Metabolic turnover of proliferation-related nuclear proteins in serum-stimulated Swiss mouse 3T3 cells

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Stimulation of quiescent Swiss mouse 3T3 cells either by serum or by pure growth factors induces DNA synthesis after a lag period of about 15 h. Following restimulation by serum or by growth factors there is an overall increase of 2–4-fold in the rate of biosynthesis of nuclear proteins. Two nuclear polypeptides show specific temporal correlations with the transition from quiescence to proliferation. The synthesis of p30 (30 kDa, p*I* 5.2) is at a maximum within 5 h of restimulation, while the synthesis of p36 (36 kDa, p*I* 4.25) is first seen at 10–12 h after restimulation. The synthesis of p36 correlates well with the initiation of DNA biosynthesis. The metabolic turnover of both of these proteins has been estimated by pulse-chase and by cycloheximide inhibition experiments. They both have a half-life of 10–15 h and appear to be cell-cycle related.

Nuclear protein synthesis *Growth stimulation* *Cell proliferation* *Protein p36* *2D gel electrophoresis* *Cyclin* *Protein p30* *Cell cycle* *[^{35}S]Methionine labeling*

1. INTRODUCTION

To understand the regulation of mammalian cell proliferation requires the elucidation of the molecular events leading up to the initiation of DNA synthesis and cell division. Cultured Swiss mouse 3T3 cells [1] provide a convenient in vitro experimental system in which to study the molecular biology of cell proliferation [3]. These cells become quiescent when grown to confluence and/or when the culture medium is depleted of growth factors. In this quiescent state they do not initiate DNA biosynthesis, but further proliferation can be induced by the addition of fresh serum or purified growth factors, and DNA replication starts after a lag phase of about 15 h [2–5]. Following restimulation by serum or by these pure hormones there is an overall increase of 2–4-fold in the rate of biosynthesis of nuclear proteins. Analysis by 2D gel electrophoresis shows that there is a relative decrease in some proteins (e.g. 200 kDa, p*I* 6.0–6.5) and increases in others (e.g. actin). From this type of analysis, two polypeptides have been identified which might participate in the regulation of cell proliferation [5]. These polypeptides show specific temporal correlations with the transition from quiescence to proliferation. The synthesis of p30 (30 kDa, p*I* 5.2) is at a maximum within 5 h of restimulation, while the synthesis of p36 (36 kDa, p*I* 4.25) is first seen at 10–12 h after restimulation. Synthesis of p36 correlates well with the initiation of DNA synthesis. The synthesis of both proteins is stimulated by serum and by the pure hormones. The 'early' protein p30, is different from the products both of *c-fos* and *c-myc* [5]. The protein p36 [5] has many properties in common with the proliferation-related cell nuclear antigen (PCNA) [6] and with the nuclear protein called cyclin [7]; it has recently [8] been shown that the latter two proteins are probably identical. It will be necessary to test for the identity of p36 and cyclin with appropriate specific antibodies.

In this study I have measured the metabolic turnover of p30 and of p36 and I find that both of these proteins have only moderate turnover rates. Pulse-chase experiments and cycloheximide inhibi-

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tion indicate that they have a half-life of about 10–15 h.

2. MATERIALS AND METHODS

2.1. *Cell culture, quiescence and restimulation*

Swiss mouse 3T3 cells [1] were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin and 10% (v/v) foetal calf serum (FCS). Subconfluent, stock cultures were grown in 90 mm Nunc petri dishes at 37°C in an atmosphere of 10% CO_2 and passaged every 3–4 days. The cultures were routinely checked for any contamination by mycoplasma. To measure DNA synthesis, cells were plated at 3×10^4 cells per 35 mm dish in 2 ml DMEM and 6% (v/v) foetal calf serum. The cultures became quiescent 3–4 days after a further medium change and were used when no mitotic cells were observed. To estimate the fraction of cells producing DNA, cells were radiolabelled with 3 μCi/ml [*methyl*-^{3}H]thymidine (3 μM) from the time of addition of 20% (v/v) FCS for varying periods up to 36 h. The cultures were subsequently processed for autoradiography [9].

2.2. *Radiolabelling and separation of nuclear proteins*

Quiescent cultures were prepared for isotopic labelling of protein as follows: when the cultures had reached confluence the medium was changed to DMEM containing 1/10 the normal concentration of methionine (final concentration 20 μM) and 2% (v/v) dialysed foetal calf serum. The cells were left for 36–48 h in this medium. Cultures prepared in this way had a labelling index of less than 1% when incubated with [^{3}H]thymidine for 25 h as described above. Addition of 20% (v/v) foetal calf serum stimulated the rate of entry into S phase with the same kinetics and to the same extent as in cultures in complete DMEM. [^{35}S]Methionine (30 μCi/ml) was added to the culture medium for 5 h, either immediately after the addition of 20% FCS or 15 h later. At the end of the 5 h labelling period the cell monolayers were washed, the cells lysed and the nuclei and chromatin structures were isolated and purified by the method of Hancock [10]. Chromatin was dissolved in lysis buffer and analysed by 2D gel electrophoresis [11,12]. The ampholine composition of the isoelectric focussing gel was modified to obtain a wider pH range. After fixation and staining the gels were prepared for fluorography and exposed to preflashed Kodak Xomat H Film at −70°C [13,14].

2.3. *Determination of protein turnover*

The turnover of nuclear proteins was determined in two ways.

(i) Pulse chase: cells were radiolabelled with [^{35}S]methionine for 5 h at zero time or at 15 h after addition of serum and then the radioactive medium was removed and the cultures were washed with complete medium (200 μM) methionine containing 20% (v/v) FCS. Complete medium with 20% FCS (v/v) was added back for the chase period; cells were harvested at various times as indicated in figs 2 and 4.

(ii) Cycloheximide inhibition: cells were radiolabelled with [^{35}S]methionine for 5 h at zero time or at 15 h after addition of serum and then the radioactive medium was removed and the cultures were washed with complete medium with 20% (v/v) FCS. Complete medium with 20% (v/v) FCS containing 50 μM cycloheximide was added to the cultures which were then further incubated for varying periods before harvesting as indicated in figs 2 and 4.

3. RESULTS AND DISCUSSION

Cultures of Swiss mouse 3T3 cells were labelled with [^{35}S]methionine for periods of 5 h before (fig.1A), immediately after (fig.1B) or 15 h after (fig.1C) the addition of serum. Nuclei were isolated and nuclear proteins separated and analysed by 2D gel electrophoresis (fig.1A–C).

There was a general increase in the rate of nuclear protein synthesis after growth stimulation. There were also a number of changes, both increases and decreases, in the rates of synthesis of specific proteins. Two proteins are of special interest; the synthesis of p30 was at a maximum at about 5 h (fig.1B, arrow), while that of p36 was seen at 15–20 h after restimulation (fig.1C, arrowhead). Synthesis of p36 correlated well with the initiation of DNA synthesis (see fig.2).

The metabolic turnover of these two proteins, p30 and p36, was then examined in turn. Two ex-

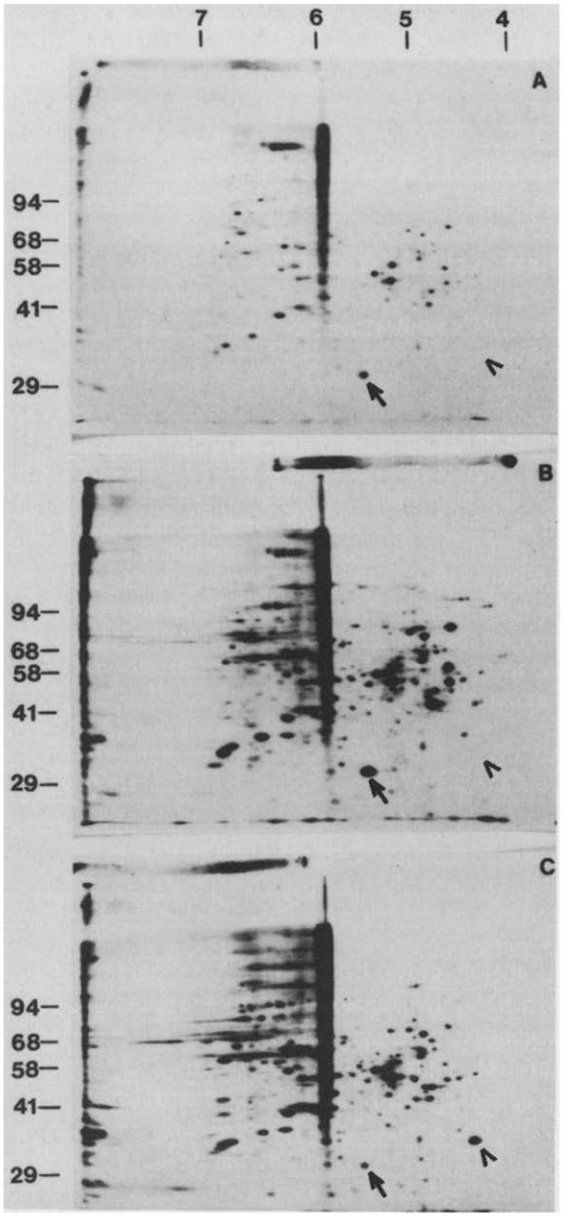

Fig.1. Analysis of nuclear proteins from quiescent and restimulated Swiss mouse 3T3 cells. (A) Quiescent cells, (B) labelled 0–5 h after restimulation with serum, (C) labelled 15–20 h after restimulation with serum. Arrow, p30; arrowhead, p36.

perimental protocols were used; these are described in section 2 and figs 2 and 4.

In the first protocol, cells were metabolically labelled with [^{35}S]methionine and then chased for

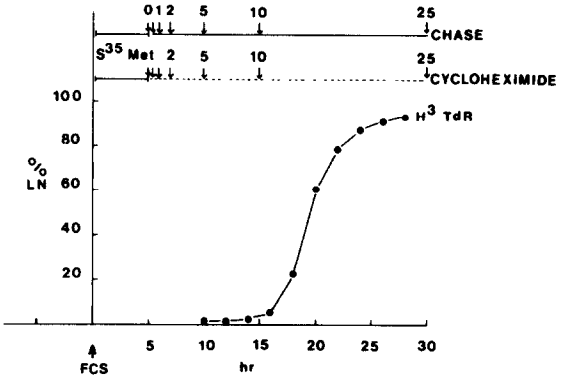

Fig.2. Quiescent Swiss mouse 3T3 cells were restimulated by 20% (v/v) foetal calf serum and labelled with [^{3}H]thymidine. The horizontal axis shows time (h) after addition of FCS; the vertical axis shows the percentage of nuclei which had entered S phase. Above the graph is shown the two alternative protocols for measuring the metabolic turnover of the protein p30.

various periods of time (fig.2). The cells were harvested, and the nuclear proteins analysed by 2D gel electrophoresis.

Fig.3 shows the results for protein p30; the lower right quadrant of each autoradiograph is displayed. Cells were incubated with [^{35}S]methionine for the first 5 h following restimulation so that p30 should be radiolabelled (fig.2). The presence of p30 is indicated by the arrow in fig.3. In all these turnover experiments (figs 3 and 5) the zero time is the end of the 5 h labelling period. The decay of the radiolabelled protein was measured by incubation of the labelled cells in fresh medium for 0.5, 1, 2, 5, 10 and 25 h. At these various times the nuclear proteins were analysed. During the 25 h of the chase there was a decrease in the amount of labelled p30 (fig.3, middle panel).

To confirm these results an analogous experiment was performed with the alternative protocol in which protein synthesis was inhibited with cycloheximide (fig.3, lower panel). This experiment confirmed the previous observation that p30 was degraded only at a moderate rate, showing a substantial decrease at 25 h.

The metabolic turnover of p36 was studied by the same two protocols (fig.4); however, in these experiments proteins were labelled with [^{35}S]methionine between 15 and 20 h after restimulation with serum; this period coincides

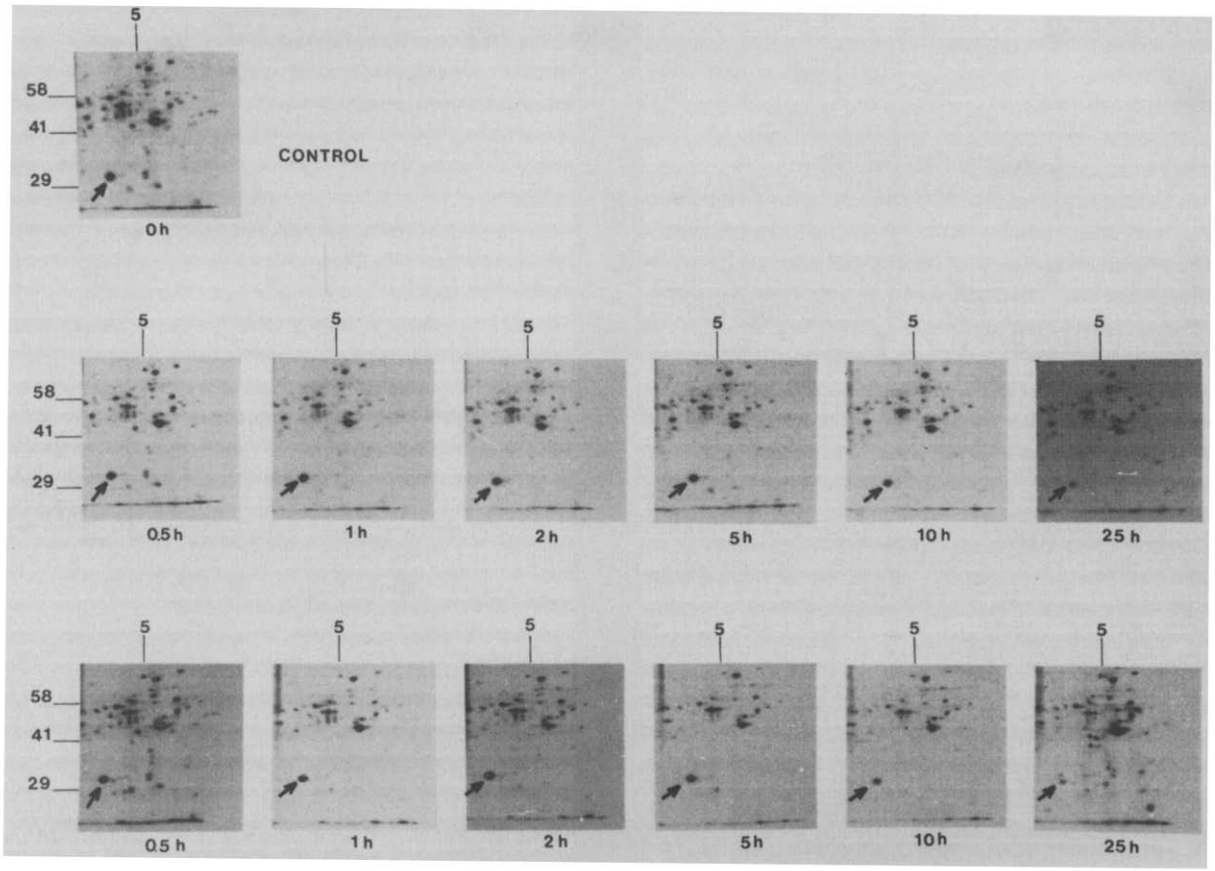

Fig.3. The metabolic turnover of the nuclear protein p30 in Swiss mouse 3T3 cells. 2D gel electrophoresis of nuclear proteins at various times after stimulation of quiescent 3T3 cells by 20% (v/v) FCS. A section of the lower right quadrant of each autoradiograph is shown. Top row, control culture, harvested at the end of the labelling period as shown diagrammatically in fig.2. Middle row, pulse-chase experiment; lower row, cycloheximide-inhibition experiment. The cells were radiolabelled for 5 h immediately after the readdition of serum, treated and harvested at the times indicated below each autoradiograph.

with the onset of DNA biosynthesis (fig.4). The decay of p36 was followed during the 25 h after labelling. During this period there was a gradual decrease in the amount of labelled p36 (fig.5, middle panel). The alternative protocol with cycloheximide gave very similar results (fig.5, lower panel).

With both protocols there was a significant decrease already evident by 10 h. This is confirmed by the almost total absence of p36 at 25 h.

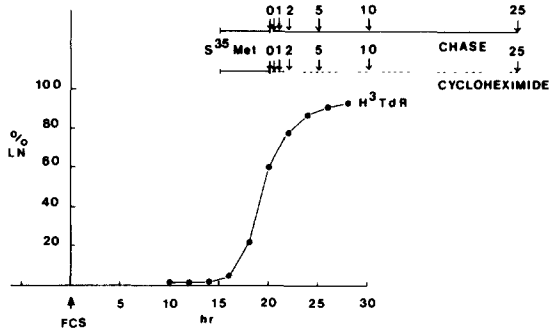

Fig.4. Quiescent Swiss mouse 3T3 cells were restimulated by 20% (v/v) foetal calf serum and labelled with [^{3}H]thymidine. Horizontal axis, time (h) after addition of FCS; vertical axis, percentage of nuclei which had entered S phase. Above the graph is shown the two protocols for measuring the metabolic turnover of the protein p36.

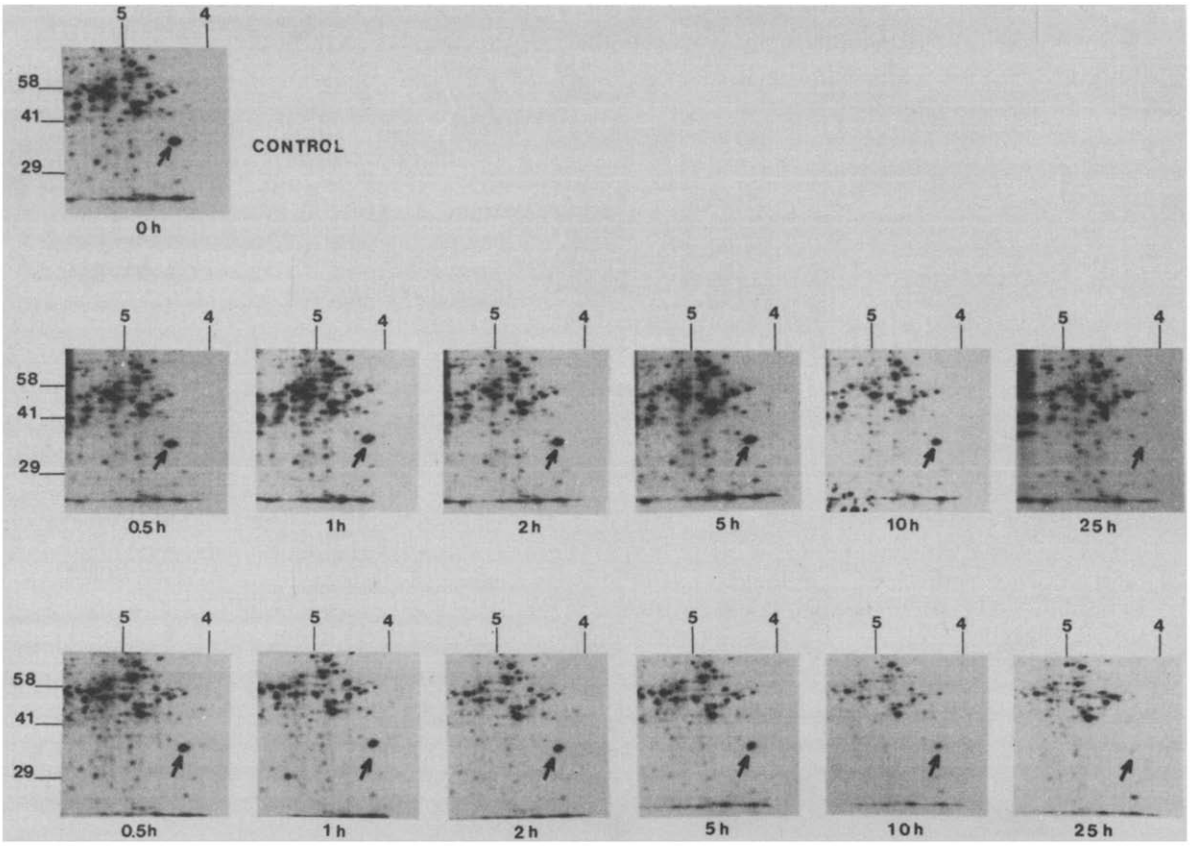

Fig.5. Metabolic turnover of the nuclear protein p36 in Swiss mouse 3T3 cells. Details are as described in the legend to fig.3, except that the cells were radiolabelled between 15 and 20 h after the readdition of serum, treated and harvested at the times indicated below each autoradiograph.

Different cellular proteins have very different turnover rates. Some proteins turn over very rapidly, for example ornithine decarboxylase (ODC), p53, c-myc and c-fos have half-lives measured in minutes. Numerous proteins have half-lives of the order of the cell cycle. Finally, some proteins are essentially totally stable, for example, the core histones. The present experiments clearly demonstrate that the two nuclear proteins studied here are neither completely stable like the histones, nor do they turn over very rapidly like the proliferation-related ODC. The half-life of p30 and p36 is of the order of 10–15 h which is about 1/2–2/3 of a cell cycle duration. The experiments with cycloheximide seem to indicate a faster turnover time than do the pulse-chase experiments. The apparent difference may be due to reutilization of radiolabelled methionine in the pulse-chase experiments. It seems that p36 may disappear faster in its last 15 h after labelling (30–45 h after additions of FCS) than during its first 10 h after the label. If this were true it would imply a biphasic turnover, perhaps because it is specifically degraded at the end of the S phase. However, this suggestion requires a much more detailed time course before a conclusion can be reached. In the experiments described in figs 1 and 5 there are some similarities seen between the behaviour of p36 and the previously described proteins PCNA [6] and cyclin [7]. It has been reported that following a hydroxyurea block, cyclin is metabolically stable over a period of 10 h [15]; that report is consistent with the present data. Figs 3 and 5 show clearly that with the longer time period used in the current experiments p30 and p36 do indeed show metabolic turnover.

We have previously suggested that p30 and p36 may be involved in the regulation of the cell cycle [5]. Both the patterns of biosynthesis of these two proteins and their intermediate rates of turnover are consistent with the notion that p30 and p36 are important in the control of cell proliferation.

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