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Enhanced transcription of mitochondrial genes after growth stimulation and glucocorticoid treatment of Reuber hepatoma H-35

Tatsuhiko Kadowaki and Yasuo Kitagawa

Institute for Biochemical Regulation, School of Agriculture, Nagoya University, Chikusa, Nagoya 464-01, Japan

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When a serum-deprived culture of Reuber hepatoma H-35 cells was stimulated by serum, cells proliferated synchronously showing a peak of [^{3}H]thymidine incorporation within 16 h and doubling of cell number within 24 h. A 5–10-fold increase of mRNAs of mitochondrial genes was observed prior to S-phase by Northern hybridization of total cellular RNA with cloned fragments of rat mitochondrial DNA as probes. In organelle transcription by mitochondria isolated from growth-arrested or growth-stimulated cells suggested that the increase of mitochondrial mRNAs was mainly due to enhanced transcription. Less enhanced transcription was observed by treating the cells with dexamethasone, which causes translocation of large amount of protein into mitochondria. The latter effect was counteracted by insulin.

Mitochondrial gene; Transcription; Dexamethasone; (Reuber hepatoma H-35)

1. INTRODUCTION

Human [1], mouse [2] and bovine [3] mitochondrial DNAs are sequenced completely. They have a common organization of 22 tRNAs, 2 rRNAs and 13 open reading frames. Proteins encoded by these open reading frames have been identified [4]. The mammalian mitochondrial genome lacks intervening sequences and is transcribed as a polycistronic message from H- and L-strand promoters present in the D-loop region [5]. For the precise initiation of the transcription, a transcription factor (TF) is important [6] in addition to a RNA polymerase [7]. In subsequent processing of the polycistronic message, tRNA sequences are considered to serve as punctuation signals of rRNA and mRNA [8].

Comparing with the extensive knowledge about the structure of mammalian mitochondrial genes and the mechanism of their transcription, relatively little is known about its regulation. The number, size and morphology of mitochondria are different in every type of differentiated cells. Copy number of mitochondrial DNA per cell is constant over repeated generations of a given type of mammalian cells. These observations suggest that the biogenesis of mitochondria is strictly regulated during the course of differentiation and proliferation, but little is known about the mechanism of such regulation. This may be partially due to the lack of an experimental system in which marked changes in mitochondrial gene transcription can be easily reproduced. Williams and co-workers [9,10] found an enhanced expression of the mitochondrial Cyt *b* gene by contractile activation of skeletal muscle, but this in vivo system is not suitable for further analysis of the regulatory mechanisms involved. Edwards and Denhardt [11] found decreased levels of certain mitochondrial poly(A)$^+$ RNAs after growth stimulation of

Correspondence address: Y. Kitagawa, Institute for Biochemical Regulation, School of Agriculture, Nagoya University, Chikusa, Nagoya 464-01, Japan

Abbreviations: BSA, bovine serum albumin; CPS-I, carbamoyl-phosphate synthetase I; Cox I and Cox II, subunits I and II of cytochrome oxidase; Cyt *b*, cytochrome *b*; Dex, dexamethasone; EtBr, ethidium bromide; FCS, fetal calf serum; H-35, Reuber hepatoma H-35 (H-4-II-EC3); TF, transcription factor; 16 S rRNA, 16 S ribosomal RNA

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murine fibroblasts, but they could not demonstrate any change in the rate of mitochondrial gene transcription. The result was similar in another type of normal cells (FR3T3 cells) when Glaichenhaus et al. [12] estimated the level of mRNA for subunit II of cytochrome oxidase (Cox II). However, after transformation of FR3T3 cells by polyoma virus DNA, a 5–10-fold increase of mRNAs encoding mitochondrial genes was found [12].

In this communication, we report that transcription of mitochondrial genes in a hepatoma cell line (Reuber hepatoma H-35) is enhanced during growth stimulation by FCS. This enhanced transcription seemed to be coincident with a stimulated expression of *c-myc* and *c-fos* in H-35 [13]. In this hepatoma cell line, treatment with glucocorticoid was also effective in enhancing mitochondrial gene transcription. These results suggest that manipulation of culture conditions of transformed cells is an appropriate in vitro system for the analysis of the transcriptional regulation in mammalian mitochondria.

2. MATERIALS AND METHODS

MEM from Nissui Pharmaceutical Co. (Tokyo), FCS from M.A. Bioproducts (Walkersville) and dishes from Nunc (Roskilde) were used for cell culture. Dex, insulin (Sigma, St. Louis), creatine kinase, BSA (molecular biology grade; Boehringer, Penzburg), DE81 filter paper (Whatman, Maidstone), nylon membrane (Pall Ultrafine Filtration Corp., Glen Cove) and restriction enzymes (Takara, Kyoto) were used. [*methyl*-^{3}H]Thymidine (25 Ci/mmol), [^{32}P]dCTP (3000 Ci/mmol), [5,6-^{3}H]UTP (60 Ci/mmol) and [^{32}P]UTP (400 Ci/mmol) were from Amersham (Buckinghamshire). Wistar rats were from Shizuoka Laboratory Animals Agricultural Co. Assoc. For dye-binding assays of protein, a kit from Bio-Rad (Richmond) was used. All other reagents were of the highest quality commercially available.

2.1. *Cell culture*

Reuber hepatoma H-35 (H4-II-EC3) cells, provided by Dr L.I. Pizer (University of Colorado, Denver), were maintained as described [14–16]. To prepare growth-arrested cells, 30–40% confluent cultures were serum-deprived for 72 h. Synchronous growth of arrested cells was triggered by adding 10% FCS. [^{3}H]Thymidine incorporation during synchronous growth was followed by incubating the cultures on 96-well plates with 50 μl of MEM containing 1 μCi of [*methyl*-^{3}H]thymidine for 1 h. To follow cell number during synchronous growth, another series of cultures on 96-well plates were used. Synchronous culture on a 140-mm dish was prepared for each extraction of RNA. Isolation of mitochondria for in organelle transcription was started from cultures on six 140-mm dishes, which were serum-deprived for 72 h or growth stimulated by FCS for 12 h. For hormone treatment, sub-confluent cultures on 140-mm dishes were incubated in MEM containing 1 μM Dex and/or 0.2 μM insulin for 12 h. NRK cells provided by Dr K. Takimoto (Yamaguchi University, Yamaguchi) were cultured as above, except that 0.5% FCS was added to the medium for growth arresting.

2.2. *Cloning of rat mitochondrial DNA fragments*

Rat liver mitochondria were isolated by differential centrifugation [17]. DNA was extracted by the SDS-phenol method [18] and its purity was checked by agarose electrophoresis. Wistar rats used for this preparation were found to have D type of mitochondrial DNA [19]. The *Bam*HI-*Hap*II fragment spanning Cyt *b* and ND6 genes [20], *Hin*dIII-*Pvu*II fragment in Cox I gene [21], and *Hin*dIII-*Sac*II fragment in 16 S rRNA gene [22] were ligated into pUC19. [^{32}P]dCTP labeled probes were prepared by nick-translation [23] of these plasmids.

2.3. *Northern hybridization*

Total cellular RNA was extracted from H-35 cultures as described [24]. Electrophoresis of total RNA (10 μg) on 1.4% agarose gel was done after denaturation [25]. RNA was transblotted onto a nylon membrane and hybridized with [^{32}P]dCTP labeled probes as described [26].

2.4. *In organelle transcription*

Mitochondria were isolated from H-35 cultures (5×10^7 cells) by the method of Tapper et al. [27]. The reaction mixture contained isolated mitochondria (10 mg protein/ml), 40 mM Tris-HCl (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, 2 mg/ml BSA, 20% glycerol, 10 mM Na_2HPO_4, 0.2 mg/ml creatine kinase, 2 mM ATP, 1 mM CTP, 1 mM GTP, 5 mM creatine phosphate, and 4 mM pyruvate in a total volume of 50 μl. The reaction was initiated by adding carrier-free [^{32}P]UTP or [^{3}H]UTP (0.2 or 0.3 mCi/ml, respectively). To estimate background radioactivity, 0.1 mg/ml EtBr was added in control assays. An aliquot of reaction mixture corresponding to 10 μl was mixed with 1 μl of 20% SDS with 10 min intervals, spotted onto DE81 filter paper, and the radioactivity on filters remained after extensive washing was counted. For the analysis of transcripts by electrophoresis, the reaction mixtures were centrifuged and RNA was extracted from packed mitochondria as described above. After electrophoresis of denatured RNA as above, autoradiography of wet gels was taken.

3. RESULTS

H-35 is known to grow in response to insulin contained in serum. Growth of the cells is arrested in the early G_1 phase in its absence, and synchronous growth can be triggered by low concentration of insulin [28]. In order to study the regulation of mitochondrial gene expression depending on cell proliferation, we took advantage of this characteristic of H-35. When growth-arrested cells were stimulated by FCS, a sharp in-

crease in [^{3}H]thymidine incorporation was observed after 8 h, reaching a peak within 16 h (fig.1). The cell number doubled 24 h later, indicating that a cell cycle had been completed within this period (fig.1). To know the levels of RNAs encoded by mitochondrial DNA during cell proliferation, total cellular RNA (10 μg) was separated by electrophoresis and hybridized with radiolabeled DNA probes having the sequences of 16 S rRNA, Cyt *b*-ND6 and Cox I (fig.2A). A marked increase in the levels of both Cyt *b* and Cox I mRNAs was observed 6 h after the addition of FCS and they reached a plateau within 9–12 h. Densitometry of the autoradiogram showed a 5–10-fold increase. It is worth noticing that this increase was observed prior to S phase, when the cells needed a large supply of energy. Hybridization with the DNA probe coding for Cyt *b*-ND6 also gave a weak band with a migration distance corresponding to ND6 mRNA (not shown). The density of this band was also increased by growth stimulation, but the signal was too weak to quantify the increased level. Another interesting observation in fig.2A was that the level of 16 S rRNA remained unchanged. Montoya et al. showed two distinct promoters for initiating the transcription of H-strand: the upstream promoter can lead to termination at the 3′-end of the 16 S gene, while initiation from the downstream promoter results in an entire polycistronic message [29]. The results in fig.2A suggest that the initiation from the two promoters is regulated unevenly by the growth stimulation. However, an unchanged pattern of in organelle transcripts produced by mitochondria from growth-arrested and growth-stimulated cells (see below) suggested that this is not the case. The unchanged level of 16 S rRNA may reflect abundance and stability of mitochondrial rRNAs.

In experiments not shown, we estimated the levels of Cyt *b* and Cox I in NRK cells under similar conditions. In these normal cells, however, we could not observe a marked increase of these mRNAs after stimulating the cells with FCS.

In order to confirm that the increase in the levels of mRNAs coded by mitochondrial genes is due to an enhanced level of transcription, we studied the transcription in organelle employing mitochondria isolated from growth-arrested or growth-stimulated H-35. As shown in fig.2B, mitochondria from growth-stimulated H-35 had higher

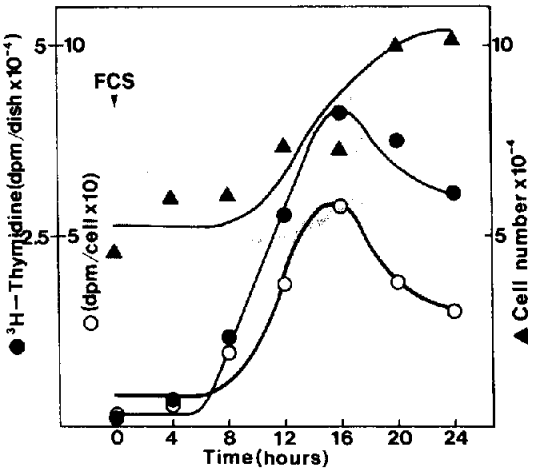

Fig.1. Synchronous growth of serum-deprived H-35 triggered by FCS addition. H-35 cells plated on 96-well plates were serum-deprived for 72 h and synchronous growth was stimulated by adding 10% FCS. Incorporated [^{3}H]thymidine (●) and cell number (▲) were counted. Incorporated [^{3}H]thymidine per cell is also indicated (○).

transcriptional activity than those from growth-arrested cells. Based on the same amount of mitochondrial protein and taking the reaction at 10 min as a comparison, the transcriptional activity was 4-fold higher in growth-stimulated cells. This result demonstrates that the increase of mitochondrial mRNAs was mainly due to enhanced transcription. On the autoradiograms of transcripts in organelle separated by electrophoresis, the density of each band increased proportionally after growth-stimulation (not shown). This result suggests that transcription from all mitochondrial promoters was enhanced in parallel depending on the growth stimulation.

Reflecting the function of hepatocytes in metabolic regulation, H-35 retains the ability to respond to many hormones. We demonstrated that the synthesis of carbamoyl-phosphate synthetase I (CPS-I) in H-35 is markedly stimulated by glucocorticoids and suppressed by insulin [14–16]. CPS-I is synthesized on cycloheximide-sensitive ribosomes [30] and is translocated into mitochondria in subsequent steps [31]. CPS-I is the major mitochondrial protein in H-35, accounting for more than 30% of total mitochondrial proteins [14]. Due to stimulated translocation of CPS-I and other enzymes into mitochondria, the amount of mitochondrial proteins of H-35 increased 1.3-fold

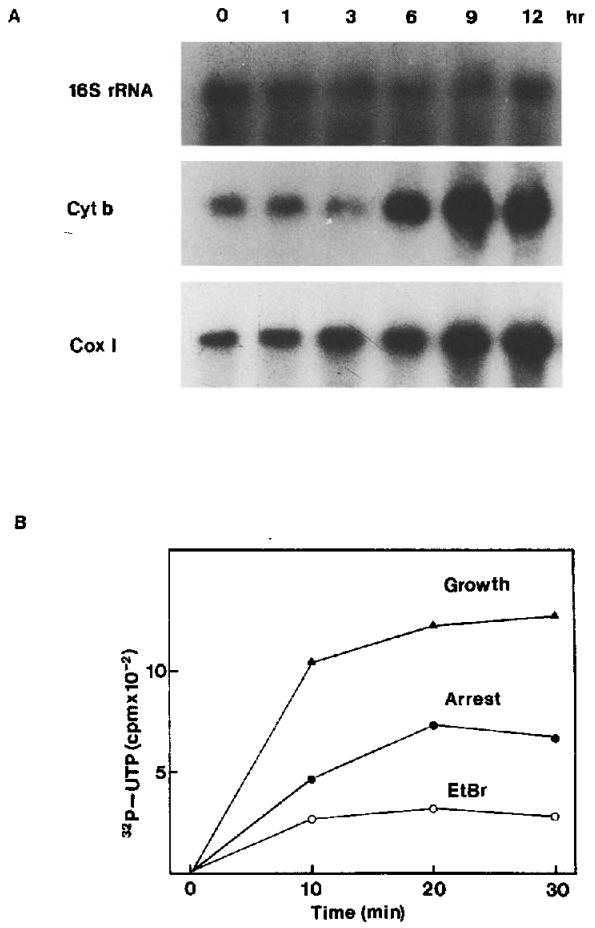

Fig.2. Enhanced transcription of mitochondrial genes by growth stimulation of H-35. (A) Total RNA was extracted from H-35 at the times indicated after growth stimulation with FCS. RNA corresponding to 10 μg was electrophoresed on a 1.4% agarose gel, trans-blotted on a nylon membrane, and hybridized with [^{32}P]dCTP-labeled probes. (B) Activity of transcription in organelle was assayed employing mitochondria isolated from growth-arrested (●) or from growth-stimulated (▲) H-35 cells. To estimate background radioactivity, 0.1 mg/ml of EtBr (○) was added to the control assay.

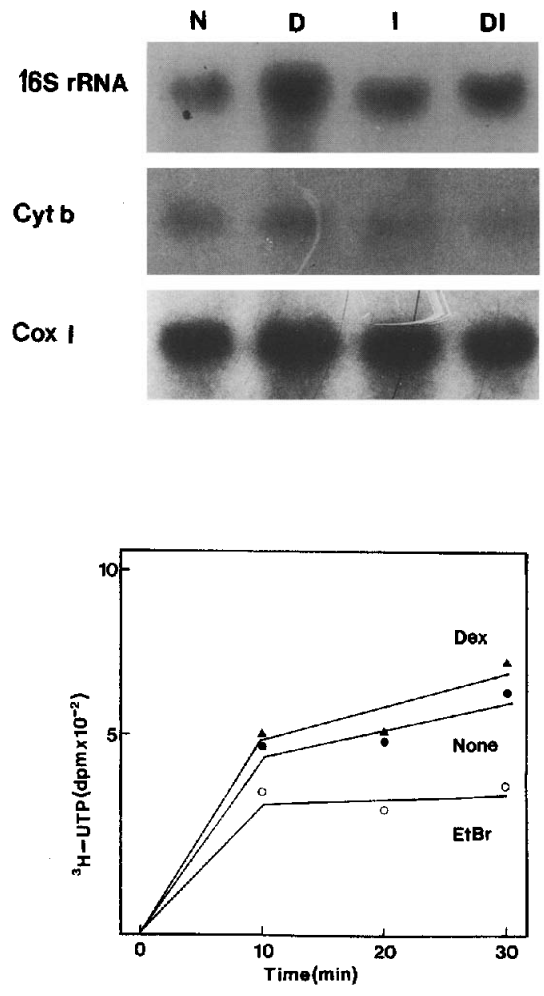

Fig.3. Effect of dexamethasone and insulin on mitochondrial gene expression in H-35. (A) Total RNA was extracted from H-35 treated with none (N), Dex (D), insulin (I), or Dex plus insulin (DI) for 12 h. (B) Activity of transcription in organelle was assayed employing mitochondria isolated from H-35 treated with none (●) or Dex (▲) for 12 h. Other details were the same as in fig.2.

after Dex treatment [14]. In such a situation, it is likely that the biogenesis of mitochondria is stimulated so as to expand their capacity of accepting these proteins. As shown in fig.3A, a slight but definite (1.5–2-fold) increase in the levels of Cyt *b* mRNA, Cox I mRNA and 16 S rRNA was observed by treating the cells with Dex for 12 h. This increase seemed to be counteracted by insulin (fig.3A). In contrast with the results after growth stimulation (fig.2A), the levels of the two mRNAs and 16 S rRNA changed virtually in parallel after hormone treatment. These results suggest a different mechanism in hormonal regulation. Based on the same amount of mitochondrial protein as a comparison, mitochondria from Dex-treated H-35 showed a slightly higher transcriptional activity in organelle than those from control cells (fig.3B). As mentioned above, Dex caused an increase of mitochondrial protein of H-35 by 1.3-fold. This suggests that the transcriptional activity per

mitochondrion is more stimulated by Dex than we see in fig.3B.

4. DISCUSSION

We found that transcription of mitochondrial genes in a hepatoma cell line is enhanced by growth stimulation. The expression of mitochondrial genes was enhanced prior to S-phase, when the cells needed a large supply of energy for proliferation. Considering the fact that initiation of L-strand transcription provides the primer for the replication from O_H [32], enhanced transcription by growth stimulation may be important for mitochondrial proliferation as well. The stimulative effect of Dex on mitochondrial gene expression may be relevant to this, because Dex stimulates translocation of large amount of proteins into mitochondria of H-35 [14], and demands biogenesis of their destination.

Possible mechanisms of enhanced transcription of mitochondrial genes are: (i) the expression of genes encoding mitochondrial RNA polymerase and/or TF is regulated at the nuclear genome level and the amount of these proteins determines the transcriptional activity after their translocation into mitochondria, or (ii) RNA polymerase and/or TF have active and inactive forms and this interconversion is somehow regulated by cellular proliferation or by the amount of proteins translocated into mitochondria. In order to explore this mechanism, we may need a cell-free transcription system from H-35 mitochondria.

In normal cells such as murine fibroblasts [11] and FR3T3 cells [12], expression of mitochondrial genes did not change remarkably depending on cell proliferation. It was also the case in our experiment with NRK cells. Glaichenhaus et al. [12] found that the steady-state level of mitochondrial mRNA encoding Cox II increased 5–10-fold in fully transformed FR3T3 cells after transfer of polyoma virus DNA, and also in immortalized cell lines established by transfer of *plt* (polyoma large T antigen), E1A (adenovirus) and *myc* oncogenes. Taub et al. [13] reported that the induction of *c-myc* and *c-fos* expression in H-35 depends on growth stimulation with insulin. It is worth noticing that the enhanced transcription of the mitochondrial gene coincides with stimulated expression of nuclear oncogenes. From this aspect, H-35 is an attractive system for analyzing interaction between nuclear and mitochondrial genomes during cell proliferation.

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REFERENCES

[1] Anderson, S., Bankier, A.T., Barrell, B.G., De Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. and Young, I.G. (1981) Nature 290, 457–465.
[2] Bibb, M.J., Etten, R.A.V., Wright, C.T., Walberg, M.W. and Clayton, D.A. (1981) Cell 26, 167–180.
[3] Anderson, S., De Bruijn, M.H.L., Coulson, A.R., Eperon, I.C., Sanger, F. and Young, I.G. (1982) J. Mol. Biol. 156, 683–717.
[4] Chomyn, A., Cleeter, M.W.J., Ragan, C.I., Riley, M., Doolittle, R.F. and Attardi, G. (1986) Science 234, 614–618.
[5] Ojala, D., Montoya, J. and Attardi, G. (1981) Nature 290, 470–474.
[6] Fisher, R.P., Topper, J.N. and Clayton, D.A. (1987) Cell 50, 247–258.
[7] Fisher, R.P. and Clayton, D.A. (1985) J. Biol. Chem. 260, 11330–11338.
[8] Clayton, D.A. (1984) Annu. Rev. Biochem. 53, 573–594.
[9] Williams, R.S., Salmons, S., Newsholme, E.A., Kaufman, R.E. and Mellor, J. (1986) J. Biol. Chem. 261, 376–380.
[10] Williams, R.S. (1986) J. Biol. Chem. 261, 12390–12394.
[11] Edwards, D.R. and Denhardt, D.T. (1985) Exp. Cell. Res. 157, 127–143.
[12] Glaichenhaus, N., Leopold, P. and Cuzin, F. (1986) EMBO J. 5, 1261–1265.
[13] Taub, R., Roy, A., Dieter, R. and Koontz, J. (1987) J. Biol. Chem. 262, 10893–10897.
[14] Murakami, A., Kitagawa, Y. and Sugimoto, E. (1983) Biochim. Biophys. Acta 740, 38–45.
[15] Kitagawa, Y. and Sugimoto, E. (1985) Eur. J. Biochem. 150, 249–254.
[16] Kitagawa, Y. (1987) Eur. J. Biochem. 167, 19–25.
[17] Kitagawa, Y., Katayama, H. and Sugimoto, E. (1979) Biochim. Biophys. Acta 582, 260–275.
[18] Koike, K., Kobayashi, M. and Fujisawa, T. (1974) Biochim. Biophys. Acta 361, 144–154.
[19] Hayashi, J., Yonekawa, H., Gotoh, O., Tagashira, Y., Moriwaki, K. and Yoshida, T. (1979) Biochim. Biophys. Acta 564, 202–211.

[20] Koike, K., Kobayashi, M., Yaginuma, K., Taira, M., Yoshida, E. and Imai, M. (1982) Gene 20, 177–185.
[21] Pepe, G., Holtrop, M., Gadaleta, G., Kroon, A.M., Cantatore, P., Gallerani, R., De Benedetto, C., Quagliariello, C., Sbisa, E. and Saccone, C. (1983) Biochem. Int. 6, 553–563.
[22] Saccone, C., Cantatore, P., Gadaleta, G., Gallerani, R., Lanave, C., Pepe, G. and Kroon, A.M. (1981) Nucleic Acids Res. 9, 4139–4148.
[23] Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237–251.
[24] Cathala, G., Savouret, J.F., Mendez, B., West, B.L., Karin, M., Martiol, J.A. and Baxter, J.D. (1983) DNA 2, 329–335.
[25] McMaster, G.K. and Carmichael, G.G. (1977) Proc. Natl. Acad. Sci. USA 74, 4835–4838.
[26] Miki, K., Sugimoto, E. and Kitagawa, Y. (1987) J. Biochem. 102, 385–392.
[27] Tapper, D.P., Van Etten, R.A. and Clayton, D.A. (1983) Methods Enzymol. 97, 426–434.
[28] Koontz, J.W. and Iwahashi, M. (1981) Science 211, 947–949.
[29] Montoya, J., Gaines, G.L. and Attardi, G. (1983) Cell 34, 151–159.
[30] Kitagawa, Y., Murakami, A. and Sugimoto, E. (1979) FEBS Lett. 102, 43–45.
[31] Kitagawa, Y., Murakami, A. and Sugimoto, E. (1984) FEBS Lett. 165, 133–137.
[32] Chang, D.D., Fisher, R.P. and Clayton, D.A. (1987) Biochim. Biophys. Acta 909, 85–91.