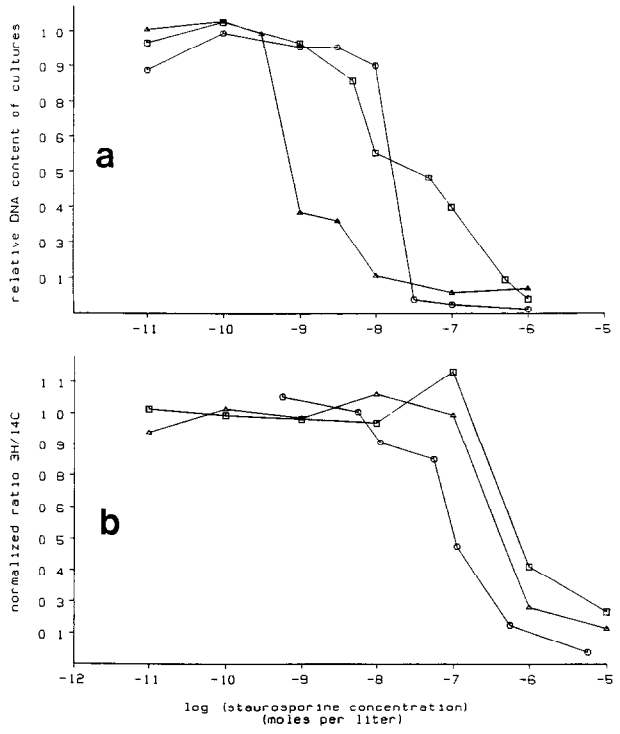

Fig. 1. Staurosporine dose–response curves. (a) Long-term response of cell growth (total DNA of small cultures after 72 h of drug treatment relative to untreated controls) (b) Response of the DNA synthesis rate 2 h after drug addition relative to untreated controls. □, Ehrlich ascites cells; ○, CCRF-CEM cells; △, BHK-21 cells.

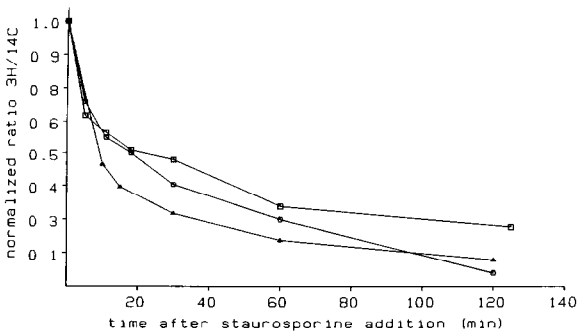

Fig. 2. Temporal course of the [^{3}H]dThd incorporation rate after addition of 5 μM staurosporine (relative to the first samples labelled at 0 min before the addition of staurosporine). Symbols as in Fig. 1.

The dose–response curves of the growth of the 3 cell lines recorded after the 72 h exposition to staurosporine are strikingly different (Fig. 1a): with Ehrlich ascites cells, the range of increasing inhibition spanned about 3 orders of magnitude, 50% inhibition was observed around 3×10^{-8} M. The curve of the CCRF-CEM cells, in contrast dropped abruptly between 10^{-8} M and 3×10^{-8} M. The 50% inhibition of the BHK-21 cells occurred at an about 30 times lower drug concentration. However, the course of the dose–response curve of BHK-21 cells is biphasic to some extent. The final drop to full suppression of growth just occurs around 3×10^{-8} M, together with the emergence of full inhibition of CCRF-CEM cells.

We suppose that the heterogeneous responses of cell growth reflect the existence of several cell growth related targets of staurosporine: staurosporine inhibits a variety of protein kinases [4] which probably exhibit different susceptibilities to the drug. Protein kinases are involved at several levels of cellular growth control [16] whose individual relevance for proliferation may be quite different in different transformed and non-transformed cell lines. Ehrlich ascites cells and CCRF-CEM cells are malignant tumor cells of very different origin and possibly bear very different defects of growth control. On the other hand, BHK-21 cells show contact inhibition and are commonly thought to have retained (despite their immortality) a largely 'normal' proliferation control and are therefore suited for studies on growth factors in serum free media [17] and on the mechanisms of neoplastic transformation [18].

The dose–response curves of the rate of DNA synthesis (Fig. 1b) exhibited, qualitatively and quantitatively, much less differences. This facilitates the supposition that the short term response of DNA synthesis might rely on a rather uniform mechanism in all 3 cell lines. At micromolar concentrations of staurosporine, a 2 h exposition caused a strong diminution of the DNA synthesis rate in all 3 cell lines which, however, exhibited a clearly discernible order.

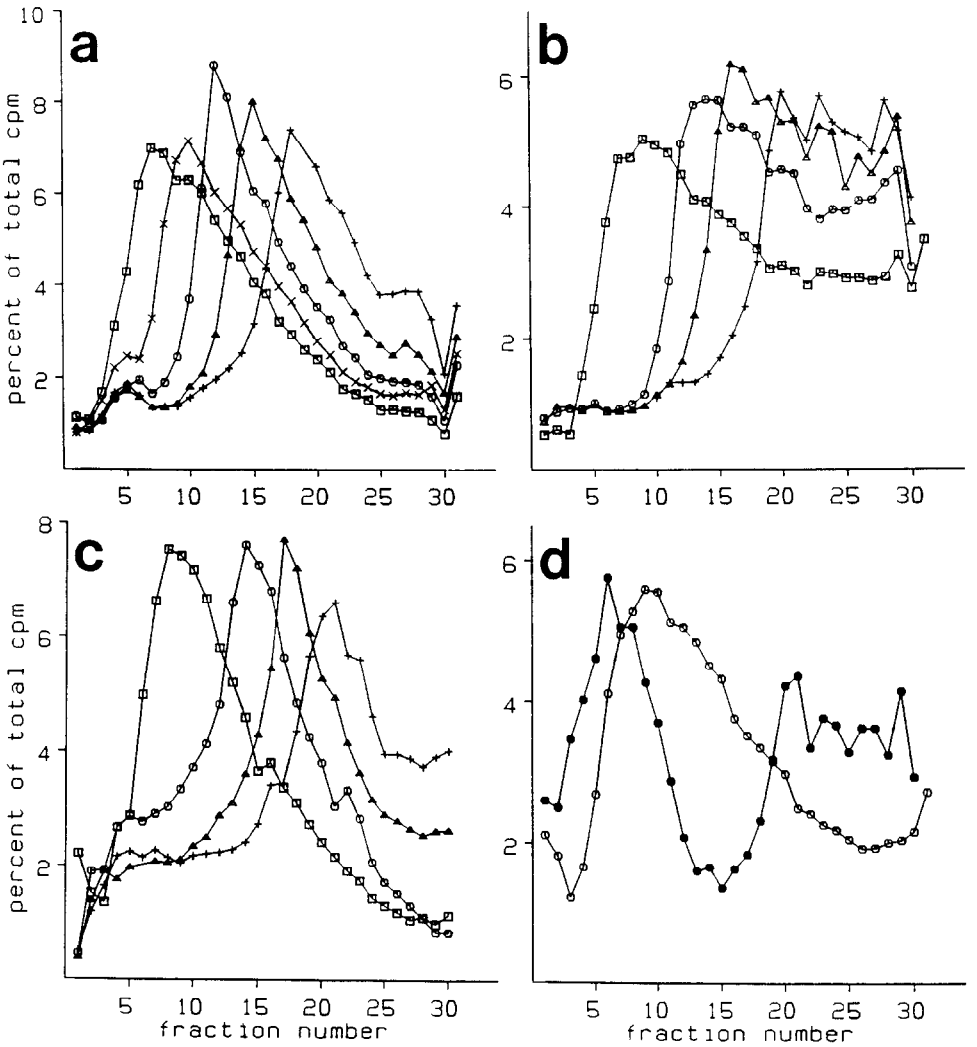

Fig. 3. Alkaline sedimentation profiles of growing daughter strand DNA pulse labeled (8 min) at different times after addition of 5 μM staurosporine (a–c): (a) Ehrlich ascites cells, (b) CCRF-CEM cells, (c) BHK-21 cells Symbols used (a–c): □, untreated; ×, 15 min; ○, 30 min; △, 60 min, +, 120 min. (d) CCRF-CEM cells labeled at 30 min (●) and 60 min (○) after wash-out of staurosporine added 60 min beforehand (dilution with 50 vol. staurosporine free medium, sedimentation and resuspension in the original volume). Sedimentation direction was from left to right. Sedimentation by one fraction corresponds to an increase of the $S_{20,w}$ by 5 4, the first 1.5 fractions are overlay ($S_{20,w} = 0$) Total cpm recovered from the gradients (in the order of peaks from left to right). (a) 225038, 127273, 71840, 32855, 20586; (b) 84024, 16829, 13786, 5315; (c) 246682, 112110, 31859, 38320; (d) 2883, 31766.

Fig. 2 shows the temporal course of the depression of the DNA synthesis rate following addition of staurosporine at micromolar concentrations. All 3 curves fit with the assumption of a common cause of the decline: According to [9,19], kinetics of this type can be expected if preferentially replicon initiation is suppressed while chain growth in already active replicons is not severely affected. Then, the temporal course of the decline of total DNA synthesis mainly reflects the decay of replicon firing caused by 'normal' termination events whose frequency in a given cell population is mainly governed by its individual replicon organisation. Thus, different replicon organisations can cause different decline kinetics in different cell lines, even though the susceptibility of replicon initiation to the drug is the same. In Fig. 2, the curves of CCRF-CEM cells and BHK-21 cells cross at about 100 min, and probably, we would have obtained another order of the dose–responses than that in Fig. 1b if we had measured the rate of DNA synthesis at 30 min after staurosporine addition instead of 2 h.

Fig. 3a–c shows the alkaline sedimentation patterns of growing daughter strand DNA labeled by 8 min [^{3}H]dThd pulses at different times after staurosporine addition. Profiles obtained with all 3 cell lines behaved very similar. The peaks and the ascending branches of the curves were progressively shifted towards higher S values with increasing time after drug addition, indicating a progressive increase of the minimal lengths of growing daughter chains. This is expected if chain growth in firing replicons continued while new short

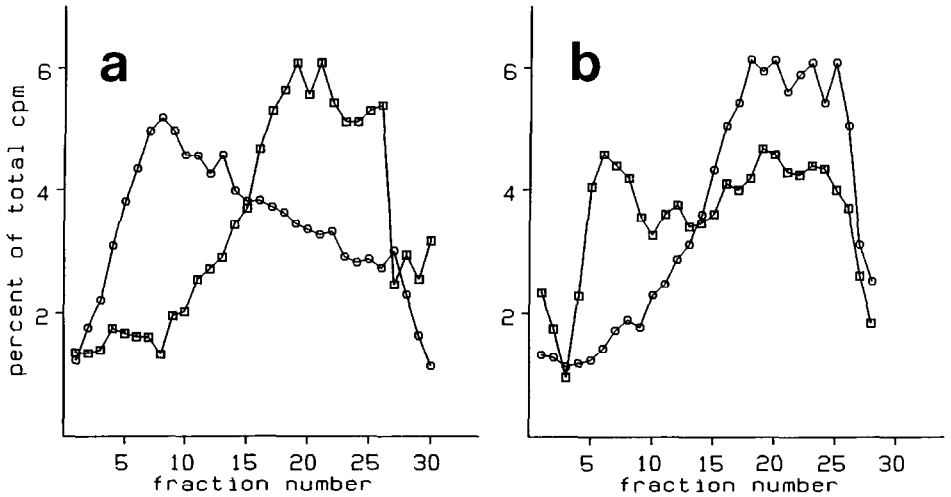

Fig. 4. Alkaline sedimentation profiles after a 90 min treatment of CCRF-CEM cells with different staurosporine concentations (a) ○, no staurosporine (control), 35817 cpm; □, 1 μM, 5823 cpm; (b) ○, 0.3 μM, 8607 cpm; □, 0.1 μM, 18194 cpm.

chains belonging to freshly inititiated replicons failed to appear. Thus, the staurosporine treatment very probably caused a fast and relatively specific suppression of replicon initiation. The observed shifts provide a measure of the chain growth rate in the presence of staurosporine. Conversion of peak S values to chain lengths according to Studier [20] yields a linear increase within the first h at following rates: Ehrlich ascites cells (Fig. 3a) 3.4 kb or 1.13 μm helix length per min, CCRF-CEM cells (Fig. 3b) 3.9 kb or 1.3 μm helix length per min, BHK-21 cells (Fig. 3c) 4.5 kb or 1.5 μm helix length per min. The increases found between 1 and 2 h after staurosporine addition lag behind this mark by about 10%. These rates are well compatible with the fork propagation rates estimated by DNA fibre autoradiography (see below) if bidirectional growth of daughter chains is assumed. Fig. 3d demonstrates that removal of staurosporine at 1 h after its application stimulated the reemergence of short daughter chains growing in recently initiated replicons (closed circles). The profile recorded 1 h after drug removal again reflected an almost normal distribution of lengths of growing chains (open circles). This indicates that the suppression of replicon initiation caused by staurosporine is reversible, at least if the period separating drug addition and removal does not exceed 1–2 h. If this period was prolonged to > 2 h, we found an increasing loss of the reversibility (results not shown). On the other hand, the suppression of initiation was only transient when relatively low staurosporine concentrations were applied. The experiment of Fig. 4 demonstrated that CCRF-CEM cells were, in a nearly identical manner, depleted of short chains when treated for 90 min either with 10^{-6} or 3×10^{-7} M staurosporine (Fig 4a □, Fig. 4b ○). However, 10^{-7} M staurosporine produced a two peaked profile (Fig. 4b □) which is qualitatively comparable to the profile obtained 30 min after removal of the drug (Fig. 3c). This indicates that a transient suppression of replicon initiation was followed by a recovery. It is not clear whether this recovery reflects instability of staurosporine under cell culture conditions or an active cellular response.

In Table I we compiled the results of the statistical evaluation of DNA fibre autoradiographs obtained by a 20/20 min 'hot/warm' labeling schedule [9,15,21,22]. We evaluated tracks produced by untreated control cells and by cells to which staurosporine was added 10 and 60 min before the start of labeling. Fig. 5 shows, as examples, photographs of patterns obtained with Ehrlich ascites cells (control and 10 min staurosporine). In order to give a representative general visual impression of the respective situation of the replication machinery, we present relatively dense regions of the microscopic slides. For quantitative evaluation, we preferably selected photographs providing rather well separated arrays of patterns. The visual impression is confirmed by the results of statistical evaluation (see below). As in prior work [7–9,15,23] and in conformity with the pertinent literature [21,22], we estimated fork propagation rates from lengths of 'hot' tracks and inter initiation distances from center to center distances of tracks of adjacent bidirectional replicons. In accordance with [7– 9,24], the ratio of the number of patterns indicative of initiation within the 20 min 'hot' pulse ('post-pulse patterns') and patterns indicating an initiation before the start of labeling (gapped 'pre-pulse patterns') was designated 'relative initiation frequency'.

As a whole, the data presented in Table I consistently confirm the conclusions drawn from the alkaline sedimentation profiles. Within less than 10 min, staurosporine reduced the relative initiation frequency by about one order of magnitude in all three cell lines. Concurrently, the mean rate of propagation of replication forks was reduced by 37% in Ehrlich ascites cells, 26% in CCRF-CEM cells and 14% in BHK-21 cells. The

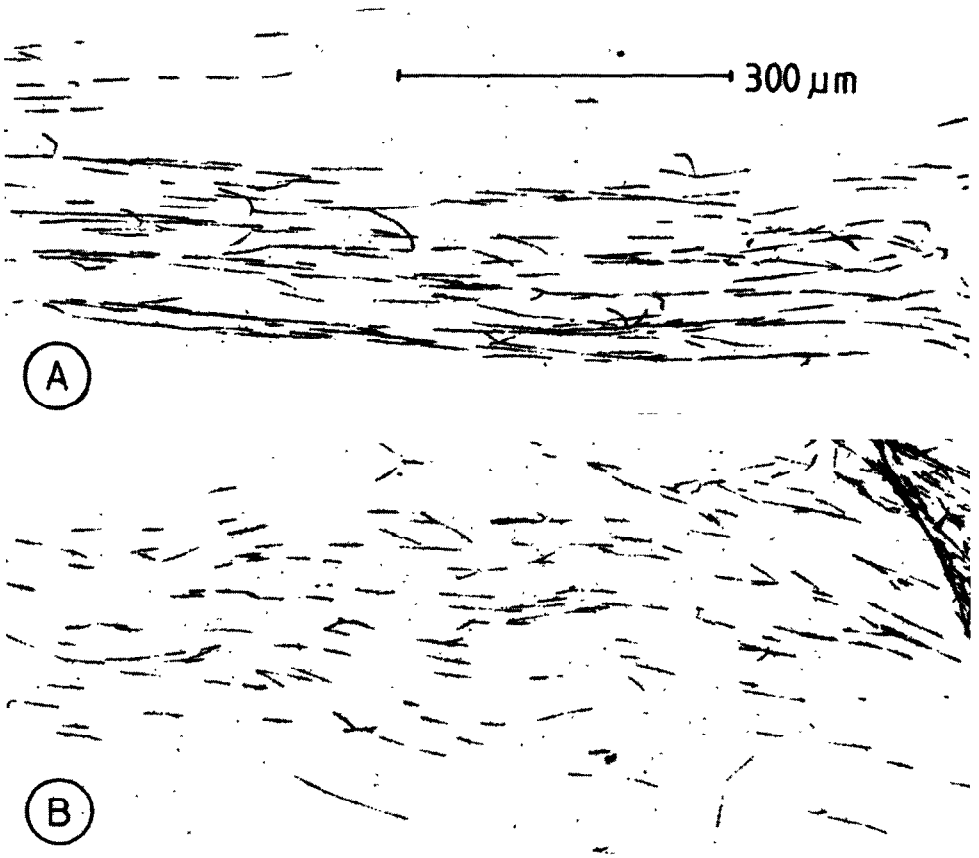

Fig. 5. Photographs of DNA fibre autoradiography patterns obtained with Ehrlich ascites cells (A) Untreated control; (B) labeling started 10 min after addition of staurosporine.

mean rates found under staurosporine fit well the above mentioned chain growth rates derived from the shifts of the sedimentation profiles (Fig. 3). An analysis of the distributions of the fork propagation rates (Fig. 6) according to [7] reveals that probably about half of this reduction is due to the disappearance of replicons with comparably fast forks which attain termination faster than slow replicons [7], therefore, the former are depleted first when new initiations fail. However, the distributions shown in Fig. 5 also indicate an additional reduction of fork progress rates in already active replicons accounting for roughly the other half of the reduc-

Table I

Results of DNA fibre autoradiography

	Fork propag. rate (µm/min)			Rel. init. frequency		Inter init. distance (µm)			
	Mean	S.D.	*n*	Ratio*	*n*	Median	Mean	S.D.	*n*
Ehrlich ascites									
Control	0.99	0.38	315	0.88	294	64.5	77.5	44.0	134
10 min stau.	0.62	0.18	675	0.05	296	60.0	68.4	39.4	87
60 min stau.	0.60	0.27	550	0.12	223	76.5	81 1	47.9	76
CCRF-CEM									
Control	0 90	0 38	555	0 64	441	76.3	93.6	59.4	223
10 min stau.	0.66	0.25	699	0.04	309	72.2	94 7	66 6	123
60 min stau.	0.69	0.23	546	0.15	274	88.6	110.0	100.2	120
BHK-21									
Control	0.92	0.32	311	1.26	271	56.2	64.1	31.5	116
10 min stau.	0.79	0.29	389	0.22	178	56.1	64.4	31.3	64
60 min. stau	0.76	0.43	341	0.14	87	45.8	67.6	39.6	19

*The relative initiation frequency is the ratio of post-pulse initiation (-■-) to pre-pulse initiation pattern (-■ ■-)

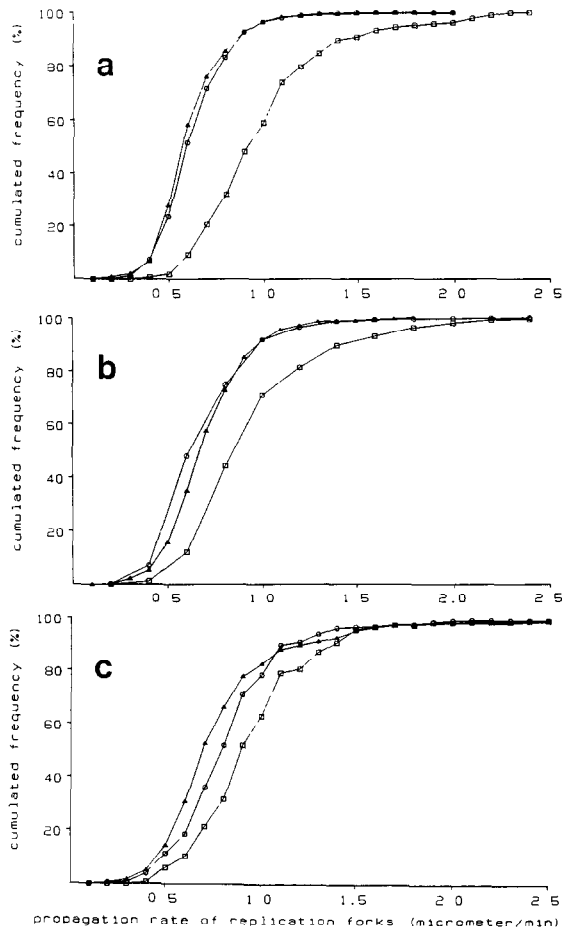

Fig. 6. Cumulated frequency distributions of fork propagation rates (estimated by DNA fibre autoradiography) 10 (□) and 60 (△) min after staurosporine addition, (○) untreated control. (a) Ehrlich ascites cells; (b) CCRF-CEM cells; (c) BHK-21 cells. Abscissa, upper limit of classes.

tion of the mean. This contrasts with the suppression of replicon initiation in Ehrlich ascites cells by hypoxia where depletion of fast forks could account for the entire concomitant reduction of the mean fork progress rate. Clearly, in this latter case, the reduction of the mean did not reflect true slowing down of working forks [7]. The spatial organisation of firing replicons as reflected by the inter initiation distances was not significantly altered by staurosporine. Control cells and 10 min drug treated cells yielded practically identical distributions of this parameter (not shown). The 60 min treated samples of Ehrlich ascites- and CCRF-CEM cells exhibited a slight (statistically not significant) shift of the middle region of the distributions towards larger distances. The observed absence of significant alterations of inter initiation distances is compatible with the following presumptions. (i) Suppression of replicon initiation occurred coordinately in whole clusters and did not abolish single interspersed initiations. (ii) The average spatial organisation of clusters terminated or initiated, respectively, during drug treatment (initiation, however, at distinctly diminished frequency) did not significantly differ from that of the clusters firing in the untreated control cells. (iii) The drug treatment caused no significant amount of interspersed replicons to cease firing prematurely (i. e. before their forks meet that of adjacent neighbours). In earlier studies, we observed a similar coordinated suppression of replicon initiations, accompanied by only insignificant changes of inter initiation distances, under controlled hypoxia [7–9] and after inhibiting protein synthesis [8,9,23].

After 60 min under staurosporine, the relative initiation frequency in Ehrlich ascites cells and in CCRF-CEM cells significantly re-increased as compared to the 10 min incubation (Table I). To interprete this phenomenon correctly, it should be considered that the relative initiation frequency, as defined above, reflects the *rate of change* of the initiation frequency. It provides no information about the quantity of the initiation frequency itself which can adopt any value for the same value of the relative initiation frequency. If we define the state of asynchronously growing control cells (representing an average of all stages of the S-phase) as 'steady' replication, then numerical values of the relative initiation frequency smaller than that of the control cells indicate a decay of firing replicons, caused by deficiency of initiations. Thereby, the actual 'rate constant' of the decay correlates with the relative initiation frequency. This (as yet undefined) correlation depends on various parameters such as the length of the 'hot' pulse and the individual replicon organisation of the cell line examined. Clearly, the opposite (i.e. growth of the number of firing replicons) holds for elevated values of the relative initiation frequency. In this sense, the observed re-increase merely indicates that the (relative) rate at which the the initiation frequency *decreased* was smaller at 60 than at 10 min after addition of staurosporine. Perhaps, this announces to the approaching recovery from the staurosporine block discussed above (experiment of Fig. 4).

4. CONCLUSION

Taken together, our data show clearly that suppression of initiation of cellular replicons is a fast response of mammalian cells to micromolar concentrations of staurosporine. In contrast to the long-term inhibition of cell growth which possibly depends on different staurosporine sensitive events in the different cell lines analyzed, the short-term response of replicon initiation to relatively high staurosporine concentrations seems to be caused by one common mechanism in Ehrlich ascites cells, CCRF-CEM cells and BHK-21 cells. The fast onset of the effect and its fast reversibility rather argue for inhibition of an event which is relatively tightly associated with replicon initiation itself than for an interfer-

ence with complicated regulatory cascades including changes of gene expression. We don't deny that staurosporine can cause the latter also, but this may rather be pertinent with respect to the loss of reversibility of the suppression of replicon initiations after prolonged exposition and with respect to the inhibition of cell growth occuring at distinctly lower concentrations of the drug.

The large T antigen of SV 40, a multifacetted multifunctional virus encoded protein, is one of the few so far known initiator proteins functioning in mammalian cells [3]. For the purpose of the replication of the viral genome, it replaces still unidentified cellular initiator protein(s). Prerequisite of the initiator function of the T antigen is a specific phosphorylation at threonine 124 [1–3]. We propose that the cellular counterpart of the initiator function of the large T antigen also has to be activated by a specific phosphorylation event which can be inhibited by staurosporine only at relatively high concentrations. It can be imagined that this phosphorylation represents the ultimate on/off switch of replicon activation situated at the end of one or several regulatory pathways ('cascades'). At paramount levels, such cascades may include other phosphorylation steps exhibiting distinctly lower sensitivity to staurosporine and partly exerting pleiotropic effects, including switching of the cellular gene expression between resting (G_0) and proliferative states. Such alterations of gene expression may e.g. be responsible for the loss of reversibility of the suppression of replicon initiation when the exposition to staurosporine lasts for several hours.

The significance of the slight reduction of fork propagation rates accompanying the staurosporine-induced suppression of replicon initiation is still unclear. Although the large T antigen functions in initiation and fork propagation as well [3], it seems too speculative to postulate, on the basis of the present data, a cellular factor harbouring both functions. An independent mechanism can by no means be excluded as yet.

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