PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 170, 155–159 (1982)

Effect of Temperature on the Production of Human Fibroblast Interferon (41411)

DONALD J. GIARD,*[,1] ROBERT J. FLEISCHAKER,† AND MICHELE FABRICANT*

*Cell Culture Center, and Department of †Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. The effect of temperature upon interferon production in human fibroblasts was examined. Kinetic studies showed that exposing the cells to 37° for one hour before lowering the temperature to 30° resulted in a more prolonged period of production in comparison to cultures maintained at either 37° or 30°. These data suggest that a temperature-dependent event early in the production phase is responsible for the increased rates of synthesis in the later stages of production. The addition of cycloheximide (10 μg/ml) to the culture medium during the 37° period did not shorten the production period indicating that the extended period of production does not depend upon protein synthesis. In view of the improved yields observed, these studies may have applications to large-scale interferon production.

A number of studies (1–4) have shown that the successful induction of human fibroblast interferon using a synthetic inducer in combination with various antimetabolites ("super-induction") is dependent upon careful control of certain critical parameters. These parameters include concentration of and the duration of exposure to antimetabolites, previous exposure to interferon (priming), and temperature. In our own work (2), we have shown that interferon production using cells grown in microcarrier culture is dramatically influenced by temperature and priming.

Since reduction in temperature during the production phase of interferon synthesis has been shown to increase interferon yields (5), we felt it would be of value to examine in greater detail the relationship between temperature and rates of interferon synthesis.

Materials and Methods. Human diploid foreskin cells (FS-4) were obtained from Dr. Jan Vilček, New York University School of Medicine, New York, and were used in all experiments. Cells were obtained at approximately the 18th population doubling. They were frozen in one batch for the entire series of experiments and were used between the 30th and 40th population doublings. Stock cultures were maintained in Corning plastic roller bottles in a walk-in incubator at 37°, and microcarrier cultures were seeded from these stocks. Cultures were seeded onto microcarriers in either 2-liter spinner vessels (Wheaton Scientific) in a 1-liter volume or a 14-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) in 5-liter volumes. The cell inoculum ranged from 3 to 4 × 10^5 cells/ml. A microcarrier concentration of 5 mg/ml was used and cultures were incubated in a humidified incubator supplied with 10% CO_2. Growth medium for stock cultures consisted of Dulbecco's modified Eagles medium (DMEM) (Flow Laboratories, Inc. Rockville, Md.) supplemented with 10% fetal bovine serum (Sterile Systems, Inc., Logan, Utah). For microcarrier growth the serum concentration of the DMEM was reduced to 5%. Antibiotics used were penicillin (100 units/ml) and streptomycin (100 μg/ml), which were obtained from Sigma Chemical Company, St. Louis, Missouri. Cultures were split using a trypsin–EDTA solution (Grand Island Biological Co., Grand Island, N.Y.).

The procedure for microcarrier preparation has been described previously (6). The initiation of microcarrier cultures consisted of the following: Microcarriers were suspended in phosphate-buffered saline (PBS) at a concentration of 10 mg/ml and steril-

[1] To whom correspondence should be addressed.

0037-9727/82/060155-05$01.00/0

ized in glass bottles by autoclaving. Microcarriers were washed twice with PBS and once with growth medium and then dispensed into spinner flasks containing growth medium to give a final concentration of 5 mg/ml.

The superinduction procedure used was a modification of the method reported by Havell and Vilček (3). The modified procedure, developed in our laboratory, has recently been described (2). For most experiments cells were grown to confluency on microcarriers in a 1-liter volume to a density of approximately 1×10^6 cells/ml. On the fifth or sixth day cells were washed two times with DMEM and transferred in 50-ml aliquots to 250-ml spinner vessels (Wilbur Scientific, Inc., Boston, Mass.). Cells were then primed at 37° for 16 hr in DMEM + 0.5% Plasmanate (Daly Hospital Supply, Lynnfield, Mass.) + 50 units/ml of fibroblast interferon. Cells were then washed two times with DMEM after which 50 ml of DMEM containing 50 μg/ml of poly I·poly C (PL Biochemical Co., Milwaukee, Wisc.) and 10 μg/ml of cycloheximide (Sigma Chemical Co.) were added. After 4 hr of incubation, actinomycin D (Sigma Chemical Co., St. Louis, Mo.) was added to give a concentration of 1 μg/ml. After 1–2 hr of further incubation, the medium was removed and the cells were washed two times with DMEM. DMEM + 0.5% Plasmanate was then added and cultures were incubated for 24 to 48 hr. In this study, a variety of temperatures was used during both induction and production. Culture fluids were collected, clarified by centrifugation at 200 rpm for 10 min, and either assayed immediately or frozen at −70° until assayed. The procedure for interferon assay has been described elsewhere (3).

Cells in microcarrier cultures were enumerated by counting nuclei, using a modification of the method of Sanford *et al.* (7) as described by VanWezel (8). Cells in roller bottles were dispersed with a trypsin–EDTA solution and counted with a hemacytometer. Cultures were thoroughly screened for the presence of mycoplasma by the culture method (isolation of PPLO colonies on artificial media), the uridine–uracil assay described by Schneider *et al.* (9), and the DNA staining method reported by Russell *et al.* (10). All results were negative.

Results and Discussion. The kinetics of interferon production were examined using different combinations of temperatures (Figs. 1A and B). These data showed that the rate of interferon synthesis increased rapidly at the beginning of the production phase, reached a peak within 4 to 6 hr and subsequently decreased. When the temperature was held constant (34°) during production but was varied during induction (30, 34 and 37°) it was noted that the initial rate of synthesis was substantially lower at 30° compared to either 34° or 37°. However, the rate of decline of interferon synthesis was approximately the same at all temperatures, and production was essentially complete by 16 hr suggesting that the rate of synthesis was controlled by the temperature during production. Figure 1B shows the kinetics of interferon production when the temperature was held constant (34°) during induction and was varied during the production phase (30, 34, and 37°). The highest rate of initial synthesis (approximately 6000 units/ml·hr), was observed at 37° and this was approximately threefold higher than the initial rates observed at 30 and 34°. Although a rapid decline in the rate of production was observed at 34 and 37°, the rate of decline at 30° was more gradual and production was still substantial after 24 hr (400 units/ml·hour). These kinetics experiments suggested that the use of a higher temperature (37°) for a short period at the beginning of the production phase followed by a lower temperature for the duration of the production period, might result in a high initial rate of synthesis which could be sustained for a longer period at the lower temperature.

Further kinetic studies were done to determine the effect of such a shift in temperature during the production phase. Results are shown in Fig. 2. When 37° was maintained throughout the production period, a peak of approximately 2000 units/ml·hr was reached at 5 hr, after which a rapid de-

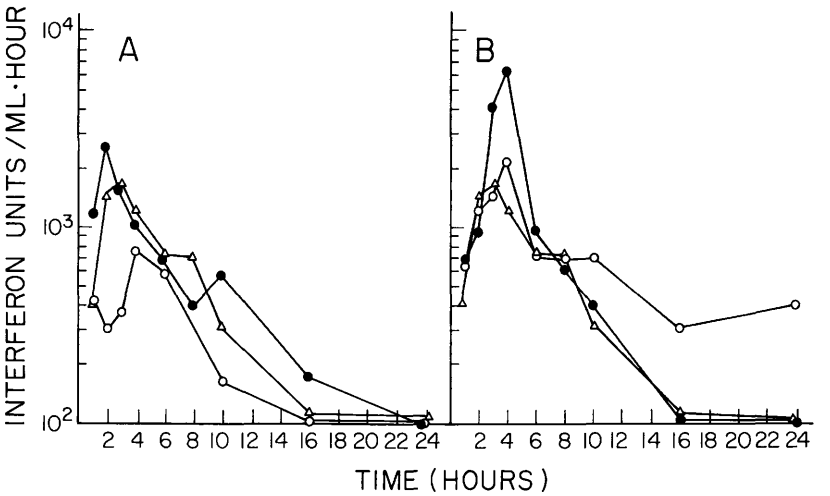

FIG. 1. Kinetics of interferon production using different temperatures during induction and production. (A) Induction. Duplicate cultures of FS-4 cells were primed for 16 hr at 37° with 50 units/ml of fibroblast interferon then induced at temperatures of 30° (○), 34° (△), and 37° (●). After inducer and antimetabolites were removed and production medium (DMEM + 0.5% Plasmanate) added, the temperature was held at 34° throughout the production period. At each of the indicated times, medium was removed from each culture vessel and fresh production medium was added. (B) Production. Culture conditions were identical to those described in (A) except that the temperature was held at 34° during induction and cultures were maintained at 30° (○), 34° (△), and 37° (●) during production.

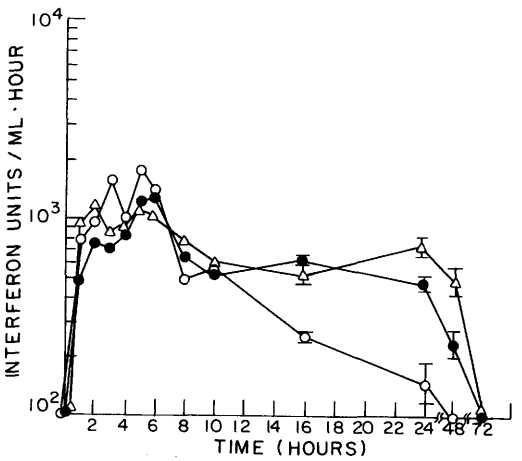

FIG. 2. Kinetics of interferon production using temperature shift during production phase. Duplicate cultures of FS-4 cells were primed for 16 hr at 37° with 50 units/ml interferon, then induced at 34°. After inducer and antimetabolites were removed and production medium (DMEM + 0.5% Plasmanate) added, the temperature was held at 37° for 1 hr, then shifted to 30° for the remainder of the production period (△). Control cultures were held at either 30° (●) or 37° (○) during the entire production period.

cline occurred in the production rate. When cultures were either maintained at 30° throughout production, or exposed to 37°, for 1 hr, followed by a shift in temperature to 30°, the production rate rose to a level slightly less than the 37° cultures, then declined less rapidly. Notably, cultures at 30° which had initially received a short exposure at 37° showed an extended period of production compared to when 30° was maintained throughout production.

In order to determine whether the extended period of production was dependent upon protein synthesis during the 37° period, the following experiment was performed: cultures were induced using standard conditions after which cycloheximide (at concentrations of either 1 or 10 μg/ml) was added during the 1 hr 37° period. Cultures were then washed, and fresh production medium was added. The temperature was maintained at 30° for the remainder of the production period. We have demonstrated (data not shown) by measuring the incorporation of [^{14}C]leucine, that concen-

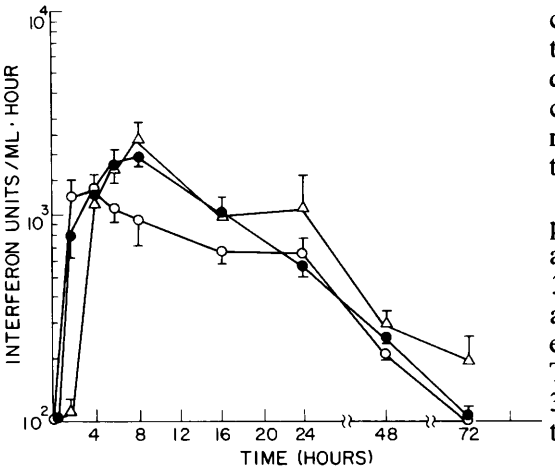

FIG. 3. Kinetics of interferon production using different concentrations of cycloheximide during the initial 37° period. Following the induction period cells were washed twice with DMEM and production medium (DMEM + 0.5% Plasmanate) containing either 0 (○), 1 (●), or 10 μg/ml (△) of cycloheximide was added. Cultures were incubated at 37° for 1 hr, after which culture fluids were removed and replaced with fresh production medium. Cultures were then incubated at 30° for the remainder of the production period. At the indicated times fluids were removed, samples were collected for assay, and fresh production medium was added.

trations of 1 and 10 μg/ml of cycloheximide inhibit >80% and >95%, respectively, of protein synthesis in FS-4 cells. Results (Fig. 3) showed that the presence of cycloheximide did not shorten the period of production relative to control cultures containing no cycloheximide, indicating that protein synthesis is apparently not required during the 37° period. In fact, at both concentrations of cycloheximide, higher rates of synthesis were observed between the 4th and 16th hr of production.

The effect of switching to different temperatures from the initial 37° period was also determined. Cultures were induced at 34°, production was initiated at 37°, and after 2 hr, the temperature was lowered to either 34, 30, or 25°. The data shown in Table I indicate that a shift to either 34 or 30° results in higher yields of interferon than either no shift or a shift to 25°.

In another study the optimal time for temperature reduction was examined. Cultures were induced at 34° and either held at 30° or 37°, or switched from 37° to 30° after 1, 2, 3, and 4 hr. As shown in Table II, higher yields were obtained using the temperature shift in comparison to control cultures at either 30° or 37°. It appears from these data that although the shift itself is important, the actual time of shift is not particularly critical.

It has previously been reported (2, 5, 11, 12) that a reduction in temperature throughout the production period results in higher yields of interferon and we have confirmed these results. One possible explanation for the effects of temperature reduction is that interferon mRNA is more stable at lower temperatures. A reduction in temperature could also exert a stabilizing effect by interfering with an inhibitor which inactivates

TABLE I. EFFECT ON INTERFERON YIELDS OF SWITCHING TO DIFFERENT TEMPERATURES DURING PRODUCTION PHASE[a]

Temperature conditions (°)		
Induction	Production	Interferon yield (units/ml)[b]
34	37	28,000 ± 10,000
34	37 → 34	43,000 ± 4,000
34	37 → 30	49,400 ± 0
34	37 → 25	22,000 ± 10,000

[a] Induction was carried out using standard procedures described under Methods. Cells were exposed to actinomycin D for 1 hr, the temperature switch was made after 2 hr and samples were collected for assay after 24 hr.

[b] Data represent the average values and the standard deviation of two samples assayed in duplicate. Since the cell density was 1×10^6 cells/ml, the data also refer to units per 10^6 cells.

TABLE II. DETERMINATION OF OPTIMAL TIME OF TEMPERATURE SHIFT DURING PRODUCTION PHASE[a]

Temperature conditions (°)		
Induction	Production	Interferon yield (units/ml)
34	37	15,200 ± 4400
34	37 → 30 (1 hr)	41,700 ± 4200
34	37 → 30 (2 hr)	37,100 ± 7600
34	37 → 30 (3 hr)	28,100 ± 12,100
34	37 → 30 (4 hr)	36,400 ± 12,700
34	30	12,400 ± 4500

[a] Induction was carried out using standard procedures described under Methods. Cells were exposed to actinomycin D for 1 hr and interferon samples assayed in duplicate. Since the cell density used was 1×10^6 cells/ml, data also refer to units per 10^6 cells.

interferon mRNA or by decreasing the rate of cellular deterioration, thereby allowing production over a longer period. In addition, it is evident from these studies that some event early in the production period influences the rate and duration of interferon synthesis. This effect is enhanced by an increase in temperature and does not appear to involve protein synthesis.

This work was supported by National Science Foundation Grant PCM 78-19497 and the Lewis and Rosa Strauss Memorial Fund. Robert Fleischaker was supported by NCI Training Grant 1-T32 CA09258-01.

1. Billiau A, Joniau M, Desomer P. Mass production of human interferon in diploid cells stimulated by poly I·poly C. J Gen Virol 19:1–8, 1973.
2. Giard DJ, Fleischaker RJ. Examination of parameters affecting human interferon production with microcarrier-grown fibroblast cells. Antimicrob Agents Chemother 18:130–136, 1980.
3. Havell EA, Vilček J. Production of high titered interferon in cultures of human diploid cells. Antimicrob Agents Chemother 2:476–484, 1972.
4. Tan YH, Armstrong JA, Ke YH, Ho M. Regulation of cellular interferon production enhancement by antimetabolites. Proc Nat Acad Sci USA 67:464–471, 1970.
5. Vilček J, Havell EA. Stabilization of interferon mRNA activity by treatment of cells with metabolic inhibitors and lowering of the incubation temperature. Proc Nat Acad Sci USA 70:3909–3913, 1973.
6. Levine DW, Wong JS, Wang DIC, Thilly WG. Microcarrier cell culture: New methods for research-scale application. Somat Cell Genet 3:149–155, 1977.
7. Sanford JK, Earle WR, Evans VJ, Waltz HK, Shannon JE. The measurement of proliferation in tissue cultures by enumeration of cell nuclei. J Nat Cancer Inst 11:773–795, 1951.
8. VanWezel AL. Microcarrier cultures of animal cells. In: Kruse PF, Patterson MK, eds. Tissue Culture Methods and Applications. New York, Academic Press, p372, 1973.
9. Schneider EL, Stanbridge EJ, Epstein CJ. Incorporation of ^{3}H-uridine and ^{3}H-uracil into RNA: A simple technique for the detection of mycoplasma contamination of cultured cells. Exp Cell Res 84:311–318, 1974.
10. Russell WC, Newman S, Williamson DH. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasma. Nature (London) 253:461–462, 1975.
11. Sypula A, Zielinska-Jenczylik J. Production of interferon in human fibroblast cultures:effect of temperature. Arch Immunol Ther Exp 26:499–501, 1978.
12. Kojima Y, Yoshida F. Enhanced production of interferon by temperature shift-down from 37°C to 25°C in rabbit cell cultures stimulated with Newcastle disease virus. Japan J Microbiol 18:217–222, 1974.

Received September 2, 1981. P.S.E.B.M. 1982, Vol. 170.