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INHIBITION BY INTERFERON OF BIOCHEMICAL TRANSFORMATION INDUCED BY CLONED HERPESVIRUS THYMIDINE KINASE GENES

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To learn whether interferon could prevent the biochemical transformations induced by cloned herpesvirus thymidine kinase (TK) genes, LM(TK$^-$) mouse fibroblast cultures were pretreated for 24 h with 2.4–40 international units (I.U.)/ml mouse $\alpha + \beta$ interferon, and subsequently transformed to the TK$^+$ phenotype with recombinant plasmids containing the herpes simplex virus type 1 (HSV-1) TK gene (pAGO and pMH110) and the marmoset herpesvirus (MarHV) TK gene (pMAR035). Mouse $\alpha + \beta$ interferon inhibuted transformation and the inhibition was interferon dose-dependent. Transformation was also inhibited when LM(TK$^-$) cells were pretreated for 2 – 5 h with 40 I.U./ml interferon. Maximal inhibitions of TK$^+$ colony formation were observed following a 9–20 h pretreatment period with interferon. In contrast, 40 I.U./ml interferon treatment for 20 h did not reduce the rate or extent of LM(TK$^-$) cell growth. Experiments in which cultures were first treated with plasmid pAGO and only afterwards treated with interferon also showed that, as the interferon concentration used, interferon did not inhibit the outgrowth of transformated colonies. Enzyme assays showed that pretreatment with interferon inhibited the induction of TK activity in cells that had been transfected with pAGO DNA.

biochemical transformation interferon thymidine kinase herpes simplex virus hybrid plasmids LM(TK$^-$) cells

INTRODUCTION

Recent studies showing that interferon blocks the biochemical transformation of thymidine kinase (TK)-deficient mouse cells by UV-inactivated herpes simplex virus type 2 (HSV-2) [23] suggested that the inhibitory effects of interferon might be partially due to the suppression of HSV TK gene expression. However, other indirect effects on the biochemical transformation process could not be excluded. When transformation of TK$^-$ cells to TK$^+$ is mediated by the entire HSV genome, a function required for viral DNA synthesis may also be required to obtain stable expression or to transfer the TK$^+$ gene, or both [24]. In lytic infections, 'immediate early' gene products are required for the ex-

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pression of 'early' genes, such as TK [9,16]. Thus, during transformation by UV-inactivated virus, immediate early genes may be targets for interferon action.

The herpesvirus TK gene has an important role in establishing and/or maintaining herpesvirus latency in vivo [3,15,20,22,28–30]. To assess the effects of interferon on the functional expression of the herpesvirus TK gene, per se, we have now studied the TK induction and the biochemical transformation of TK^- mouse fibroblast cells by cloned herpesvirus TK genes. In cells biochemically transformed by hybrid plasmids, the herpesvirus TK genes are expressed constitutively, although TK expression can often be enhanched by superinfection of transformed cells with TK^- herpesvirus mutants [9,32]. The data to be presented demonstrate that i) interferon can inhibit induction of TK enzyme activity in transfected cells, and ii) interferon can indeed block biochemical transformations by plasmids containing HSV-1 and MarHV TK genes.

MATERIALS AND METHODS

Cells

Bromodeoxyuridine (BrdUrd)-resistant LM(TK^-) mouse fibroblast cells [8] were propagated in Eagle's minimal essential medium (APMEM, Flow Laboratories, Rockville, MD) supplemented with 10% calf serum and with 25 μg/ml BrdUrd, except that BrdUrd was omitted from the medium in the passage immediately preceding an experiment.

Plasmid DNAs

Plasmid DNAs were isolated from antibiotic-resistant *Escherichia coli* K-12 cells and purified by sucrose gradient centrifugation as described [11,12,14,19]. Hybrid plasmid pAGO, a derivative of pBR322, contains a 2 kbp PvuII fragment of HSV-1 DNA inserted at the pBR322 PvuII site [2]. Plasmid pMH110 is a deletion mutant derived from pAGO [12,14] by eliminating the 1689 bp pBR322 nucleotide sequence of pAGO, extending from the BamHI to the PvuII cleavage site, and the 250 bp HSV-1 nucleotide sequence of pAGO, extending from the PvuII to the BglII cleavage site which contains the promoter region of the HSV-1 TK gene [4,18,31]. Plasmids pAGO and pMH110 can transform LM(TK^-) cells to the TK^+ phenotype, indicating that both plasmids contain the functional coding region of the HSV-1 TK gene. Plasmid pMAR035 contains a 3 kbp PstI fragment of marmoset herpesvirus (MarHV) DNA which codes for MarHV TK. pMAR035 is identical to pMAR034 [19], except that the 3 kbp PstI fragment of MarHV DNA is inserted at the PstI cleavage site of plasmid pKH47 rather than pBR322. Plasmid pKH47 is a derivative of pBR322 containing an oligo(dA-dT) homopolymer sequence at the PvuII cleavage site of pBR322 [6].

Interferon preparations

Mouse $\alpha + \beta$ interferon (25,000 I.U./ml, 8000 I.U./mg protein) was prepared from Newcastle disease virus-Bl-infected mouse L929 cell cultures and was generously supplied by S. Baron, University of Texas Medical Branch, Galveston, TX. Mock interferon

preparations were prepared from the culture fluid of uninfected L929 cells. Human lymphoblastoid interferon (HuIFN-α; 1.1 X 10^6 I.U./mg protein) was purchased from Sigma Chemical Co., St. Louis, MO. The human interferon was produced in Burkitt lymphoma cells (Namalva) by induction with Sendai virus.

Growth of interferon-treated LM(TK$^-$) cell cultures

Three day old cultures of actively growing LM(TK$^-$) mouse fibroblast cells in 8 oz prescription bottles were pretreated for 24 h with mouse α + β interferon (or mock interferon), trypsinized, and planted at 5 X 10^5 cells/dish in 60 mm plastic Petri dishes. Five ml medium (APMEM supplemented with 10% calf serum) was added and the cultures were incubated for 5 days at 37°C, with media changes on days 1 and 3 after planting. To measure cell growth, replicate dishes were trypsinized and the number of cells per dish was determined using a Coulter counter.

Biochemical transformation experiments

LM(TK$^-$) mouse fibroblast cells were biochemically transformed with DNAs from plasmids pAGO, pMH110, or pMAR035 (plus carrier LM(TK$^-$) cell DNA) as described previously [10,13]. To study the effects of interferon on the biochemical transformation process, the following standard procedure was used. Three day old cultures of LM(TK$^-$) cells in 8 oz prescription bottles were treated for 24 h with mock interferon or mouse α + β interferon (2.5–40 I.U./ml), trypsinized, and planted in 60 mm Petri dishes at 5 X 10^5 cells/dish. Five ml APMEM plus 10% calf serum were added and the cultures were incubated for 24 h at 37°C. Then, 0.5 ml calcium phosphate-plasmid DNA precipitate containing 20–40 ng of restriction nuclease-cleaved plasmid DNA/dish was added for 5h; the precipitate was removed; 5 ml of fresh medium were added; and the cultures were further incubated 24 h at 37°C, at which time the medium was changed to HATG (hypoxanthine, 10^{-5} M; aminopterin, 10^{-6} M; thymidine, 4 X 10^{-5} M; glycine, 10^{-5} M) selective medium. Incubation in HATG medium was continued for 2–3 weeks with media changes every 3 days. The colonies in the dishes were fixed with ethanol, stained with Giemsa, and counted.

RESULTS

Interferon inhibition of plaque formation and biochemical transformation

In preliminary experiments, LM(TK$^-$) mouse fibroblast cells were pretreated for 24 h with 40 I.U./ml mouse α + β interferon. The cells were then infected with serial dilutions MarHV DNA (0.21 and 0.42 μg DNA/dish), or with plasmid DNAs. The effects of interferon on virus plaque formation and on the biochemical transformation of LM (TK$^-$) cells to the TK$^+$ phenotype were assayed. These experiments showed that 24 h pretreatment of LM(TK$^-$) cells with 40 I.U./ml interferon reduced plaque formation by virus particles and by infectious viral DNA by 83% or more, and inhibited biochemi-

cal transformation by pAGO and pMH110 DNAs by more than 90%. Control experiments demonstrated that biochemical transformation was not inhibited when mouse cell cultures were pretreated with a mock interferon preparation, with human lymphoblast interferon 40–160 I.U./ml), or with mouse $\alpha + \beta$ interferon which had been predigested with trypsin to hydrolyze the proteins.

Fig. 1 shows that the inhibition of biochemical transformation resulting from mouse $\alpha + \beta$ interferon pretreatment of LM(TK$^-$) cells was concentration dependent. Regardless of whether the biochemical transformations were mediated by plasmids pAGO or pMH110, which contain the coding sequences for the HSV-1 TK gene, or by pMAR035, which contains the MarHV TK gene, the transformation process was inhibited by pretreatment of the cells with interferon. Inhibition was detectable after pretreatment of cells with 2.5 I.U./ml mouse $\alpha + \beta$ interferon. At 20–40 I.U./ml interferon, the inhibitions increased to 80–90%.

To ascertain the minimum interferon pretreatment time required to inhibit biochemical transformation, the experiments depicted in Fig. 2 were carried out. Biochemical transformation of LM(TK$^-$) cells by pAGO DNA was partially inhibited when the cultures were pretreated for only 2 h with 40 I.U./ml mouse $\alpha + \beta$ interferon. The inhibi-

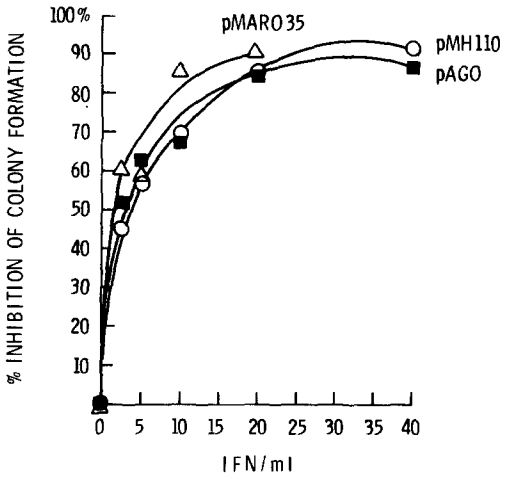

Fig. 1. Effect of pretreatment of LM(TK$^-$) cells with different concentrations of interferon on the biochemical transformation of the cells by plasmids pAGO, pMH110, and pMAR035. LM(TL$^-$) cell cultures were pretreated for 20 h with mouse $\alpha + \beta$ interferons at the concentrations shown in the figure, trypsinized, and planted at 5 X 10^5 cells/dish in 60 mm Petri dishes. 24 h later calcium phosphate-plasmid DNA precipitates were added to the cultures and the biochemical transformation of LM(TK$^-$) cells to the TK$^+$ phenotype was assayed. For biochemical transformation the LM(TK$^-$) cell cultures were treated with 20 ng/dish of PvuII-cleaved pAGO DNA, 40 ng/dish of HindIII-cleaved pMH110 DNA, or 20 ng/dish of HindIII-cleaved pMAR035 DNA. The control dishes without interferon showed 184 colonies/dish, 302 colonies/dish, and 78 colonies/dish, respectively, for the pAGO-, pMH110-, and pMAR035-transfected cells.

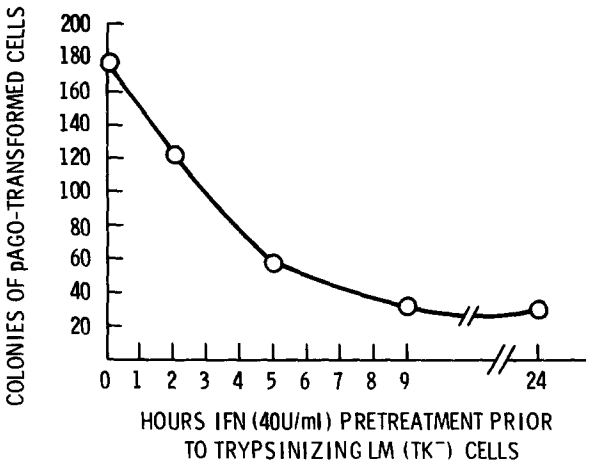

Fig. 2. Effect of duration of pretreatment of LM(TK$^-$) cells with mouse $\alpha + \beta$ interferon on the biochemical transformation of the cells to the TK$^+$ phenotype by hybrid plasmid pAGO DNA. LM(TK$^-$) cell cultures in 8 oz prescription bottles were pretreated with interferon for the times shown in the figure, trypsinized, and planted at 5×10^5 cells/dish in 60 mm Petri dishes. 24 h later calcium phosphate-plasmid pAGO DNA precipitate was added to the cultures and the biochemical transformation of the LM(TK$^-$) cells to the TK$^+$ phenotype was assayed.

tions increased to over 80% as the pretreatment period was increased to 5 and 9 h. The inhibition of biochemical transformation obtained by 24 h pretreatment of cells was about the same as that obtained with 9 h pretreatment.

Post-treatment of cells with interferon

The preceding experiments demonstrated that interferon treatment of cells *prior* to the addition of hybrid plasmid DNA inhibited the formation of colonies of biochemically transformed cells. To learn whether the addition of interferon *after* the addition of hybrid plasmid DNA also inhibited the formation of transformed TK$^+$ colonies, the experiments shown in Table 1 were carried out. Cultures of LM(TK$^-$) cells were pretreated with mock interferon for 24 h, trypsinized, and planted in plastic Petri dishes at 5×10^5 cells/dish. Twenty-four hours later, pAGO DNA was added to the dishes as a calcium phosphate precipitate for 5 h. The medium was then changed to fresh growth medium and incubation of the cultures was continued. At 24, 48, or 96 h after the addition of the pAGO DNA to the cells, selective HATG medium plus mouse $\alpha + \beta$ interferon was added. The cultures were incubated for *12 more days* and the biochemically transformed colonies were enumerated. The first two lines of Table 1 show that pretreatment of LM (TK$^-$) cells in the usual way with mouse $\alpha + \beta$ interferon inhibited biochemical transformation by over 90%. In contrast, when the addition of the mouse $\alpha + \beta$ interferon was delayed until 24 h after the addition of the pAGO DNA, the number of transformed

TABLE 1

Effects of pretreatment or post-treatment of LM(TK$^+$) cells with mouse interferon (IFN) on biochemical transformation of cells by pAGO DNA

Pretreatment of cells[a]	Post-treatment of cells after addition of DNA-calcium phosphate precipitate		Time after DNA-calcium phosphate to add HATG (h)	Colonies/dish
	HATG	HATG + IFN		
Mock IFN	+		24	113
IFN	+		24	11
Mock IFN		+	24	84
Mock IFN		+	48	103
Mock IFN		+	96	114

[a] Cells were treated for 24 h with mock IFN or IFN (40 I.U./ml), trypsinized, and planted at 5×10^5/dish in 60 mm Petri dishes. 24 h later, a calcium phosphate-DNA precipitate was added for 5 h, the precipitate was removed, 5 ml medium was added, and cultures were incubated at 37°C. HATG medium ± IFN (40 I.U./ml) was added 24–96 h after the DNA precipitate.

colonies was reduced by only 26%, which is within the limits of experimental error. When the addition of interferon was delayed until 48 or 96 h after the addition of pAGO DNA to the cells, biochemical transformation was not inhibited at all. The experiments indicate that at the interferon concentrations used, interferon inhibits an early step in the pAGO transformation of LM(TK$^-$) cells to the TK$^+$ rather than the outgrowth of colonies of transformed cells. The effect of mouse $\alpha + \beta$ interferon on the growth of LM(TK$^-$) cells was also studied under conditions similar to those used to assay for biochemical transformation. Table 2 shows that 24 h pretreatment with interferon had no significant effect on the rate or extent of LM(TK$^-$) cell growth.

TABLE 2

Growth of LM(TK$^-$) cells after pretreatment for 24 h with interferon (IFN)[a]

Days after planting in 60 mm Petri dishes	Cells/dish ($\times 10^6$)	
	Mock IFN	IFN (40 I.U./ml)
0	0.50	0.50
1	0.76	0.78
2	2.28	2.14
3	4.68	4.16
5	6.24	6.04

[a] Cultures of LM(TK$^-$) cells were pretreated for 24 h with IFN, trypsinized, and planted at 5×10^5 cells/dish in 60 mm Petri dishes. Five ml medium were added and cultures were incubated for 5 days at 37°C, with media changes on days 1 and 3.

Effect of interferon on induction of enzyme activity

Biochemical transformation by cloned herpesvirus TK genes to cells that can form colonies in HATG medium implies that TK enzyme activity has been induced early in the transformation process. To verify this by direct measurements, extracts were prepared 3 days after cultures of LM(TK$^-$) cells had been transfected with pAGO DNA and the extracts were assayed with ATP and [^{3}H]thymidine for TK activity. TK activity was observed in the transfected cells. The induced enzyme activity was about one-tenth that found in a cloned population of fully transformed cells, as would be expected if only a few cells were expressing normal amounts of TK activity [8–14] or if a majority of the cells were expressing low levels of TK activity. Table 3 shows that pretreatment of the mouse LM (TK$^-$) cells with mouse $\alpha + \beta$ interferon almost completely prevented the induction of TK activity in the transfected cells.

DISCUSSION

The preceding experiments have shown that pretreatment of LM(TK$^-$) mouse fibroblast cultures with mouse $\alpha + \beta$ interferon inhibited the biochemical transformation of the cells to the TK$^+$ phenotype by recombinant plasmids containing the HSV-1 and Mar-HV TK genes. Enzyme assays also demonstrated that the induction of TK activity in transfected cells was inhibited by interferon. Interferon inhibitions of colony formation were concentration dependent and detectable when the cells were pretreated with only 2.5 I.U./ml mouse $\alpha + \beta$ interferon for 20 h. When the LM(TK$^-$) cells were pretreated

TABLE 3

Inhibition by interferon (IFN) of HSV-1 TK induction in LM(TK$^-$) cells transfected with plasmid pAGO DNA[a]

LM(TK$^-$) cells transfected with pAGO DNA	TK activity[b]	
	Mock IFN	Mouse $\alpha + \beta$ IFN
–	0.08	0.10
+	0.34	0.13

[a] Two million LM(TK$^-$) cells were planted in 8 oz prescription bottles in 20 ml growth medium with 10% donor calf serum and incubated for 3 days at 37°C. The medium was removed and fresh medium containing either mock interferon or 40 I.U./ml mouse $\alpha + \beta$ IFN was added. After 18 h, the cells were trypsinized and 5×10^5 cells were planted in 60 mm Petri dishes in 5 ml growth medium. 24 h later half of the cultures were transfected with superhelical pAGO DNA (0.5 μg DNA/dish) by the calcium phosphate precipitation method (see Materials and Methods). The cultures were incubated for 3 days in normal growth medium (without HATG), harvested, enzyme extracts were prepared, and assayed for TK activity [8–14]. [^{3}H]Thymidine at 0.04 mM and 829 c.p.m. per picomole was utilized as nucleoside acceptor in the enzyme assay.

[b] Picomoles [^{3}H]dTMP formed in 30 min at 38°C per μg protein.

with 40 I.U./ml mouse $\alpha + \beta$ interferon, significant inhibition of TK^+ colony formation was observed after only 2–5 h of interferon treatment. Maximum inhibitions were obtained after 9 h. The observation that only a few hours of pretreatment of LM(TK^-) cells with interferon is sufficient to inhibit the biochemical transformation process is consistent with previous observations on the kinetics of induction of the antiviral state by interferon. In cell cultures exposed to interferon, induction of antiviral proteins, such as 2′,5′-oligoisoadenylate synthetase, occurred after about a 3 h lag period. The increase in the antiviral proteins were dependent upon the concentration of interferon and the time of treatment [1,7,27]. Antiviral proteins were synthesized exponentially for about 5 h and maximum levels were reached about 10 h after interferon treatment. The mRNA for 2′,5′-oligoisoadenylate synthetase increased in interferon-treated mouse L cells with the same time course as enzyme itself. In particular, there was about a 3 h lag period between interferon addition and the onset of mRNA accumulation [27]. When anti-mouse interferon antibodies were added to L cells 3 h after interferon, induction of 2′,5′-oligoisoadenylate synthetase and its mRNA was prevented. The induction of the enzyme and its mRNA proceeded for about 8 h and then stopped. Also, actinomycin D, added 2 h after interferon, did not block the induction of 2′,5′-oligoisoadenylate synthetase in L cells, showing that antiviral proteins are elicited after only brief interaction of interferon with the cell surface [27].

The data presented suggest that interferon can inhibit transformation by cloned herpesvirus TK genes under conditions where outgrowth of transformed colonies are not inhibited. During biochemical transformation of LM(TK^-) cells by the calcium phosphate precipitation method, which entails the use of carrier LM(TK^-) DNA, donor fragments containing the selectable herpesvirus TK gene often become covalently linked to carrier DNA molecules in concatameric structures ('transgenomes') [21,25,26]. The carrier DNA may act passively to aid DNA transfer by forming a 'good' calcium phosphate precipitate or by acting as a sink for nucleases, or it may supply functions to the 'transgenomes'. Under selective conditions, the 'transgenomes' can eventually become associated with recipient cell chromosomes to yield stable transformants [10,21,25,26].

In the experiments where biochemical transformation was mediated by pAGO or pMAR035 DNA, expression of the herpesvirus TK genes could have occurred from the donor plasmid DNA, from the concatemeric structures formed by the covalent linkage of donor DNA to carrier LM(TK^-) DNA, or from the donor DNAs stably integrated in recipient cell chromosomes. However, in experiments where plasmid pMH110 was the donor DNA, transcription of the TK gene probably occurred only after linkage of the pMH110 DNA to carrier LM(TK^-) DNA or to chromosomal DNA. Transcription of the TK gene from the input pMH110 DNA is highly improbable because this plasmid lacks the HSV-1 TK gene promoter sequence and the mRNA start site, which are 5′ to the BglII cleavage site in the intact HSV-1 TK gene [2,18,31]. In cells biochemically transformed by plasmid pMH110 DNA, cellular promoters are probably utilized for TK gene transcription, and hybrid messenger RNAs consisting of 5′ flanking cellular nucleotide sequences covalently joined to the coding sequences of the HSV-1 TK gene may be form-

ed [12,14]. The experiments with pAGO and pMH110 DNA indicate that interferon can inhibit biochemical transformation by the HSV-1 TK gene regardless of whether or not the gene contains normal virus promotor sequences.

The present experiments do not disclose the detailed mechanism of action of interferon in inhibiting biochemical transformation and enzyme induction by herpesvirus TK genes. Assuming that the interferon inhibition is in part at the translational level, a role for the interferon-induced endoribonuclease activity could be hypothesized [5,7, 17,27]. However, regardless of whether the mechanism of action of interferon is at the translational or at some other level, the observations suggest that the herpesvirus TK gene may be an important target for interferon action.

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