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Biochemistry

Induction of an antiviral response by interferon requires thymidine kinase

(double-stranded RNA/DNA-mediated gene transfer/gene expression/oligo[(2′-5′)A] polymerase/eukaryotic initiation factor 2 protein kinase)

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ABSTRACT Mouse fibroblastoid cells (Ltk^-) that lack thymidine kinase (tk) activity are unable to respond to murine β-interferon by establishing antiviral activity or inducing the double-stranded RNA-dependent enzymes, oligo[(2′-5′)A] polymerase and M_r 68,000 protein kinase. In contrast, the parental L-929 cell line or clonal derivatives of Ltk^- cells into which the herpes virus *tk* gene was introduced by DNA-mediated gene transfer respond normally to interferon in developing resistance to viral infection and in inducing double-stranded RNA-dependent enzymes. Further evidence for a role of tk in the response to interferon was obtained by isolating revertants of tk^+ clones that lost the herpes virus *tk* gene during growth in BrdUrd-containing medium. In such revertant sublines both tk enzyme activity and viral *tk* genes were undetectable and treatment with interferon failed to produce an antiviral effect or induce synthesis of the double-stranded RNA-dependent enzymes. Our results indicate that the ability of mouse L cells to respond to β-interferon is dependent upon the presence of a functional *tk* gene. We propose that the induction of antiviral responses by interferon stringently requires a metabolite, the level of which is determined by tk activity. The system described may provide a means for elucidating the mechanisms by which responses to interferon are induced.

Interferons are secretory proteins and glycoproteins that modulate a variety of cellular functions, including sensitivity to virus infection (1), the ability to proliferate (2), and, in certain cases, immune responses (3). The mechanisms by which interferons mediate these several effects are not fully understood but interaction of the interferon with specific cell-surface receptors (1) is assumed to lead to derepression of specific genes, as indicated by the appearance of a variety of specific polypeptides between 6 and 12 hr after treatment with interferon (4–6). The establishment of antiviral activity in the cells follows similar kinetics (7) and requires both RNA and protein synthesis (8), suggesting that some of these induced proteins are involved in preventing viral replication. Two of the proteins induced by interferon, oligo[(2′-5′)A] polymerase and eukaryotic initiation factor 2 (eIF 2) protein kinase, have been generally considered as likely mediators of the inhibition of viral protein synthesis (1, 9), although there is not a direct correlation between their inducibility and antiviral functions (10, 11). In the presence of double-stranded RNA (ds RNA), oligo[(2′-5′)A] polymerase generates a series of 2′-5′-linked adenine oligonucleotides that activate a latent endonuclease capable of degrading a wide spectrum of RNA species (12, 13). The eIF 2 kinase, again in the presence of ds RNA, phosphorylates the α subunit of eIF 2, thereby impairing its function (14). The mechanisms involved in decreasing cellular growth rates or modulating immune responses are almost completely unknown.

It is important to establish how interferon modulates cellular genetic programs in such a variety of ways. In particular, are all of the responses to interferon (antiviral, cytostatic, and immunomodulatory) dependent on the same induction pathway and what intracellular mediator(s) is involved?

We describe here observations that may provide means for examining these questions. We have found that a derivative of the mouse L-929 cell line (Ltk^-) that lacks the enzyme thymidine kinase (tk) is unable to respond to mouse β-interferon, as measured by the lack of an antiviral response and a failure to produce oligo[(2′-5′)A] polymerase or eIF 2 kinase. In contrast, normal L-929 cells and clonal lines derived from Ltk^- cells by DNA-mediated introduction of the herpes simplex virus type I *tk* gene are highly sensitive to interferon, both in terms of antiviral responses and induction of ds RNA-dependent enzymes.

MATERIALS AND METHODS

Growth of Cells and DNA-Mediated Gene Transfer. Mouse L-929 fibroblastoid cells and their various derivatives were grown in minimal essential medium supplemented with 10% newborn calf serum, penicillin at 100 units/ml and streptomycin at 50 μg/ml. Ltk^- cells were obtained from A. Pellicer (New York University). BrdUrd (30 μg/ml) or HAT (hypoxanthine at 15 μg/ml/aminopterin at 1 μg/ml/thymidine at 5 μg/ml) was added as required. Introduction of herpes simplex type I virus *tk* gene into Ltk^- cells was carried out as described by Wigler *et al.* (15) by using high molecular weight Ltk^- DNA as carrier and 5 ng of plasmid ptk_2 DNA (16) cleaved with *Bam*HI restriction endonuclease. After selection in HAT-containing medium, clones were picked with cloning cylinders and grown into mass culture for storage and further analysis.

Interferon Sensitivity. Cells (2×10^4) were seeded in 96-well microtiter plates and, after 24 hr, were treated with serial 1:1 dilutions of mouse β-interferon (4×10^7 units/mg of protein; purchased from the late K. Paucker). After a further 24 hr the cells were infected with vesicular stomatitis virus (VSV) at a multiplicity of infection of 0.1 plaque-forming unit per cell in minimal essential medium containing 0.5% newborn calf serum. Cytopathic effect was determined 40 hr later by staining viable cells with methyl violet or crystal violet.

Synthesis of VSV Proteins. Cells (2×10^5) were seeded in 24-well microtiter plates and treated with interferon for 24 hr. Cells were infected (10 plaque-forming units per cell) with VSV in the presence of actinomycin D (5 μg/ml) and minimal es-

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Abbreviations: ds RNA, double-stranded RNA; tk, thymidine kinase; HAT, hypoxanthine/aminopterin/thymidine; VSV, vesicular stomatitis virus; eIF 2, eukaryotic initiation factor 2.

sential medium containing 0.1% serum. Cells were labeled with [^{35}S]methionine (10 μCi/ml; 600 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; Amersham) 3.5 hr after infection for 1 hr, washed with cold phosphate-buffered saline, and lysed in 10 mM Tris·HCl, pH 7.4/15 mM NaCl/1.5 mM $Mg(OAc)_2$/1% Triton X-100/0.25% Na deoxycholate/1 mM phenylmethylsulfonyl fluoride/Trasylol at 15 units/ml. Extracts were analyzed by $NaDodSO_4$/10% polyacrylamide gel electrophoresis by using the buffer system of Laemmli (17) and autoradiography with Kodak XAR-5 film.

Activity of Oligo[(2'-5')A] Polymerase and Protein Kinase. Cells (2×10^6) were seeded in 6-cm culture dishes and were treated with interferon (0 or 250 units/ml) for 24–48 hr. The monolayers were rinsed with 35 mM Hepes, pH 7.5/140 mM NaCl/3 mM $MgCl_2$ and were scraped into buffer B (20 mM Hepes, pH 7.5/120 mM KCl/5 mM $MgCl_2$/10% glycerol/1 mM dithiothreitol) containing 0.5% Nonidet P-40. After sedimenting nuclei at $5{,}000 \times g$ for 5 min, the extracts were used for enzyme and protein assays (18) or stored at −70°C. For assaying oligo[(2'-5')A] polymerase activity, 75 μl of extract was incubated with 50 μl of a 50% (vol/vol) suspension of AG-POLY(I)·POLY(C) (agarose beads) (P-L Biochemicals) for 20 min at 4°C. The beads were washed three times with buffer B and then were incubated for 20 hr at 30°C in buffer B containing 0.5 mM [^{3}H]ATP (100 μCi/ml; 200 Ci/mol; New England Nuclear). Conversion of ATP to 2'-5'-linked oligoadenylates was assayed by batchwise adsorption to DEAE-cellulose, removal of the unbound ATP by five washes in 0.09 M KCl/10 mM Tris·HCl, pH 7.4, and elution of oligo[(2'-5')A] in 0.35 M KCl/20 mM Tris·HCl, pH 7.4. Aliquots were assayed for radioactivity in Aquasol (New England Nuclear) and were analyzed by polyethyleneimine/cellulose chromatography (19). Protein kinase activity was assayed by incubating extracts (15 μl) in the absence or presence of poly(I)·poly(C) (400 ng/ml) and [γ-^{32}P]ATP (400 μCi/ml; 800 Ci/mol; Amersham) at 30°C for 60 min, followed by $NaDodSO_4$/10% polyacrylamide gel electrophoresis and autoradiography.

tk Activity. Cells (2×10^6) were seeded in 10-cm culture dishes and, after 48 hr, extracts were prepared by lysis in 20 mM Hepes, pH 7.5/120 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol/10% glycerol/0.5% Nonidet P-40/10 μM thymidine. Aliquots (2–5 μl) were incubated at 37°C for 30 min in a final volume of 20 μl containing 10 μM [^{3}H]thymidine (40 μCi/ml; 4 Ci/mmol; New England Nuclear), 10 mM NaF, 8 mM $MgCl_2$, 5 mM ATP, and 10 mM 2-mercaptoethanol. Conversion of thymidine to phosphorylated derivatives was measured by spotting aliquots on Whatman DE 81 paper discs, washing the discs in 5 mM NH_4 formate at pH 4.0, and assaying bound radioactivity in a toluene-based scintillation fluid.

Analysis of Cellular DNA for Herpes Virus *tk* Gene. High molecular weight cellular DNA was extracted (15) and cleaved with *Hin*dIII restriction endonuclease (Bethesda Research Laboratories). After electrophoresis on 0.7% agarose gels in 50 mM Tris acetate/20 mM Na acetate/2 mM EDTA, pH 8.0, DNA fragments were transferred to nitrocellulose paper (20) and were hybridized to 8×10^6 cpm of plasmid ptk_2 DNA nick-translated (21) with [α-^{32}P]dCTP and [α-^{32}P]dATP (2 mCi/ml; 800 Ci/mmol; New England Nuclear). Hybridizations were performed at 69°C for 24 hr in the presence of 10% Na dextran sulfate (22). After thorough washing, the blots were exposed to x-ray film with Cronex Lightning Plus image intensifier screens at −70°C.

RESULTS

Sensitivity of cells to interferon was established by titration on microtiter dishes and by measuring cytopathic effect after chal-

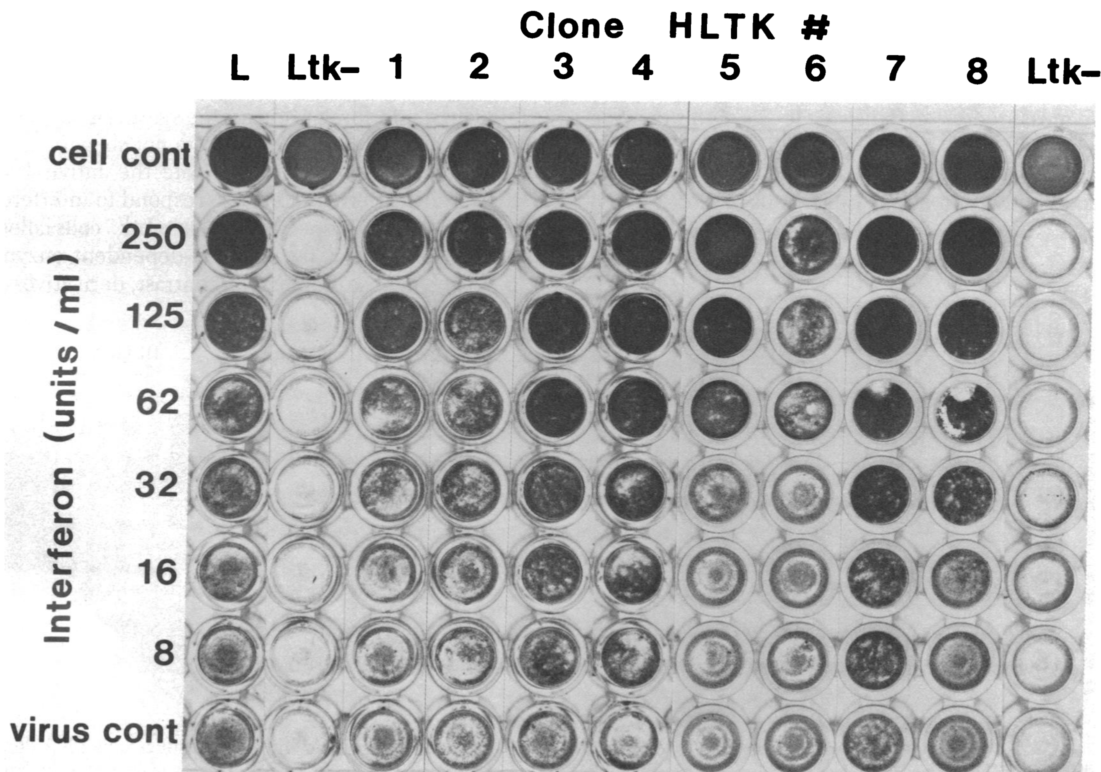

FIG. 1. Inhibition of cytopathic effect induced by VSV in tk⁻ and tk⁺ cells treated with interferon. Cells were treated with serial 1:1 dilutions of interferon at the titers indicated and after 24 hr were challenged with VSV. After 48 hr viable cells were stained. Cell controls (cell cont) received neither interferon nor virus, whereas virus controls (virus cont) received only virus. Clonal lines 1–8 are derived from Ltk⁻ cells by DNA-mediated transfer of the herpes virus *tk* gene.

Table 1. tk activity in cloned tk^{+} cells

Cells	tk activity, pmol/mg of protein per 30 min
L-929	4,140
Ltk^{-}	94
HLTK 1	1,544
HLTK 2	7,096
HLTK 3	11,512
HLTK 4	6,809

Cells (2×10^6) were seeded in 10-cm culture dishes in either neutral (L-929 and Ltk^{-}) or HAT (HLTK 1–4) medium containing 10% newborn calf serum. After 48 hr extracts were prepared and enzyme activity was assayed.

lenge with VSV. Although treatment of L-929 cells with interferon at 60–125 units/ml provides total protection against the cytopathic effect of VSV, Ltk^{-} cells remain unprotected at 250 units/ml (Fig. 1) or, indeed, up to 2,000 units/ml (not shown). Because the Ltk^{-} cells that we used were derived from the parental L-929 cells 19 years ago (23), this lack of response to interferon might not be due simply to the absence of a functional *tk* gene. The availability of purified herpes virus *tk* gene (15) provided a simple means to test this and, therefore, we derived clones of tk^{+} cells from the Ltk^{-} line by calcium phosphate-mediated transfer of the *Bam*HI 3.4-kilobase fragment of plasmid ptk$_2$ (15). Of 25 tk^{+} derivatives isolated, all showed sensitivity to mouse interferon at levels similar to that of L-929 cells (Fig. 1). Comparison of VSV yields in different cell lines by plaque assay on Vero cells also showed that interferon failed to block virus replication in Ltk^{-} cells but inhibited virus production by 1,000-fold in tk^{+} clones. Identical results were obtained whether Ltk^{-} or calf thymus DNA was used as the carrier material for gene transfer or when the chicken *tk* gene (24) was used instead of the herpes virus gene. These results indicate that the presence of a functional *tk* gene is required for an antiviral response to interferon in mouse L cells.

Because interferon activity appeared to require the presence of *tk* genes, we measured the cytoplasmic levels of this enzyme in various cell lines (Table 1). Conversion of thymidine to phosphorylated derivatives by extracts of Ltk^{-} cells was barely detectable, whereas activity in either L-929 cells or clonal derivatives of Ltk^{-} cells containing the herpes virus *tk* gene was 20- to 100-fold higher, as might be expected from their ability to grow in HAT-containing medium. Cell lines that exhibited activity corresponding to approximately 1,000 pmol/mg of protein per 30 min were all sensitive to the antiviral effect of interferon.

Because interferon causes an inhibition of viral protein synthesis (1), we examined the production of VSV-specific polypeptides in interferon-treated tk^{-} and tk^{+} cells. As shown in Fig. 2, interferon fails to prevent viral protein synthesis in Ltk^{-} cells, even at 250 units/ml. In L-929 cells and in clonal lines derived from Ltk^{-} cells by introduction of the herpes virus *tk* gene, the synthesis of VSV polypeptides is essentially abolished by pretreatment with interferon at 50 units/ml and host protein synthesis is restored to normal. Thus, expression of tk activity permits cells to respond to interferon by selectively blocking viral protein synthesis, whereas cells lacking the enzyme are no longer able to inhibit the translation of viral mRNA. Similarly, interferon prevents the synthesis of Mengo virus proteins in tk^{+} but not tk^{-} cells (data not shown).

We next attempted to correlate the antiviral sensitivity of these cells with their ability to respond to interferon by induction of synthesis of specific proteins. Ltk^{-} cells failed to produce enhanced levels of the ds RNA-dependent enzyme oligo[(2'-5')A] polymerase (Table 2). In contrast, derivatives of these cells

Table 2. Induction of oligo[(2'-5')A] polymerase activity by interferon

Cells	Oligo[(2'-5')A] polymerase activity, pmol/mg of protein: Without interferon	With interferon
L-929	39	1,514 (37.8)
Ltk^{-}	37	53 (1.4)
HLTK 1	96	1,923 (19.2)
HLTK 2	116	2,378 (19.8)
HLTK 3	108	1,521 (13.8)
HLTK 4	99	1,086 (10.9)

Cells (2×10^6) were seeded in 6-cm culture dishes in the absence or presence of murine β-interferon (250 units/ml). After 48 hr extracts were prepared and assayed for oligo[(2'-5')A] polymerase activity. Values in parentheses show the stimulation of activity due to interferon treatment.

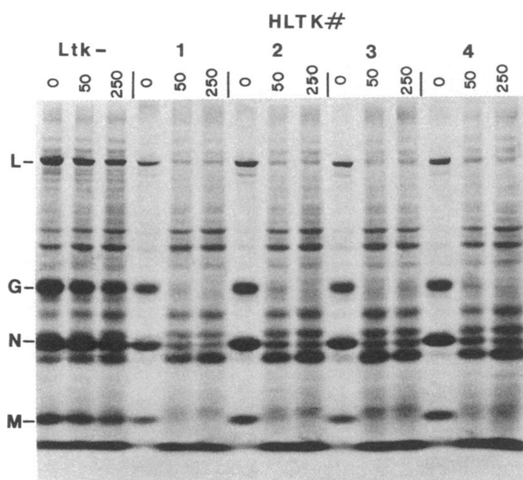

FIG. 2. Inhibition of VSV protein synthesis by interferon in tk^{-} and tk^{+} cells. Cells were treated with interferon at the doses indicated (units/ml) for 24 hr, infected with VSV, and labeled with [^{35}S]methionine for 60 min at 4 hr after infection. Extracts were analyzed by NaDodSO$_4$ gel electrophoresis and autoradiography. Positions of the four major viral polypeptides (L, G, N, and M) are indicated.

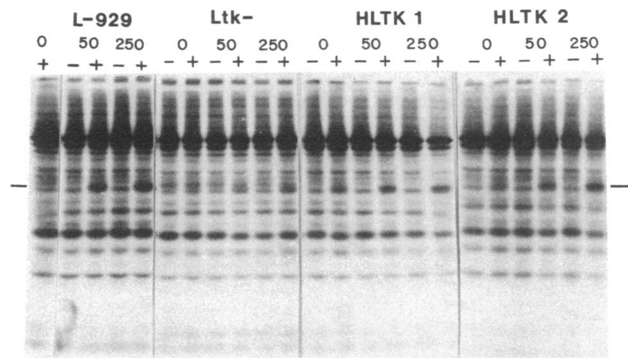

FIG. 3. Activity of interferon-induced, ds RNA-dependent protein kinase in tk^{-} and tk^{+} cells. Extracts were prepared from cells treated for 48 hr with interferon at the doses indicated (units/ml) and were incubated with [γ-^{32}P]ATP for 60 min in the absence (−) or presence (+) of poly(I)·poly(C) at 400 ng/ml. Phosphorylation was detected by NaDodSO$_4$ gel electrophoresis and autoradiography. The migration position of the M_r 68,000 polypeptide is indicated by the marker.

Table 3. tk activity in revertant subclones

Cells	tk activity, pmol/mg of protein per 30 min
Ltk⁻	94 (8)
C6	1,133 (100)
B9	160 (14)
B10	182 (16)
B12	70 (6)

Cells (2 × 10^6) were seeded in 10-cm culture dishes in medium containing 10% newborn calf serum and either BrdUrd or HAT (C6). After 48 hr extracts were prepared and assayed for tk activity. Values in parentheses show percentage of activity in clone C6 from which sublines B9, B10, and B12 were derived by passage in BrdUrd-containing medium.

that contain the herpes virus *tk* gene displayed a 10- to 20-fold stimulation of this enzyme on treatment with interferon, as observed previously for L-929 cells (Table 2). The oligo[(2'-5')A] synthesized by extracts of these cells was analyzed by chromatography on polyethyleneimine-coated glass plates and found to migrate in the position expected for authentic oligo[(2'-5')A] nucleotides.

Similarly, interferon failed to induce the production of a ds RNA-dependent protein kinase in Ltk⁻ cells. To detect this activity we have relied on the enhancement of phosphorylation of a protein kinase with M_r 68,000 that has been shown previously to be correlated with eIF 2 phosphorylation (25). No increase in the phosphorylation of this protein was observed in extracts of Ltk⁻ cells treated with interferon at 50 units/ml and only a very slight increase was detected after treatment with interferon at 250 units/ml (Fig. 3). In contrast, L-929 cells or tk⁺ clones showed a strong stimulation, even after treatment with interferon at 50 units/ml. Four other tk⁺ clones tested showed identical responses to interferon.

If sensitivity to interferon requires tk, cells that cease to express this gene should become resistant to interferon. Hence,

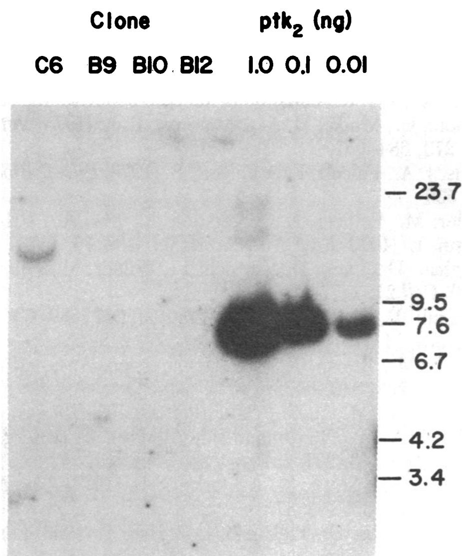

FIG. 4. Presence of a herpes virus *tk* gene in a tk⁺ clone and its absence in revertant tk⁻ sublines. High molecular weight DNA (20 μg) extracted from the tk⁺ clone C6 and its tk⁻ revertants (B9, B10, and B12) was cleaved with *Hin*dIII and analyzed by agarose gel electrophoresis, Southern blotting, hybridization to ^{32}P-labeled plasmid ptk$_2$ DNA, and autoradiography. In parallel, 0.01, 0.1, and 1.0 ng of unlabeled plasmid DNA were mixed with 20 μg of Ltk⁻ DNA, cleaved with *Hin*dIII and were subjected to electrophoresis. The migration position of phage λ DNA markers (sizes in kilobases) is shown.

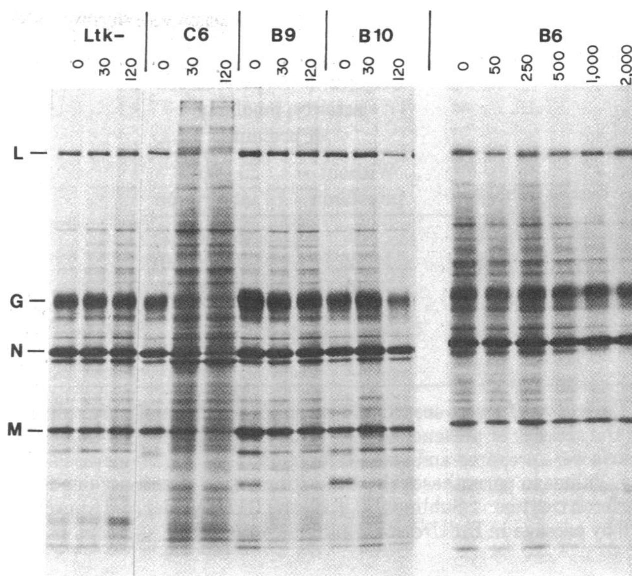

FIG. 5. Inhibition of VSV protein synthesis by interferon in the tk⁺ clone C6 and its revertant tk⁻ sublines. Cells were treated with interferon at the doses indicated (units/ml) for 24 hr, infected with VSV, and labeled with [^{35}S]methionine for 60 min at 4 hr after infection. Extracts were analyzed by NaDodSO$_4$ gel electrophoresis and autoradiography. Sublines B6, B9, and B10 were obtained by selection in BrdUrd-containing medium and were derived from clone C6. Positions of the four major viral polypeptides (L, G, N, and M) are indicated.

clonal lines expressing the herpes virus *tk* gene were subcultured in medium containing BrdUrd (30 μg/ml) and resistant clones were isolated. Subclones derived in this fashion were unable to grow in HAT medium and were considered to be phenotypically tk⁻. To ensure that they in fact lacked this enzyme, cytoplasmic levels were assayed (Table 3) and were found to be between 6% and 16% of that found in the maternal clone, C6, and were similar to levels observed in Ltk⁻ cells. DNA was isolated from these cells, cleaved with *Hin*dIII restriction endonuclease, and analyzed by Southern blotting and hybridization to a ^{32}P-labeled probe containing the herpes virus *tk* gene. The results (Fig. 4) showed that DNA from clone C6 contained a readily detectable *tk* sequence in a fragment 15 kilobases in size and at a level of approximately one copy per cell, whereas the revertant sublines B6, B9, and B12 did not. These results indicate that the exogenously acquired *tk* gene of clone C6 was lost during subculture in BrdUrd.

Treatment of the tk⁻ sublines B6, B9, and B10 with interferon at doses up to 2,000 units/ml failed to produce a reduction in the levels of VSV protein synthesis (Fig. 5), whereas the parental clone C6 was completely resistant to VSV infection at a dose of interferon of 30 units/ml. Thus, the loss of a functional *tk* gene is accompanied by the loss of responsiveness to the antiviral effect of interferon. Similarly, the tk⁻ sublines derived from C6 failed to respond to interferon by enhanced synthesis of oligo[(2'-5')A] polymerase (Table 4), whereas their parental tk⁺ clone, C6, exhibited a 33-fold enhancement of oligo[(2'-5')A] polymerase levels in response to interferon treatment (250 units/ml).

DISCUSSION

We have described here evidence that interferon sensitivity of mouse L cells is dependent on the presence of a functional *tk* gene, whether of murine or viral origin. This finding is based on the resistance of Ltk⁻ cells to interferon, the acquisition of interferon sensitivity in Ltk⁻ cells expressing the herpes virus

Table 4. Induction of oligo[(2'-5')A] polymerase activity by interferon in revertant cell lines

Cells	Oligo[(2'-5')A] polymerase activity, pmol/mg of protein: Without interferon	With interferon
L929	31	587 (19.2)
Ltk⁻	12	11 (0.9)
C6	10	322 (33.2)
B9	11	12 (1.1)
B10	16	17 (1.1)
B12	12	9 (0.7)

Cells (2×10^6) were seeded in 6-cm culture dishes and were cultured in the absence or presence of interferon (250 units/ml) for 24 hr. Extracts were prepared and assayed for oligo[(2'-5')A] polymerase activity. Values in parentheses show the stimulation of activity due to interferon treatment. Sublines B9, B10, and B12 were derived from clone C6 by passage in BrdUrd-containing medium.

tk gene, and the loss of response to interferon in subclones that lose the viral gene. DNA-mediated gene transfer involves the random integration into chromosomal DNA of relatively large concatameric complexes of the specific gene and carrier DNA sequences (26). The DNA used in our transfer procedure contained only the herpes virus *tk* gene, plasmid pBR322 DNA, and homologous (Ltk⁻) DNA as carrier, and because different clones expressing resistance to HAT will have integrated different sequences, it does not seem likely that the development of interferon sensitivity is due to the introduction of another gene or DNA sequence. Moreover, identical results have been obtained by using a chicken *tk* gene. Thus, the ability of Ltk⁻ cells transformed with *tk* gene to respond to interferon is most likely due to the presence of tk activity. This is supported by the finding of greatly enhanced enzyme levels in all clones, showing that resistance to HAT was due to incorporation of the *tk* gene, and by the loss of sensitivity to interferon that accompanies reversion to the tk⁻ phenotype. In the latter case, both enzymatic activity and gene sequences were lost from the three revertants analyzed showing that the modifications involved loss of the gene and not alterations in substrate specificity or, for instance, in the transport of BrdUrd across the cell membrane.

The lack of an antiviral response to interferon observed in tk⁻ cells is accompanied by a deficiency in the induction of ds RNA-dependent enzymes. Introduction of the herpes virus *tk* gene leads to restoration not only of the antiviral response but also of the capacity to induce synthesis of these ds RNA-dependent enzymes. Our results can be explained most simply by proposing that the requirement for tk operates at a step occurring early in the induction pathway so that, in the absence of the enzyme, synthesis of a variety of new proteins is lacking, including those required to block viral protein synthesis.

The failure of interferon to induce a response in tk⁻ cells may reflect the absence, or relatively low amount, of some metabolite related to tk, presumably a uridine or thymidine nucleotide. Ltk⁻ cells utilize the *de novo* pathway for the production of dTMP from uridine diphosphate and their rapid growth rate indicates that the supply of dTTP, which is derived from dTMP, is not noticeably decreased when compared to L-929 cells. Thus, it seems unlikely that dTMP itself is the required metabolite. It is possible that tk acts by sparing a uridine nucleotide that, in tk⁻ cells, is depleted owing to a drain of UDP for production of dTMP or that the enzyme catalyzes the synthesis not only of dTMP but also of some as yet unknown metabolite. Identification of a nucleotide or some other product required for a response to interferon would facilitate the elucidication of the pathways involved in the initial steps of interferon function.

We have extended our findings to human cell lines and to a comparison of other interferon species. Preliminary observations show that human fibroblastoid cells lacking tk do not respond to α-interferon. Use of cells lacking tk and derivatives containing the herpes virus gene may permit us to establish whether induction of antiviral and cytostatic effects involve separate pathways. In addition, comparison of the responses of tk⁻ and tk⁺ cells to α- and β-interferons with those to γ-interferon may indicate whether or not γ-interferon, which appears to interact with a receptor distinct from that of α- and β-species (27), induces its response by a different pathway. These studies may throw light on the events involved in the induction of the antiviral and cytostatic responses by different interferon species.

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