Thymidine kinase genes and the induction of anti-viral responses by interferon

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A mouse fibroblast cell-line deficient in thymidine kinase (Ltk(−) aprt(−)) fails to show an anti-viral response when treated with interferon. After introduction of a viral *tk* gene into these cells the resultant clones showed normal responses to interferon. However, one such *tk*-containing clone (C6) spontaneously lost its ability to respond to interferon by inducing an antiviral state although it retained its ability to induce the enzyme oligo(2′–5′ A)-synthetase. This sub-clone (6A) still expressed thymidine kinase activity but restriction endonuclease analysis indicated an alteration in the sequences flanking the exogenous viral *tk* gene. Our results suggest that a modification in the exogenous viral DNA sequences led to a loss of interferon sensitivity.

Interferon *Thymidine kinase* *Oligo(2′–5′ A)-synthetase*

1. INTRODUCTION

Interferons mediate a variety of responses in cultured cells including the induction of an anti-viral state and the inhibition of cell growth [1]. We reported recently that a line of thymidine kinase (tk)-deficient mouse fibroblast (Ltk(−) aprt(−)) cells were unable to respond to murine β-interferon by activation of an anti-viral state [2]. Incorporation of the Herpes simplex type 1 virus *tk* gene into these cells by DNA-mediated gene transfer gave rise to cloned tk(+) cell lines which showed sensitivity to interferon at levels similar to those of the original L-929 cell line [2]. Furthermore, sub-clones selected for loss of the Herpes virus *tk* gene by continuous passage in bromodeoxyuridine (BUdR)-containing medium were no longer able to respond to interferon by inducing an anti-viral state [2]. These results suggest that, at least in certain cells, *tk* genes may be necessary to mediate some responses to interferon.

In this report we describe observations made with a spontaneously arising sub-clone of an Ltk(+) cell-line which had been derived by introduction of the Herpes virus *tk* gene into Ltk(−) aprt(−) cells. In this sub-clone an apparent genetic rearrangement occurring in the region of the inserted *tk* gene is accompanied by a loss of the ability of interferon to induce an anti-viral response, further suggesting some relationship between thymidine kinase and the capacity of cells to respond to interferon.

2. METHODS

2.1. *Cells and cell culture*

Ltk(−) aprt(−) cells obtained from Dr A. Pellicer (New York University Medical Center) were used in all the experiments described. These cells were originally obtained from Dr R.G. Hughes (Roswell Park Memorial Institute, Buffalo

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NY) who derived them as an adenine phosphoribosyl transferase-deficient (aprt(−)) sub-clone of the LMTK(−) clone 1D of Kit et al. [3]. Cells were grown in minimal essential medium (Gibco F15) supplemented with 10% newborn calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in an atmosphere of 95% air and 5% CO_2. For tk(−) cells, bromodeoxyuridine (30 μg/ml) and/or 2,6 diaminopurine (50 μg/ml) were also added. For tk(+) lines, HAT (15 μg/ml hypoxanthine; 1 μg/ml aminopterin; 5 μg/ml thymidine) was added. The clone C6 was derived from Ltk(−) aprt(−) cells by calcium phosphate-mediated introduction of the HSV-type 1 3.6 kb DNA fragment (cloned in pBR322) in the presence of calf thymus DNA as carrier as in [2].

2.2. *Analysis of anti-viral effects of interferon*

Cells were grown to confluency in 6 cm culture dishes and treated for 24 h with 0, 50 or 500 reference units/ml of murine β-interferon (4×10^7 units/mg protein; purchased from the late Dr K. Paucker). Monolayers were infected with vesicular stomatitis virus (multiplicity of infection = 1 plaque forming unit/cell) and the supernatants harvested after 24 h. Virus titres were assayed by plaque formation on confluent L-929 monolayers covered with 1% agar.

2.3. *Enzyme assays*

Cells were grown in either 6 cm or 10 cm culture dishes and extracts prepared and assayed for thymidine kinase or oligo(2′–5′ A)-synthetase as in [2]. Protein content of extracts was estimated by the procedure of Bradford [4].

2.4. *Analysis of cellular DNA*

High molecular mass cellular DNA was extracted [5] and subjected to cleavage with restriction endonucleases (2 units/μg DNA) for 2–3 h at 37°C under the conditions suggested by the supplier (Bethesda Res. Labs., Gaithersburg MD). Fragments of DNA were analyzed by electrophoresis on 0.7% agarose gels in 50 mM Tris–acetate (pH 8.0), 2 mM EDTA buffer. After electrophoresis, DNA was transferred to nitrocellulose paper (Schleicher and Schuell BA 85, Keene, New Hampshire) by blotting with 20 × standard saline citrate (SSC, 0.15 M NaCl, 0.03 M Na citrate (pH 7.0)). The position of DNA fragments containing the HSV *tk* gene was identified by hybridization to ^{32}P-labelled, nick-translated ptK_2 DNA [6] in the presence of 3 × SSC and 10% dextran sulphate [7] at 69°C for 18 h. After washing several times at progressively higher degrees of stringency (to 0.1 × SSC), filters were dried and exposed to Kodak XAR-5 X-ray film with Cronex Lightning Plus intensifier screens at −70°C.

3. RESULTS

The cell-line designated C6 was derived from murine Ltk(−) aprt(−) cells by calcium phosphate-mediated uptake of a 3.6 kb fragment of HSV type I DNA containing the viral *tk* sequence [8] as described in [2]. During routine culture in HAT-containing medium, a spontaneous variant of this line was obtained, cloned and designated 6A. As a control, the original C6 line was re-cloned by plating at low density and an isolate designated 6M used for further comparative studies. As shown in table 1, interferon treatment of cell lines C6 and 6M causes a 3–4 log reduction in vesicular stomatitis virus yield, indicating a sensitivity to interferon similar to that observed in mouse L-929 cells. By contrast, line 6A was markedly resistant to induction of an anti-viral state even at fairly high doses (500 reference units/ml) of interferon. Similarly, the parental line from which C6 was derived, Ltk(−) aprt(−),

Table 1

Reduction of virus yield by interferon

Cell-line	VSV titre (p.f.u. × 10^{-7}/ml)		
	0 units/ml	50 units/ml	500 units/ml
L-929	2.0	0.006	<0.001
Ltk(−) aprt(−)	7.5	4.2	2.4
C6	4.3	0.002	0.001
6M	5.2	0.001	<0.001
6A	2.8	0.84	0.8

Cells growing in 6 cm culture dishes were treated with interferon at the doses shown for 24 h and then infected with vesicular stomatitis virus (m.o.i. = 1 p.f.u./cell). After 24 h, culture supernatants were harvested and virus yield determined by plaque-assay on L-929 cells

showed only a slight reduction in virus yield after treatment with interferon. Likewise Ltk(−) aprt(−) and 6A cells were resistant to the anti-viral effects of interferon when challenged with Mengovirus, whereas C6 and 6M were essentially completely protected from the cytopathic effects of this virus at low doses of interferon (not shown).

Previously we showed that, like anti-viral responses, induction of the double-stranded RNA-dependent enzyme oligo(2′−5′ A)-synthetase by interferon did not occur in Ltk(−) aprt(−) cells but could be readily observed in tk(+) derivatives of this line [2]. Therefore, we monitored induction of this enzyme by interferon in cell-lines C6, 6M and 6A (table 2). As expected, the level of oligo(2′−5′ A)-synthetase was enhanced about 10-fold in C6 and 6M cells after interferon treatment. Somewhat to our surprise, however, treatment of line 6A with interferon also resulted in the same level of induction of enzyme activity despite the absence of an accompanying anti-viral response (table 1). In 4 separate experiments, treatment with 250 units/ml of interferon gave the same level of oligo(2′−5′ A)-synthetase activity in 6A cells as in 6M. The Ltk(−) aprt(−) cells showed no significant increase (1–2-fold) in enzyme levels after interferon treatment.

Although line 6A was capable of growth in HAT-containing medium, we decided to ensure that it retained the inserted viral *tk* gene and continued to express enzyme activity. To ensure that the Herpes virus gene was still present in these cells, we analyzed total cellular DNA by restriction endonuclease cleavage, agarose gel electrophoresis, Southern blotting and hybridization with a ^{32}P-labelled ptK$_2$ probe. As shown in fig.1, the restriction endonuclease patterns for C6 and 6M DNA were identical, indicating the presence of a unique insertion site for the *tk* gene which was not affected by re-cloning. Since neither *Hin*dIII nor *Bam*HI cut within the viral 3.6 kb sequence, the fragments produced (16.5 kb and 7.5 kb, respectively) represent cleavage within host or carrier DNA flanking sequences. The enzyme *Sma*I cuts at 5 positions within the 3.6 kb viral sequence [9] but none of the 3 cellular fragments which hybridize to ptK$_2$ DNA (2.8 kb, 6.2 kb and 8.4 kb) correspond in size to the expected cleavage fragments. It is probable that the most intensely radioactive band (6.2 kb) corresponds to the *tk* coding sequence with the 5′-proximal *Sma*I site of viral sequence replaced by host or carrier DNA sequences. The restriction pattern obtained with 6A DNA was quite distinct from that seen with C6 or

Table 2

Induction of oligo(2′−5′ A)-synthetase by interferon

Cell line	Effect of IFN	(2′−5′ A)-polymerase activity after interferon (pmol A incorp./mg protein)	Stimulation (cpm + IFN/cpm − IFN)
L-929	+	1075	13.6
Ltk(−) aprt(−)	−	45	1.2
C6	+	1693	9.9
6M	+	917	9.1
6A	−	907	12.5

Cells growing in 6 cm culture dishes were treated with interferon (250 units/ml) for 24 h, harvested and assayed for (2′−5′ A)-synthetase activity. Untreated cells showed basal levels identical to values obtained without addition of cell extract (⩽72 pmol A incorp./mg protein) which were subtracted to give the activity due to interferon treatment. The fold stimulation of interferon-treated extracts over the background level is shown. The ability of interferon (IFN) to induce an anti-viral effect is indicated for each cell type

Table 3

Thymidine kinase activity in extracts of C6, 6M and 6A

Cell line	Effect of IFN	pmol [^{3}H]thymidine incorp. . mg protein^{-1} . 30 min^{-1}
Ltk(−) aprt(−)	−	7
C6	+	467
6M	+	639
6A	−	691

Cells growing in 10 cm culture dishes were harvested when sub-confluent and assayed for thymidine kinase activity as in section 2. The ability of interferon to induce an anti-viral effect is indicated

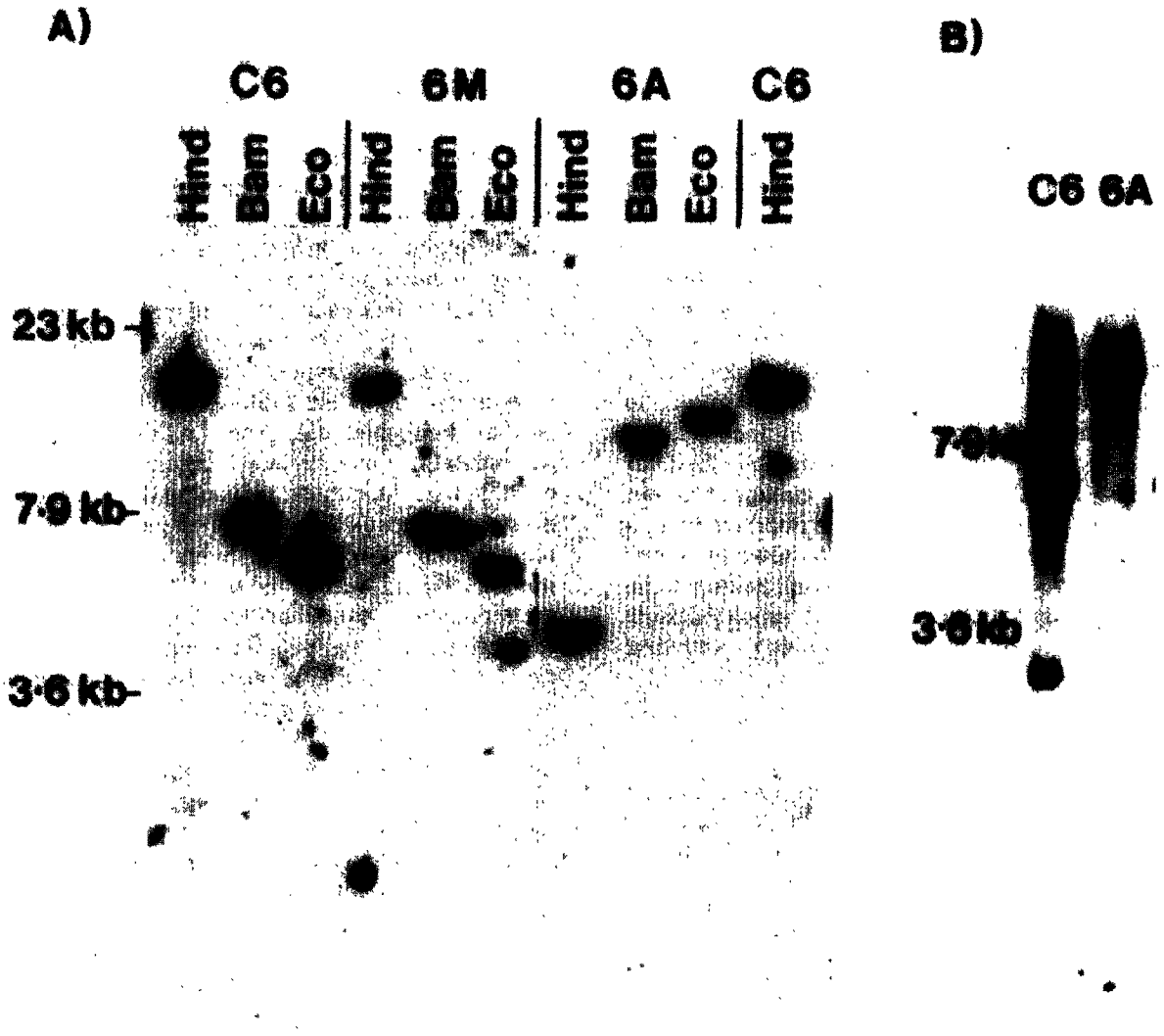

Fig.1. Restriction endonuclease digestion of cellular DNA containing HSV DNA sequences. High relative molecular mass cellular DNA (20 μg) extracted from cells C6, 6M and 6A was cleaved with restriction endonucleases and analyzed by electrophoresis on 0.7% agarose gels. Fragments containing HSV-specific DNA sequences were detected by transfer to nitrocellulose paper and hybridization with nick-translated [^{32}P]ptk$_2$ DNA. Sizes of fragments were determined from the migration of phage λ DNA (cut with *Hin*dIII) markers: (A) cleavage with *Hin*dIII, *Bam*HI and *Eco*RI; (B) cleavage with *Sma*I.

6M DNA. Each of the 4 restriction endonucleases tested gave rise to fragments of 6A DNA containing sequences hybridizing to the ptK$_2$ probe which differed from the corresponding fragments of C6 and 6M DNA. These results indicate that the DNA sequences flanking the HSV insert must have been modified when the 6A line arose. Whether or not the viral *tk* coding sequence has also been altered is not clear from our present data.

As seen from table 3, the levels of thymidine kinase activity detectable in lysates of C6, 6M and 6A were very similar, indicating continued expression of a *tk* gene in all 3 cell types. These cell lines were also capable of incorporating [^{3}H]thymidine

into acid-insoluble material (not shown) indicating that this metabolic pathway is utilized during normal growth.

4. DISCUSSION

The spontaneous derivation of line 6A was accompanied by the death of most of the cells in the culture. Presumably, the surviving cells which gave rise to line 6A had undergone a spontaneous genetic rearrangement which included the site at which the Herpes virus *tk* gene had previously been inserted. We are currently comparing this site in C6 and 6A in more detail in order to clarify the nature of the rearrangement. The loss of interferon responsiveness with respect to anti-viral activity appears to correlate with this alteration in the genome and lends support to our previous observations [2] linking the *tk* gene and interferon response. However, in contrast to our previous findings, 6A is a tk(+) cell and yet fails to respond to interferon by activation of an anti-viral state. Two possible explanations can be suggested:

(i) Since we have used the 3.6 kb fragment of the HSV genome to derive our tk(+) cells, it is possible that some sequence other than the tk coding region (1.3 kb) is responsible for the activation of interferon sensitivity and is co-selected in all the clones we have examined. In 6A this region could be modified without loss of the *tk* gene to give an interferon resistant cell.

(ii) The *tk* gene of line 6A has been slightly altered during the genomic rearrangement and expresses a modified enzyme which is still able to phosphorylate thymidine but lacks some secondary activity which is normally associated with the enzyme.

We are currently investigating these possibilities.

The observations described here showing the presence of thymidine kinase activity in 6A cells suggest that possession of thymidine kinase activity alone is not the determining factor of interferon sensitivity in these cells. Other results (J.A.L., in preparation) show that not all Ltk(−) cells are resistant to interferon. Nonetheless, it seems clear that some defect in the interferon response pathway of the Ltk(−) aprt(−) strain of cells which we have used can be corrected by insertion of the HSV type I 3.6 kb DNA fragment and that this effect may be lost by modification of this sequence.

Curiously, loss of sensitivity to the anti-viral effect of interferon was not accompanied by an inability to synthesize oligo(2′–5′ A)-synthetase in response to interferon. It is possible that different pathways are responsible for activation of different responses to interferon. The experiments also show that the presence of oligo(2′–5′ A)-synthetase is not sufficient to determine an anti-viral response to either vesicular stomatitis virus or Mengovirus. This finding agrees with reports of cells which fail to synthesize the enzyme yet show an anti-viral effect [10,11] or of cells with high basal levels of the enzyme which support viral replication [10].

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REFERENCES

[1] Lewis, J.A. (1982) in: Hormone Receptors, Horizons in Biochemistry and Biophysics (Kohn, L.D. ed) vol.6, pp.357–384, Wiley, London, New York.

[2] Lewis, J.A., Mengheri, E. and Esteban, M. (1983) Proc. Natl. Acad. Sci. USA 80, 26–30.

[3] Kit, S., Dubbs, D.R., Piekarski, L.J. and Hsu, T.C. (1963) Expt. Cell Res. 31, 297–312.

[4] Bradford, M. (1976) Anal. Biochem. 72, 248–254.

[5] Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. and Chasin, L. (1979) Proc. Natl. Acad. Sci. USA 76, 1373–1376.

[6] Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237–251.

[7] Wahl, G.M., Stern, M. and Stark, G.R. (1979) Proc. Natl. Acad. Sci. USA 76, 3683–3687.

[8] Hanahan, D., Lane, D., Lipsich, L., Wigler, M. and Botchan, M. (1980) Cell 21, 127–139.

[9] Enquist, L.W., Vande Woude, G.F., Wagner, M., Smiley, J.R. and Summers, W.C. (1979) Gene 7, 335–342.

[10] Hovanessian, A.G., Meurs, E. and Montagnier, L. (1981) J. Interferon Res. 1, 179–190.

[11] Meurs, E., Hovanessian, A.G. and Montagnier, L. (1981) J. Interferon Res. 1, 219–232.