# Quantitation of proinsulin mRNA sequences in hamster insulinoma cells in culture by molecular hybridization[1]

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*Summary.* The content of proinsulin mRNA sequences was measured in a cultured cell line established from a transplantable hamster islet cell tumor, by hybridization with proinsulin cDNA. The cells cultured in vitro were found to contain a significant amount of proinsulin mRNA sequences, compared with non-insulin-producing hamster tissues, and seem to be a useful system for the study of insulin gene expression.

Transplantable insulinomas have now been established in hamster[3] and rat[4], but no permanent cell line which produces insulin has so far been reported. Recently, Uchida et al. found that BK virus induces islet cell tumors in the golden hamster and succeeded in establishing transplantable islet cell tumor lines[5]. From these islet cell tumors, they also established cell lines which grow rapidly in vitro without loss of transplantability. Such in vitro cell lines may be useful materials in research on insulin biosynthesis and secretion, if the cells express the genes which are directed to produce insulin.

In our laboratory, proinsulin mRNA was purified from chemically induced rat B-cell adenomas, and DNA complementary to the purified proinsulin mRNA was synthesized[6,7]. Using the cDNA as a probe, a sensitive and reliable method for the quantitation of proinsulin mRNA sequences has been established[7]. In order to clarify the question whether or not a cultured cell line established from a transplantable hamster islet cell tumor line exhibits active insulin gene expression, we undertook the present study to determine proinsulin mRNA level in cells cultured in vitro, by the nucleic acid hybridization method.

*Materials and methods.* In-111$R_1$ cells, a subclonal line in culture established from a transplantable hamster islet cell tumor, were kindly supplied by Dr Uchida, Institute of Medical Science, University of Tokyo. The culture medium consisted of RPMI-1640 (Nissui Seiyaku Co., Tokyo) supplemented by 10% fetal calf serum. Cells grew in monolayers in glass bottles and were dislodged by trypsin. In order to estimate the growth rate of the cells, replicate cultures were performed in glass test tubes. Each tube, containing $1 \times 10^5$cells in 2 ml medium, was incubated at 37 °C. Cells were scraped off with a rubber-policeman at the appropriate time, stained with 0.05% crystal violet – 2.1% citric acid and counted. As materials for transplantation, $5\text{-}10 \times 10^6$ In-111$R_1$ cells or similar amounts of dissected fragments of preceding transplants were suspended in 0.5 ml phosphate-buffered saline and implanted s.c. in the flank regions of recipient 3-week-old male golden hamsters. Tumors usually grew to the size of 1 cm in diameter within 2 weeks.

Nucleic acids were extracted as previously described[8] from In-111$R_1$ cells in culture, transplanted tumors of the same subclonal line, hamster Langerhans islets, hamster liver, hamster kidney and rat Langerhans islets. The islets were

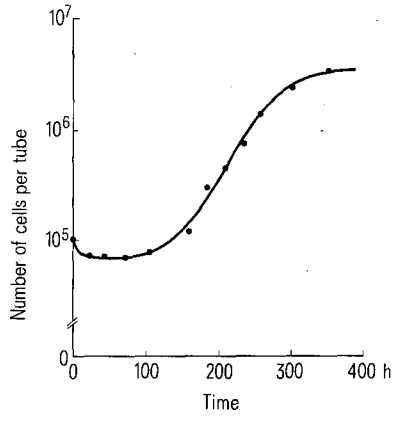


Fig. 1. Growth curve of In-111$R_1$ cells. Replicate cultures were carried out in small test tubes, each of which initially contained $1 \times 10^5$cells in 2 ml medium and was incubated at 37 °C. Cells were dislodged at the appropriate time, stained and counted. Each point indicates the cell number per tube averaged from 4 separate tubes at the given time. From the trend of the logarithmic phase, doubling time was estimated to be around 29 h.

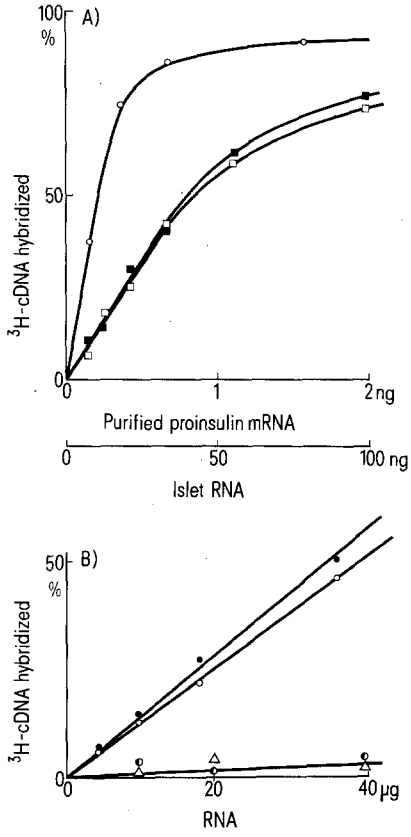


Fig. 2. Proinsulin mRNA titration with cDNA. $^3$H-proinsulin cDNA and various amounts of RNA were incubated in 50 µl of hybridization buffer for 20 h at 65 °C. $S_1$ nuclease resistant hybrids were acid-precipitated, collected on filters and counted. The content of proinsulin mRNA sequences in each RNA preparation was calculated by superimposing the data on the purified proinsulin mRNA-cDNA titration curve. *A* ○, Proinsulin mRNA; ■, rat islet RNA; □, hamster islet RNA. *B* ●, Cultured In-111$R_1$ cell RNA; ○, transplanted tumor RNA; △, hamster liver RNA; ◐, hamster kidney RNA.

isolated by the method of Okamoto et al.[9]. Total cellular RNA was prepared by LiCl precipitation from extracted nucleic acids as described by Palmiter[10] and used for the hybridization study. The proinsulin cDNA was synthesized with avian myeloblastosis virus reverse transcriptase, $^3$H-dCTP and purified rat proinsulin mRNA[6]. The method of hybridization was adapted from the procedure of Monahan et al.[11]. $^3$H-cDNA and an appropriate amount of RNA were mixed in hybridization buffer containing 0.5 M NaCl, 1 mM EDTA, 25 mM HEPES-NaOH, pH 7.3, 0.1% sodium dodecyl sulfate, 5 µg sonicated yeast DNA and 5 µg yeast RNA. The hybridization reaction was carried out in a final volume of 50 µl. Samples were initially placed at 100 °C for 2 min and then incubated at 65 °C for 20 h. To assay the extent of hybridization, 0.4 ml of a solution containing 0.4 M NaCl, 0.2 M sodium acetate, pH 4.5, 2 mM $Zn(OAc)_2$ and 200 units of $S_1$ nuclease (Sigma, St. Louis) was added to each reaction mixture and incubated at 37 °C for 1 h. The $S_1$ resistant hybrids were then precipitated with cold 10% trichloroacetic acid, collected on millipore filters and processed for scintillation counting.

*Results and discussion.* The growth curve of In-111$R_1$ cells obtained from the replicate culture is shown in figure 1. The logarithmic phase of 150–200 h duration was followed by a stationary phase at a density of $1.5–2.0 \times 10^6$ cells/ml. Doubling time was estimated to be around 29 h. The ability of the cells for in vitro proliferation was marked; this was compatible with the rapid tumor formation following transplantation described in materials and methods section.

Based on the titration curves of cDNA-RNA hybridization shown in figure 2, the content of proinsulin mRNA was determined in each RNA preparation; 0.0050 ng/ng in rat islet RNA, 0.0048 ng/ng in hamster islet RNA, 0.0073 ng/µg in In-lll$R_1$ cell RNA, 0.0067 ng/µg in transplanted tumor RNA and below 0.0003 ng/µg in both hamster liver and kidney RNAs.

The content of proinsulin mRNA sequences in rat and hamster islet RNAs was similar when determined with rat proinsulin cDNA as a probe, suggesting that there exists considerable homology in base sequences between rat and hamster proinsulin mRNAs. Moreover, it seems evident that rat proinsulin cDNA used in the present study can be employed as a nucleic acid probe for proinsulin mRNA estimation in cells or tissues from a certain range of mammalian species including hamster.

In-111$R_1$ cells were found to contain a significant amount of proinsulin mRNA sequences in comparison with the control (hamster liver and kidney), though the level was as low as about 1/700 of the level in Langerhans islets in which proinsulin mRNA is considered to occupy the majority of the whole mRNAs[6,8]. Additionally, the proinsulin mRNA content was similar in tumors developed in recipient hamsters from implanted In-111$R_1$ cells and in the cells cultured in vitro, suggesting that the tumors have homogeneously grown from the implants and that the cells express insulin gene in a similar degree both in vivo and in vitro.

Humphries et al. reported that low amounts of globin mRNA sequences were encountered in non-erythroid tissues (mouse brain and liver, and some kinds of cultured cells originated from non-erythroid tissues)[12]. In the present study, hamster liver and kidney RNAs were used as a control in the molecular hybridization study and found to contain no detectable level of proinsulin mRNA sequences, even at the RNA/cDNA ratio of $7 \times 10^5$, as high as that employed by Humphries et al. It is considered that there exist essentially no proinsulin mRNA sequences in non-insulin-producing tissues. Therefore, even though it is very low, the level of proinsulin mRNA in In-111$R_1$ cells is significant.

Our finding of a low proinsulin mRNA content in the cells may be compatible with the observation by Okeda et al. that In-111$R_1$ cells secreted much less insulin than normal islets of Langerhans and had few B-granules on an electronmicrogram[13]. They also studied the secretion of insulin from the cells in the presence of some chemicals which are known to stimulate insulin secretion from normal islets. Among the agents tested, theophylline and tolbutamide stimulated insulin release from In-111$R_1$ cells, though to a smaller degree than from normal islets, whereas glucose and other agents did not increase the amount of insulin secreted[13]. We studied the effects of 3-isobutyl-1-methylxanthine, a compound closely related to theophylline, dibutyryl-cAMP and glucose on the proinsulin mRNA level in In-111$R_1$ cells by incubating the cells for several hours with these chemicals, and found that the level was unaffected by any of them (data not shown).

Despite the fact that the original tumors had considerable amounts of immunoreactive insulin and were well-granulated[5], the established cell line was found to have a decreased function of insulin production. Among the original tumor cells, presumably only those with low insulin-synthesizing and secreting ability may have grown in culture conditions, and have been selected in the processes of serial cloning.

The recent development of cell-free translation systems has revealed the details of insulin biosynthesis at the translation level[7,8]. On the other hand, little information is available at the level of transcription. In the present study, In-111$R_1$ cells were found to grow rapidly in vitro and contain low but significant amounts of proinsulin mRNA sequences. Such an in vitro insulinoma cell line may provide clues toward elucidating the conditions under which the insulin gene operates.

1 This work was supported in part by Grants-in-Aid Scientific Research (248129, 387065, 337013, 378052) and a Grant-in-Aid for Cancer Research (301538) from the Ministry of Education, Science and Culture, Japan.

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3 T.A.I. Grillo, A.J. Whitty, H. Kirkman, P.P. Foa and S.D. Kobernick, Diabetes *16*, 409 (1967).
4 W.L. Chick, S. Warren, R.N. Chute and V. Lauris, Diabetes *25*, suppl. 1, 344 (1976).
5 S. Uchida, S. Watanabe, T. Aizawa, K. Kato, A. Furuno and T. Muto, Gann *67*, 857 (1976).
6 N. Itoh, K. Nose and H. Okamoto, Eur. J. Biochem. *97*, 1 (1979).
7 H. Okamoto, K. Nose, N. Itoh, T. Sei and H. Yamamoto, in: Proinsulin, Insulin and C-Peptide, p. 27. Ed. S. Baba, T. Kaneko, A.H. Rubenstein, D.F. Steiner and N. Yanaihara. Excerpta Medica, Amsterdam 1979.
8 N. Itoh, T. Sei, K. Nose and H. Okamoto, FEBS Lett. *93*, 343 (1978).
9 H. Okamoto, Y. Noto, S. Miyamoto, H. Mabuchi and R. Takeda, FEBS Lett. *54*, 103 (1975).
10 R.D. Palmiter, Biochemisty *13*, 3606 (1974).
11 J.J. Monahan, S.E. Harris, S.L.C. Woo, D.L. Robberson and B.W. O'Malley, Biochemistry *15*, 223 (1976).
12 S. Humphries, J. Windass and R. Williamson, Cell *7*, 267 (1976).
13 T. Okeda, J. Ono, A. Todo and R. Takaki, Proceedings of the Japanese Cancer Association, 37th Annual Meeting, p. 106. 1978.