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Arrest of Cell Cycle Progression of HeLa Cells in the Early G1 Phase in K^+-Depleted Conditions and Its Recovery Upon Addition of Insulin and LDL

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Abstract Cell cycle progression of synchronized HeLa cells was studied by measuring labeling of the nuclei with [^{3}H]thymidine. The progression was arrested in a chemically defined medium in which K^+ was replaced by Rb^+ (Rb-CDM) but was restored upon addition of insulin and/or low density lipoprotein (LDL). Cells started DNA synthesis 12 hr after addition of insulin and/or LDL, regardless of the time of arrest, suggesting their arrest early in the G1 phase. After incubation of cells in Rb-CDM containing insulin or LDL singly for 3, 6, or 9 hr, replacement of the medium by that without an addition resulted in marked delay in entry of cells into the S phase, but in its replacement by medium containing both agents, the delay was insignificant. Synthesis of bulk protein, estimated as increase in the cell volume, was not strongly inhibited. From these results we conclude that cell cycle progression of HeLa cells in K^+-depleted CDM is arrested early in the G1 phase and that the arrest is due to lack of some protein(s) required for entry into the S phase that is synthesized in the early G1 phase.

Key words: K^+-depleted cells, insulin, LDL, HeLa cells, cell cycle

There are many reports on the essential roles of intracellular K^+ in cell growth and protein or DNA synthesis of cultured cells [Wyatt, 1961; Lubin, 1967; Cuff and Lichtman, 1975; Kimelberg and Mayhew, 1975; Cahn and Lubin, 1978; Takagi, et al., 1986]. Intracellular K^+ has been shown to be necessary for amino acid-transfer from aminoacyl-soluble RNA to polypeptide and for aminoacyl-tRNA binding to ribosomes [Lubin and Ennis, 1964; Pestka and Nirenberg, 1966], and also to be required in the regulations of various enzymes [Kernan, 1980].

DNA synthesis by Swiss 3T3 cells cultured in serum-free medium has been reported to be stimulated by addition of growth factors, possibly dependent on the intracellular K^+ concentration [Lopes-Rivas et al., 1982]. The addition of growth factors seems to change the K^+ concentration of hamster fibroblasts, as demonstrated by activation of $^{86}Rb^+$ uptake mediated by a bumetanide-sensitive Na^+, K^+, Cl^--cotransport pathway [Paris and Pouysségur, 1986].

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On the other hand, there are reports suggesting that intracellular K^+ is not always essential for cell growth. Decrease in the K^+ concentration is reported not necessarily to prevent DNA synthesis in fibroblasts [Moscatelli et al., 1979] and that there is no marked change in the K^+ concentration of quiescent Swiss 3T3 cells when their DNA synthesis is stimulated by addition of growth factors [Amsler et al., 1985]. Moreover, DNA synthesis of quiescent BALB/3T3 cells is stimulated by serum or platelet-derived growth factors even when the K^+ concentration is decreased by treatment with ouabain, and the cells enter the S phase without increase in the K^+ concentration [Frantz et al., 1981]. These findings imply that DNA synthesis of cultured cells can be stimulated by addition of growth factors independently of the intracellular K^+ concentration.

There are reports that when K^+ in chemically defined culture medium (CDM) is replaced by Rb^+, cell growth and protein synthesis of HeLa cells are strongly inhibited and that the inhibition is not due to a toxic effect of Rb^+, but to the depletion of intracellular K^+ [Sakai et al., 1981, 1985]. The growth inhibition by substitution of Rb^+ for intracellular K^+ was significantly re-

stored by addition of dialyzed serum, without any change in the intracellular K^+ concentration, suggesting that stimulation of some K^+-independent mechanism(s) by serum compensates for the inhibition due to K^+ depletion. The inhibition by Rb^+-containing chemically defined medium has been found to be partially eliminated by addition of insulin and/or LDL as by addition of dialyzed serum, and significant restoration of cell growth and macromolecular synthesis occur [Hosokawa et al., 1990]. These agents caused marked stimulation of DNA synthesis, but did not influence synthesis of bulk protein significantly within 24 hr after the start of Rb^+-substitution [Yonezu et al., 1992]. Therefore, the restoration of cell growth by these agents is related to either stimulation of DNA synthesis or inhibition of degradation of protein with short lives [Sakai et al., 1985]. The promotions of cell growth and DNA synthesis would reflect stimulation of synthesis of some protein(s) essential for progression in the G1 phase and DNA synthesis. The necessity for synthesis of some labile protein(s) for progress to the S phase has been suggested in different types of cells [Rossow et al., 1979; Medrano and Pardee, 1980; Campisi et al., 1982; Murray et al., 1991].

In the present study, we examined the stage of the cell cycle at which synthesis of this putative protein(s), if present, occurred for progression from the G1 to the S phase. For this purpose, we measured the stimulating effects of insulin and LDL on the labeling index of synchronized HeLa cells whose growth had been inhibited in Rb^+-substituted medium.

MATERIALS AND METHODS

Cell Cultures

HeLa S3 cells were cultivated serially in glass culture flasks in medium consisting of modified Eagle's minimum essential medium [Miyamoto et al., 1976] supplemented with 10% (v/v) calf serum. Growing cells in cultures were detached from the culture flasks and dispersed in culture medium by treatment with 0.5% trypsin (1:250, Difco Laboratories, Detroit, MI). The cells were inoculated at a density of 10^5 cells/ml into Roux flasks in 70 ml of culture medium and placed in a CO_2 incubator for 72 hr at 37°C. Then they were resuspended at the same density by tryptic digestion and inoculated into plastic culture dishes (150-mm diameter, Nunc, Roskilde, Denmark) containing 36 ml of culture medium and kept in a CO_2 incubator for 48 hr to increase their cell number.

Cell Synchrony

Colcemid from a stock solution (10 μg/ml, Wako Pure Chemical Co., Osaka, Japan) was added to each culture dish at a concentration of 25 ng/ml, and the dishes were placed in a CO_2 incubator for 1.5 hr. The culture medium in one culture dish was gently replaced by 12 ml of fresh culture medium to dilute the Colcemid and harvest mitotic cells. The culture dish was rotated manually so that the medium moved in a wave around the dish at each gyratory movement as described elsewhere [Yamaguchi et al., 1977]. In this way, most of the mitotic cells, especially those in metaphase, were selectively detached from the culture dish, leaving nonmitotic cells attached to the dish. After 15 rotations of the culture medium, 4–5 × 10^5 mitotic cells per dish were obtained in suspension and transferred to the next dish. This procedure was carried out on 5–6 culture dishes to accumulate mitotic cells. The cell suspension was diluted with fresh medium to a cell density of 8–10 × 10^4 cells/ml. Volumes of 2 ml of the cell suspension were introduced into plastic culture dishes (35-mm diameter, Sumitomo Bakelite Medical, Tokyo, Japan) not coated with any biological material. The culture dishes were placed in a CO_2 incubator for 1.5–2 hr to permit cell attachment and then washed twice with isotonic NaCl solution containing 25 mM HEPES buffer (pH 7.4). Then 2 ml of chemically defined medium (CDM) or normal culture medium containing calf serum was added. The CDM was the same with that used in a previous study [Hosokawa et al., 1990]. K^+ (5.4 mM) in the CDM was replaced by the same concentration of Rb^+ when required. The normal and Rb^+-substituted CDM were named as K-CDM and Rb-CDM, respectively.

Autoradiography

To prepare samples for micro-autoradiography, we placed 8 slide glasses (38 × 18 mm^2, Matsunami Glass Co., Tokyo, Japan) on the bottom of each plastic dish (150-mm diameter) and inoculated synchronized cells into the dishes at a density of 5–6 × 10^4 cells/ml. The cells were allowed to become attached to the slide glasses for 1.5 hr and then the medium was replaced by K-CDM or Rb-CDM. The slide glasses were removed at intervals of 2 hr as described in Re-

sults, for labeling for 30 min with [^{3}H]thymidine (185 GBq/mmol, Amersham International, Bucks., England) at a radioactivity of 0.1 MBq/ml and washed twice with cold isotonic NaCl solution containing 25 mM HEPES (pH 7.4). They were fixed overnight in a 3:1 mixture of ethanol and acetic acid, and microautoradiographs were prepared as described previously (Yamaguchi et al., 1977). Cells with 5 grains or more on their nuclei were regarded as labeled. The number of labeled cells as a percentage of the total number of cells observed, i.e., the labeling index, was determined by counts of 100–200 cells per preparation.

Other Assays

The number of cells and cell volume were determined electronically after dispersion of the cells with 0.5% trypsin. The electronic assay system developed in collaboration with members of the Department of Electrical and Electronic Engineering, Faculty of Engineering of this University consisted of a modified electronic cell counter (Sysmex, Toa Medical Electronics Co., Tokyo, Japan) equipped with an electronic apparatus for determining cell volume and a personal computer (PC 9801 UX, NEC, Tokyo, Japan) with software to analyze data. The system was calibrated with polystyrene microspheres with mean diameters of 5.55–25.7 μm and pollen of paper mulberry with a mean diameter of 15.9 μm (Polysciences, Inc., Warrington, PA). Cell volume is expressed as units of pl. Cellular protein was assayed by the method of Lowry et al. [1951] with bovine serum albumin (fraction V) as a standard, expressed as 10 pg/cell.

RESULTS

Time-dependent changes in the labeling index after inoculation of mitotic HeLa cells are shown in Figure 1. The labeling index of cells in K-CDM remained at a low level of 5–15% for 8–10 hr, started to increase from 10–12 hr, attained a maximum of about 65–67% between 14 and 16 hr, and then decreased, returning to a low level in 24 hr (Fig. 1A). No marked stimulation of cell cycle progression was noted when insulin (5 μg/ml) and LDL (2.5 mg/ml) were added singly or in combination. In the presence of insulin, the labeling index started to increase again after 35 hr and reached a second maximum at 42 hr (data not shown). The lengths of the G1, S, and G2 phases were estimated as 12.0, 6.1, and 7.4 hr, respectively, from the time course of change in the labeling index in K-CDM.

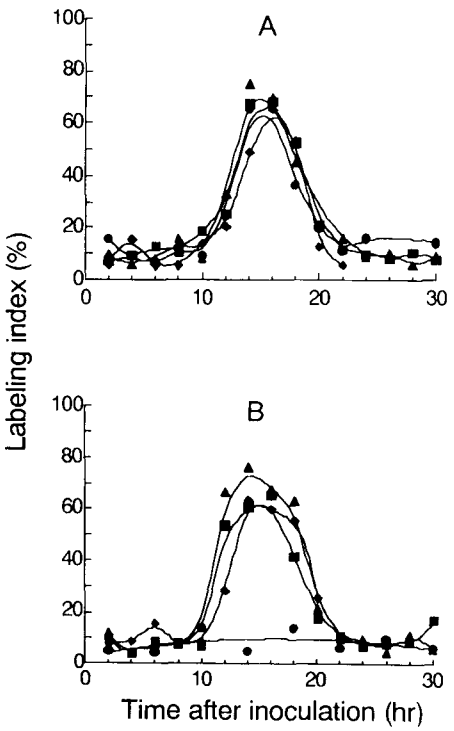

Fig. 1. Time-dependent changes in the labeling index of synchronized HeLa cells. Cells in mitosis were inoculated into culture dishes containing normal culture medium at time 0 and were allowed to become attached to the dishes for 1.5 hr. Then, the medium was replaced by K-CDM or Rb-CDM with or without a growth-promoting agent. **A:** K-CDM. **B:** Rb-CDM. ●, no addition; ■, 5 μg/ml insulin; ▲, 2.5 mg/ml LDL; ◆, 5 μg/ml insulin and 2.5 mg/ml LDL.

The labeling index of synchronized cells cultured in Rb-CDM remained almost unchanged at the low value of 4–13% throughout the experiment for 30 hr (Fig. 1B). Upon addition of insulin or LDL alone or insulin and LDL in combination to the Rb-CDM, the labeling index increased after 10 hr as in K-CDM. The lengths of the G1 and S phases were estimated to be 11.9 and 7.5 hr, indicating slight delay of the S phase compared with that in K-CDM. The maximum labeling index in the presence of LDL (77%), was slightly higher than that in the presence of insulin. But, the difference was not reproducible in a repeated experiment (data not shown).

Insulin was added at various times within 12 hr after inoculation of cells in mitosis into Rb-CDM, and the labeling index was determined after incubation for various periods (4, 8, 12, and 16 hr) (Fig. 2A). The results showed that the index remained (low less than 10%) for 8 hr after addition of insulin, but then increased to

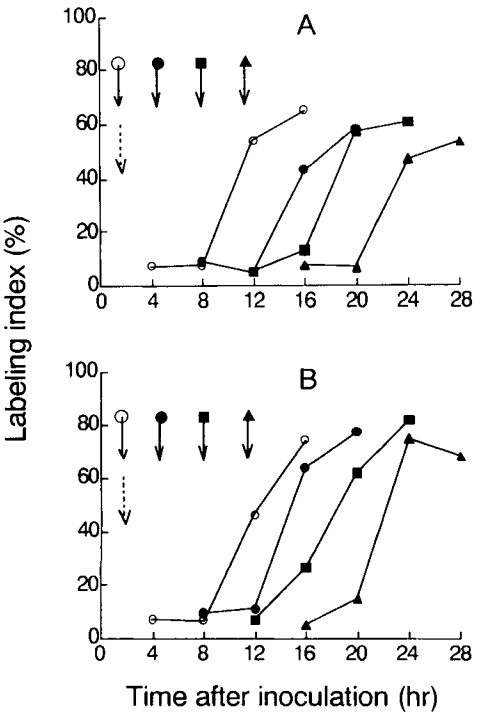

Fig. 2. Effects of addition of insulin and/or LDL on time-dependent changes in the labeling index of synchronized HeLa cells incubated in Rb-CDM. The culture medium was replaced by Rb-CDM at the time shown by the arrow with a broken line. The medium was again replaced by Rb-CDM containing insulin or LDL at the times shown by arrows with solid lines. **A:** Rb-CDM with 5 μg/ml insulin. **B:** Rb-CDM with 2.5 mg/ml LDL. Times of medium replacement after inoculation of mitotic cells: ○, 1.5 hr; ●, 4 hr; ■, 8 hr; ▲, 12 hr.

about 50% at 12 hr after insulin addition. These results suggest that progression of the cell cycle is arrested in the G1 phase in Rb-CDM but that cells enter the S phase within 12 hr after addition of insulin, regardless of the time of its addition. Similar results were obtained when LDL was added to Rb-CDM (Fig. 2B), but the labeling indices after 12 and 16 hr were usually higher than those after addition of insulin. These differences of the indices may be related to differences in activation of cell growth.

The results in Figures 1 and 2 suggest that the synthesis of some protein(s) that is specifically required for DNA synthesis is completely arrested in Rb-CDM, and that its synthesis in the G1 phase is significantly restored upon addition of insulin or LDL. To determine the time of synthesis of this putative protein in the G1 phase, we first added insulin and LDL singly or in combination after the cells had become to be attached to the culture dishes in normal medium for 1.5 hr; i.e., 3, 6, and 9 hr after inoculation of mitotic cells (Fig. 3). Then, the medium was replaced by Rb-CDM without the growth promoting agent. The labeling indices of control cells in normal medium and cells incubated for 6 hr in Rb-CDM with insulin (6-hr cells) were very low after 8 hr (Fig. 3A). These results imply that the index of cells after 3 hr must also be very low. The index of control cells increased to more than 60% after 12 hr, but was still only about 30% or less in cells after 3, 6, and 9 hr. At 16 hr, the labeling indices of cells treated with insulin after 3, 6, and 9 hr attained levels close to that of the control. The index of control cells started to decrease at 20 hr and returned to the original low level at 24 hr due to entry of the cells into the G2 and M phases. However, the index of cells treated with insulin after 6 and 9 hr did not seem to decrease at 20 hr, indicating delay of the S phase. Similar changes in the labeling index to those in Figure 3A were observed when LDL was added to Rb-CDM (Fig. 3B). On addition of insulin and LDL in combination, the labeling indices increased to higher values than did those on addition of either agent singly and approached the control level at 12 hr. However, the S phase was prolonged in cells treated with the agents for 3 and 6 hr (Fig. 3C). Changes in the labeling index in the presence of insulin and LDL in combination in K-CDM did not differ significantly from those of control cells (Fig. 3D).

The cellular protein content was found to show a highly significant correlation ($P < 0.01$) with the cell volume (Fig. 4). Therefore, we used the cell volume as a parameter of cell growth instead of cellular protein in the following experiments, because it could be determined rapidly with the electronic system using a much smaller number of cells than those necessary for protein assay.

Changes in the cell volume of synchronized cells grown in normal culture medium and Rb-CDM in the presence and absence of a growth promoting agent(s) are shown in Figure 5. The cell volume was approximately halved 2 hr after inoculation of mitotic cells. The volume of control cells started to increase and attained a maximum of somewhat less than the initial volume after 24 hr. Thereafter, the cell volume began to decrease again due to onset of the next cell division. When cells were incubated in Rb-CDM, the cell volume decreased slightly to a minimum after 12 hr. Then, it started to increase and reached about 70% more than the minimum

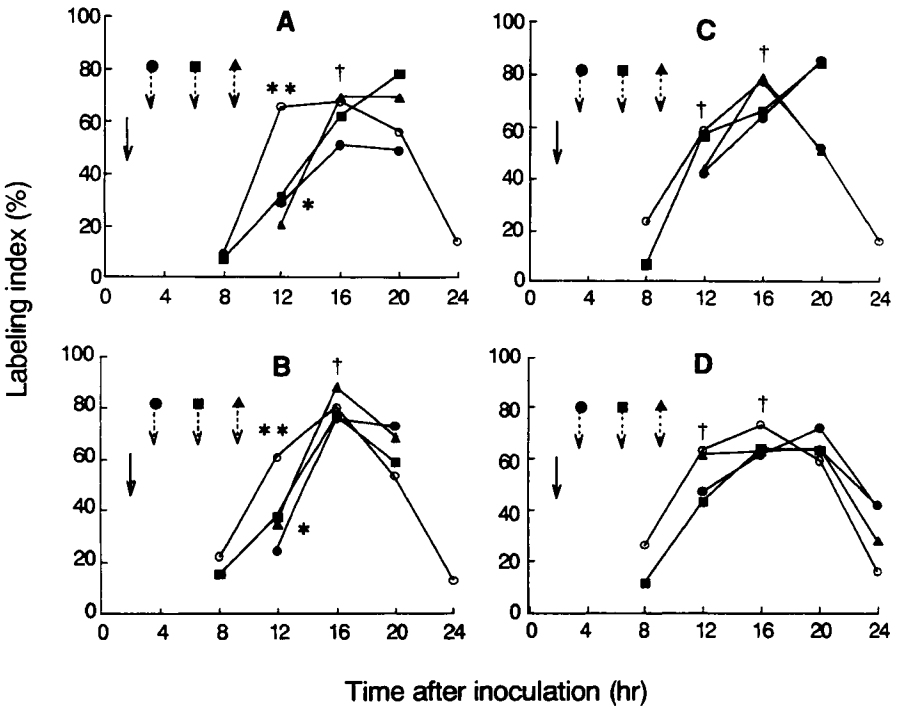

Fig. 3. Effects of depletion of insulin and/or LDL on time-dependent changes in the labeling index of synchronized HeLa cells. The culture medium was replaced by Rb-CDM or K-CDM containing the growth promoting agent(s) at the time shown by an arrow with a solid line. Then, the medium was replaced by Rb-CDM or K-CDM with no agent at the times shown by arrows with broken lines. **A:** Rb-CDM with 5 μg/ml insulin. **B:** Rb-CDM with 2.5 mg/ml LDL. **C:** Rb-CDM with 5 μg/ml insulin and 2.5 mg/ml LDL. **D:** K-CDM with 5 μg/ml insulin and 2.5 mg/ml LDL. Time of medium change after inoculation of cells in mitosis; ○, control (no medium change); ●, 3 hr; ■, 6 hr; ▲, 9 hr. *, No significant difference between any two of the indices for 3, 6, and 9 hr according to the chi-square test ($P > 0.05$). **, Significantly different from the indices for 3, 6, and 9 hr ($P < 0.01$). †, No significant difference between any two of the four indices ($P > 0.05$).

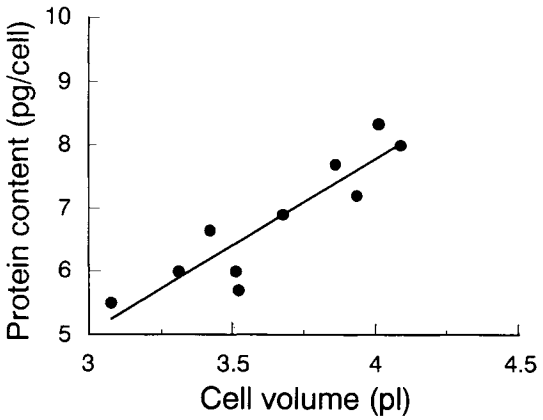

Fig. 4. Relationship between the cell volume and cellular protein content of HeLa cells. Correlation coefficient r = 0.9063. The correlation was significant at $P < 0.01$. The regression line is expressed by the equation $y = 2.75 \times -3.2132$.

volume after 30 hr. Similar changes in the cell volume were observed after addition of insulin and LDL singly and in combination to Rb-CDM, though the cell volume started to increase after 6 hr in the presence of LDL. The cell volumes in these cases were between those in normal medium and Rb-CDM without additions, but the rates of increase in the cell volume in Rb-CDM were not markedly different from that of control cells after 12 hr. The decrease in cell volume from 24 hr as observed in control medium, did not occur in Rb-CDM, irrespective of the presence or absence of additions, implying a delay of progression of the cell cycle. The increase in the cell volume corresponds to increase in the cellular protein content (see Fig. 4) and contrasts with the complete arrest of DNA synthesis in Rb-CDM (see Fig. 1B). Therefore, protein synthesis took place without DNA synthesis when intracellular K^+ was depleted.

DISCUSSION

Proliferation of HeLa cells is almost completely inhibited by replacement of K^+ in a chemically defined culture medium by Rb^+, and the inhibition is significantly restored by addition of insulin and LDL singly or in combination [Hosokawa et al., 1990]. In the present study, using

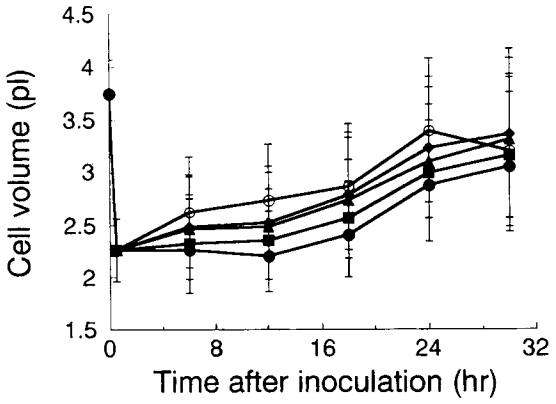

Fig. 5. Effects of additions of insulin and/or LDL on time-dependent changes in the cell volume of synchronized HeLa cells cultured in Rb-CDM. Cells in mitosis were inoculated into culture medium at time 0, and the medium was replaced by Rb-CDM with or without one or both agents at 1.5 hr. ○, control (normal culture medium); ●, Rb-CDM only; ■, Rb-CDM with 5 μg/ml insulin; ▲, Rb-CDM with 2.5 mg/ml LDL; ◆, Rb-CDM with 5 μg/ml insulin and 2.5 mg/ml LDL. Points and bars are means and SDs.

synchronized HeLa cells, we showed that progression of the cell cycle is completely arrested in the G1 phase in Rb^+-substituted medium (Rb-CDM). The arrest of cell cycle progression was completely reversed by addition of growth promoting agents (Fig. 1B). In another type of cells (mouse 3T3-4A fibroblasts) whose growth stops in the G0 or G1 phase in medium depleted in low molecular nutrients, addition of insulin is reported to stimulate DNA synthesis and cell division [Kamley and Rudland, 1976]. LDL and HDL are known to stimulate DNA synthesis of arterial smooth muscle cells in the presence of a platelet-derived growth factor [Libby et al., 1985]. Interestingly, DNA synthesis is reported to be strongly inhibited in Rb-CDM and significantly restored by addition of these agents, whereas synthesis of cellular bulk protein is not markedly influenced in this medium [Yonezu et al., 1992]. Synthesis of bulk protein was also demonstrated to occur in Rb-CDM in synchronized cells. Hence, the inhibitions of cell cycle progression and DNA synthesis could be due to inhibition of some process(es) independent of synthesis of bulk protein.

As stated above, HeLa cells arrested in the G1 phase could enter the S phase by addition of a growth-promoting agent. Regardless of the time from arrest in Rb-CDM, DNA synthesis started consistently 8–12 hr after the addition of the agent (Fig. 2), indicating that cells are arrested at an early stage of the G1 phase. Therefore, some protein(s) specifically required for entry into the S phase might be synthesized in the early G1 phase. Its synthesis may be suppressed in Rb-CDM and restored by addition of the agent. A similar conclusion has been reached from preliminary studies on the effect of a growth-promoting agent on HeLa cells growing parasynchronously in Rb-CDM [Yonezu et al., 1992]. To determine the stage of protein synthesis in the cell cycle precisely, we incubated cells for various periods in Rb-CDM containing the agents singly or in combination (Fig. 3). When we removed the agents from Rb-CDM, the cells proceeded from the G1 to S phase. After incubation with insulin or LDL singly for 1.5 hr after cell attachment, i.e., 3 hr after inoculation of mitotic cells, cell cycle progression was markedly delayed compared to that of control cells, but the cells could enter the S phase. When these agents were present until 6 or 9 hr after inoculation, progression was still delayed. The delay was dramatically reduced by addition of these agents in combination. These findings suggest the synthesis of a specific protein(s) required for entry into the S phase in the early part of the G1 phase.

Cell cycle progression of normal cells has been reported to stop in the G0 or G1 phase upon depletion of low molecular weight nutrients including amino acids and deprivation of serum or serum factors [Ley and Tobey, 1970; Brooks, 1974; Stiles et al., 1979; Zetterberg and Larsson, 1985]. On the other hand, transformed cells are thought not to show a specific arrest point [Pardee, 1974; Paul et al., 1974; Martin and Stein, 1976]. Growth of polyma transformed hamster cells (PyBHK) and SV40 transformed mouse cells (SV3T3) is reduced in serum-free conditions with proportional increases of cells in the S and non-S phases [Moses et al., 1980]. However, there are reports of growth arrest of chemically transformed cells (AKR-MCA, C3H/MCA-58) and sarcoma virus transformed BHK cells in the G1 phase upon depletion of chemical nutrients [Moses et al., 1978; Moskowitz and Cheng, 1979]. Nontransformed mouse embryo cells (AKR-2B) have been shown to have two different arrest points: a growth factor (EGF) deficiency arrest point, and a nutrient deficiency arrest point [Moses et al., 1980]. The latter point is suggested to be very similar to the arrest point of transformed cells (AKR-MCA). However, the growth factor and nutrient deficiency arrest points are thought to be located in the middle

and late G1 phase, respectively, in contrast to the early arrest point of HeLa cells in Rb-CDM.

Cell growth of mammalian cells has been proposed to be regulated by a process leading to a restriction point in the late G1 phase [Pardee, 1989], and cells must accumulate a labile protein before they can pass this restriction point [Rossow et al., 1979; Croy and Pardee, 1983]. The process is highly sensitive to moderate inhibition of protein synthesis by cycloheximide in untransformed cells such as mouse 3T3 cells, human fibroblasts and Chinese hamster CHEF/18 cells [Medrano and Pardee, 1980]. Transformed cells (BPA31) seem less sensitive than untransformed cells (A31) to cycloheximide, since the inhibition of synthesis of the labile protein necessary for passing the restriction point can be compensated for by a lower rate of degradation of the protein [Campisi et al., 1982]. The half-life of the protein is thus in between untransformed and transformed cells, but synthesis of the protein is reported to start at the beginning of the G1 phase in both types of cell [Campisi et al., 1982]. The starting point of synthesis is similar to that of the presumed protein required for DNA synthesis in HeLa cells. However, the restriction point must be accumulated before passing the restriction point, whereas the protein of HeLa cells seemed to be synthesized mainly in a short period of 3 hr after inoculation of mitotic cells. These results suggest that the properties of these proteins are different, but further studies are needed to distinguish them.

Of the expression of cell cycle-dependent genes, those of so called "immediate early genes" are insensitive to cycloheximide [Gadeau et al., 1991]. The expression of these genes characterize hormone-induction of an activated state of cells named the "competence state" [Lau and Nathaus, 1987]. Addition of lipoprotein is reported to allow complete entry of cells in the "competence state" into the S phase [Clemmons, 1984; Libby et al., 1985]. These findings would explain the significant restoration of progression to the S phase on addition of insulin and LDL in combination. Without their addition, progression must be arrested in the G1 phase in Rb-CDM.

We drew the following conclusions from the present results. In Rb-CDM, cell cycle progression of synchronized HeLa cells was completely arrested at the beginning of the G1 phase, whereas synthesis of bulk protein was inhibited only in part. The arrested progression is reversed by addition of insulin or LDL singly, and more especially in combination, and the cells entered the S phase. Results on incubation of cells with a growth-promoting agent(s) for various periods indicated that some proteins(s) necessary for entry into the S phase is synthesized early in the G1 phase. Cells can enter the S phase, even in the K^+-depleted medium, provided that the growth promoting agent(s) is present for the earliest 3 hr after mitosis.

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