Specific Induction of Cellular Gene Transcription in Herpes Simplex Virus Type 2-Transformed Cells

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SUMMARY

In spite of much work, the mechanism of oncogenic transformation by herpes simplex virus (HSV) is as yet unknown. It has been proposed that HSV type 2 (HSV-2) can transform cells by a 'hit and run' mechanism. In the past we have demonstrated that several polypeptides can be immunoprecipitated from HSV-2-transformed cells, but not from control cells or adenovirus-transformed cells, by rabbit hyperimmune sera to HSV-2. It is possible that the expression of these proteins might be the result of activation of cellular genes during transformation. We have now isolated cDNAs representing transcripts of genes that are expressed at higher levels in HSV-2-transformed hamster embryo fibroblasts than in the parental cells. Cytoplasmic transcripts and genomic sequences homologous to three clones (pAA8, pHD1 and pLC7) were analysed. Northern blot analyses showed that 0.75 kb transcripts which hybridize to the three cDNAs were present in HSV-2-transformed cells and were completely absent or present at low levels in control hamster fibroblasts. These transcripts were not present in mouse cells transformed by other DNA viruses or by a chemical carcinogen. The expression of these transcripts seemed to be confined to certain HSV-2-transformed cell lines. Southern blot analysis suggested that the 0.75 kb transcripts corresponding to these cDNAs may have arisen from a single gene. Nuclear run-off experiments indicated that activation occurred at the level of transcription. The activation of the gene or genes corresponding to these cDNAs may be an integral part of the mechanism of transformation by HSV-2.

INTRODUCTION

The oncogenic potential of herpes simplex virus type 2 (HSV-2) has been clearly demonstrated by many laboratories (Duff & Rapp, 1971a, b; Galloway & McDougall, 1981; Hayashi et al., 1985; Kessous et al., 1979). Two different regions of the HSV-2 genome have been reported to be capable of transforming primary rodent cells (Galloway et al., 1984; Jariwalla et al., 1980; Reyes et al., 1979). Moreover, it has also been demonstrated that HSV itself can act as a mutagen (Pilon et al., 1985, 1986; Schlehofer & zur Hausen, 1982). However, the mechanism of transformation by HSV-2 is not well understood since transformed cells fail to express or even retain any consistent portion of viral DNA (Galloway et al., 1984). There is growing evidence that cells can remain transformed in the absence of detectable herpesvirus sequences and that a viral protein may not even be needed to initiate transformation (Cameron et al., 1985). These observations are in keeping with the hypothesis that HSV could act in a 'hit and run' manner (Galloway & McDougall, 1983; Skinner, 1976). Transformation by certain viruses has been shown to be accompanied by an altered expression of cellular genes (Majello et al., 1985; Scott et al., 1983; Singh et al., 1985). This also seems to be the case for cells transformed by HSV-2.

In the past, many laboratories have concentrated on identifying the viral products that persist in HSV-2-transformed cells. The search for such products has been fruitful in the case of small

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DNA viruses (Tooze, 1980) but has not contributed to the understanding of the basis of HSV-induced tumourigenicity. Studies on genes differentially expressed in normal and transformed cells are important for our understanding of growth control and tumourigenesis. Here we report the characterization of three cDNA clones isolated from an HSV-2-transformed cell line. The expression of the transcripts related to these cDNA clones is confined to certain HSV-2-transformed cell lines. These results may provide evidence for a possible mechanism of HSV-2-induced cell transformation.

METHODS

Cell culture. All cell lines were cultivated in a medium supplemented with penicillin, streptomycin, fungizone and 8% foetal calf serum. The 333-8-9 cell line was kindly provided by Dr F. Rapp, RE-99 by Dr J. Macnab, and AH and A by Dr E. Frost. Cell lines SVT2, C3H and C3H-MCA were obtained from the American Type Culture Collection. Details concerning the origin of the different cell lines have been described previously (Kessous et al., 1979; Duff & Rapp, 1971a, b; Macnab, 1974; Suh & Frost, 1986).

RNA purification. Polyosomal poly(A)⁺ RNA was obtained from hamster embryo fibroblasts (HEF) and 620-7 cells by streptomycin sulphate precipitation according to Skup et al. (1981). Total RNA was isolated by the guanidine thiocyanate-caesium chloride procedure (Chirgwin et al., 1979). Poly(A)⁺ RNA was purified from polyosomal RNA or from total RNA by two successive passages through oligo(dT)-cellulose and quantified by spectrophotometry and by agarose gel electrophoresis.

Isolation of cDNA clones. Double-stranded cDNA was synthesized from poly(A)⁺ RNA prepared from HEF and 620-7 cells according to the procedure of Gubler & Hoffman (1983). dC-tailed cDNA was inserted at the dG-tailed PstI site of pUC8 (Moss & Vieira, 1982) and used to transform Escherichia coli JM103 by the technique of Hanahan (1983, 1985). Purified plasmid cDNA (450 µg) (Colman et al., 1978) obtained from the HEF cDNA bank was covalently coupled to cellulose (Moss et al., 1981), and 50 µg poly(A)⁺ RNA from 620-7 cells was hybridized at 25 °C for 10 days to the cDNA-cellulose matrix in 300 µl of hybridization buffer containing 40%, denatured formamide, 0.9 M-NaCl, 10 mM-PIPES pH 6.5, 4 mM-EDTA, 3 mM-dithiothreitol (DTT), 500 µg/ml RNA in order to obtain an enriched population of transcripts specific to 620-7 cells. Reactions achieved Rₚ values of 3000 or greater. A 10-fold enrichment was obtained after five successive cycles of hybridization [91% of poly(A)⁺ RNA from 620-7 hybridized to HEF cDNA coupled to cellulose]. The unhybridized 9% of poly(A)⁺ RNA from 620-7 cells was purified by one passage through oligo(dT)-cellulose. This enriched RNA was used to construct a cDNA bank which was screened with a reverse-transcribed ³²P-labelled cDNA probe made with the enriched RNA. Hybridization was carried out in the presence of a 100-fold excess of a single-stranded cDNA made from HEF poly(A)⁺ RNA. Each positive recombinant was analysed by Northern blotting in order to verify the pattern of differential expression.

Northern blot analysis. RNAs (2-5 µg) were denatured with formaldehyde and electrophoresed on a 1-4% agarose gel containing 2.2 M-formaldehyde and 10 mM-sodium phosphate pH 7.0 (Maniatis et al., 1982). RNA was transferred to Pall Biodyne membranes and the filters were hybridized in 50% formamide, 1 x SSC in 0.15 M-NaCl, 15 mM-sodium citrate pH 7.0, 100 mM-sodium phosphate pH 7.0, 1 x Denhardt’s solution (Denhardt, 1966), 0.5% SDS, 200 µg/ml salmon sperm DNA and 10% dextran sulphate with ³²P-labelled nick-translated (Rigby et al., 1977) plasmid cDNA probes (sp. act. 1.5 × 10⁸ to 3.0 x 10¹⁰ c.p.m./µg DNA) for 18 h. Blots were washed twice in 2 x SSC, 0.1% SDS for 15 min at room temperature and four times in 0.1 x SSC, 0.1% SDS at 50 °C, then exposed overnight to Kodak XAR film with two intensifying screens.

Southern blot analysis. High Mₗ genomic DNA was extracted from HEF and 620-7 cells by the method of Gross-Bellard et al. (1973). Ethanol-precipitated DNA was solubilized in Tris-EDTA buffer and quantified by spectrophotometry. The DNAs were digested to completion with BamHI, EcoRI and PstI and fractionated on a 0.7% agarose gel in Tris-acetate-EDTA buffer. DNA was transferred to Pall Biodyne membranes and the filters were hybridized as for the Northern blots. Blots were washed twice in 2 x SSC, 0.1% SDS at room temperature for 15 min and four times in 0.1 x SSC, 0.1% SDS at 65 °C. Filters were autoradiographed for 2 to 5 days with two intensifying screens.

In vitro transcription in isolated nuclei. Seventy percent confluent HEF and 620-7 cells were harvested with rubber scrapers in cold phosphate-buffered saline (PBS). The cells were then washed twice in cold PBS. Nuclei were prepared by lysing the cells in 5.0 ml lysing buffer containing 0.3 mM-sucrose, 10 mM-Tris-HCl pH 7.5, 5 mM-MgCl₂, 0.4% Nonidet P40, 0.5 mM-DTT (Wang et al., 1985). Purified nuclei were stored at -80 °C in 40% glycerol, 50 mM-Tris-HCl pH 8.3, 5 mM-MgCl₂, 100 µM-EDTA.

In vitro transcription experiments were performed with 2 x 10⁷ nuclei in a total reaction volume of 200 µl of 16% glycerol, 20 mM-Tris-HCl pH 8.3, 2.5 mM-MgCl₂, 70 mM-KCl, 200 µCi [³²P]UTP (600 Ci/mmol; Amersham). Reactions were carried out at 26 °C for 45 min. Labelled RNA was purified according to McKnight & Palmietter (1979). RNA polymerase II inhibition experiments were performed by adding α-amanitin to the reaction mixture at a final concentration of 1 µg/ml. Hybridizations of labelled RNA were performed as described
for the Northern and Southern blots with denatured dot-blotted plasmid DNA linked to Pall Biodyne membranes. Hybridizations were performed in 15 ml tubes with 2 ml hybridization solution and $5 \times 10^6$ c.p.m. per filter for 72 h. Washes were done as for Northern blots except that filters were incubated with 10 $\mu$g/ml RNase A and 1 $\mu$g/ml RNase T1 for 45 min at 37 °C. The filters were exposed to Kodak XAR film with two intensifying screens for 3 days.

**RESULTS**

Analysis of the expression of cellular genes induced by HSV-2 transformation

The isolation of cellular genes differentially expressed in HSV-2-transformed cells was made possible by a cDNA cloning strategy that permitted the screening of a relatively low number of recombinants. Four-thousand clones from the enriched cDNA library were picked, grown on filters and screened as described in Methods. We isolated 26 cDNA clones that showed a pattern of differential expression. Here we focus on the characterization of three cDNA clones: pAA8, pHDI and pLC7.

Fig. 1 (a) is a Northern blot showing that a 750 nucleotide RNA homologous to pAA8 was more abundant in the HSV-2-transformed cell line 620-7 (Kessous *et al.*, 1979) than in the parental HEF. The increase was five- to 20-fold depending on the growth state of the transformed cells. A transcript of the same size was also expressed in a tumour cell line (620-7T; Fig. 1a, lane 4) derived from 620-7 and in 333-8-9 cells (Duff & Rapp, 1971a, b), another hamster cell line transformed by u.v.-inactivated HSV-2 (lane 5).

Another cDNA clone, pHDI, hybridized to RNA present exclusively in the transformed cell line used to construct the cDNA library. This expression was relatively independent of growth conditions. In fact, this 0.75 kb transcript was present only in cell line 620-7 and absent from 333-8-9, A, AH (derived from BALB/c 3T3 cell lines by morphological transformation with HSV-2 DNA spanning map units 0.524 to 0.644) or RE-99 cells (Fig. 1 b). Thus, the genotype or histories of different cell lines may also affect the expression of certain genes (Singh *et al.*, 1985). Fig. 1 (c) shows the pattern of expression of pLC7 RNA. Here again, we found differential expression of a 0.75 kb transcript in 620-7 cells. The confluence of the cell culture did not influence the expression of pLC7 mRNA. With this probe 333-8-9 cells showed a faint signal. One other hamster cell line, S-111T (transformed by whole inactivated HSV-2, obtained from Dr R. Jariwalla, Linus Pauling Institute of Science and Medicine, Palo Alto, Ca., U.S.A.) has also shown a 0.75 kb transcript hybridizing with pLC7 (data not shown). Complementary RNA was also expressed in a cell line transformed by a cloned DNA fragment of HSV-2 (AH cells; lane 9).

Fig. 1 shows that the genes represented by pAA8, pHDI and pLC7 and expressed in some HSV-2-transformed cell lines were not activated in simian virus 40 (SV40)-transformed cells (lane 11) (and adenovirus-transformed cells, not shown) nor in C3H-MCA cells, a cell line transformed by a chemical carcinogen (lane 13). Thus, the expression of these clones is not a general characteristic of transformation of fibroblasts. The pAA8 and pLC7 probes hybridized to a low extent to a transcript in the control HEF line. This might be due to the heterogeneity of HEF, as hybridization to RNA from baby hamster kidney (BHK) cells gave no signal (not shown). Macnab *et al.* (1985) have recently demonstrated that infection by HSV-2 promotes the expression of some cellular genes which are also expressed in HSV-2-transformed cell lines. We have investigated the expression of the genes corresponding to pAA8, pHDI and pLC7 in HSV-2-infected HEF. As can be seen in Fig. 1 (lane 6), infection by HSV-2 led to complete repression of the low levels of expression of these genes normally observed in the HEF (Fig. 1, lane 1). This repression may be due to the action of viral regulatory proteins on the cellular promoters involved.

Thus, the expression of the mRNAs detected with these cDNA probes was restricted to HSV-2-transformed cell lines, independently of the protocol and host cell used for transformation.

**Analysis of genomic sequences homologous to pAA8, pHDI and pLC7**

To determine how many of these activated genes exist in the hamster genome, and whether any amplification or rearrangement of the genes occurred in HSV-2-transformed cells, DNAs from HEF and 620-7 cells were analysed by Southern hybridization.
Fig. 1. Northern blot analysis of poly(A)+ RNAs from normal and transformed cell lines. The poly(A)+ RNAs (2.5 µg of each) were analysed by Northern blotting with 32P-labelled cDNA clones (a) pAA8, (b) pHD1 and (c) pLC7. RNA samples were: lane 1, HEF; lane 2, 620-7 cells at 70 to 80% confluence; lane 3, 620-7 super-confluent cells; lane 4, 620-T cells, a tumour cell line produced by the injection of 620-7 cells in a newborn hamster; lane 5, 333-8-9 cells; lane 6, HSV-2 (strain HG52)-infected HEF; lane 7, RE-99 cells, rat embryo cells transformed by HSV-2; lane 8, NIH 3T3 cells; lane 9, AH cells, a BALB/c 3T3 cell line transformed by a cloned HSV-2 DNA fragment (map units 0.524 to 0.644); lane 10, A cells, a BALB/c 3T3 cell line transformed by a cloned HSV-2 DNA fragment (map units 0.416 to 0.644); lane 11, SVT2 cells, an NIH 3T3 cell line transformed by SV40; lane 12, C3H, an established mouse embryo cell line; lane 13, C3H-MCA, a C3H cell line transformed by 3-methyl cholangrene. The size standards used (lane M, with sizes in kb indicated alongside) were products of digestion of pAT153 with EcoRI and pUC8 with TaqI.

In DNA from both HEF and 620-7 cells, and using any of these probes, a single DNA fragment was detected after digestion with any of the three restriction enzymes used, suggesting that each 0.75 kb transcript might be encoded by a single gene (Fig. 2a, b, c). The surprising result was that each clone yielded the same hybridization pattern, even though they had different spectra of hybridization on Northern blots. Neither rearrangement nor gene amplification of these activated genes had occurred in HSV-2-transformed cells. However, we cannot exclude the possibility of mutation of these genes since such modification would not necessarily be detectable by the Southern blot technique.
Cellular genes in HSV-2-transformed cells

Fig. 2. Southern blot analysis of genomic DNA from HEF and 620-7 cells. Ten μg of genomic DNA extracted from HEF (lanes 1, 2 and 3) and from 620-7 cells (lanes 4, 5 and 6) was digested with EcoRI (lanes 1 and 4), BamHI (lanes 2 and 5) and PstI (lanes 3 and 6) and electrophoresed on a 0.7% agarose gel. Blots were hybridized with pAA8 (a), pHD1 (b) and pLC7 (c). Size standards used were from a HindIII digest of λ phage DNA. Sizes in kb are indicated at the left of each blot.

Fig. 3. (a) Run-off transcription analysis of the genes corresponding to pAA8 (row 2), pHD1 (row 3) and pLC7 (row 4) in HEF (column 1) and 620-7 (column 2) isolated nuclei. pUC8 plasmid (row 1) was included to evaluate the non-specific binding and a β-actin cDNA clone (row 5) was used as a positive control. Nuclei from approximately 2 × 10⁷ cells were prepared and nuclear transcription reactions were then performed in the presence of 200 μCi [α-³²P]UTP (600 Ci/mmol; Amersham) for 45 min at 26 °C. (b) RNA polymerase II inhibition experiments were performed with 620-7 cell nuclei by adding α-amanitin (1 μg/ml) to the reaction mixture (column 2). The ³²P-labelled nuclear transcripts were then isolated and 5 × 10⁶ c.p.m. were used in each hybridization to 5 μg of the DNAs indicated for (a) immobilized on Pall Biodyne membranes. A control series (no α-amanitin) is shown in column 1.
Run-off transcription studies in isolated nuclei

In order to determine whether differences in RNA levels were due to different transcriptional activities, nuclei of exponentially growing HEF and 620-7 cells were isolated and assayed by run-off transcription. This technique has found widespread application since the RNA that is synthesized accurately reflects the transcriptional state of the cell (Greenberg & Ziff, 1984; Groudine et al., 1981).

We used the run-off RNA transcripts to probe identical dot blots carrying plasmid DNAs pAA8, pHD1 and pLC7 (Fig. 3). Included on these blots were a β-actin cDNA clone (Farmer et al., 1983) as a positive control and pUC8 plasmid to evaluate the non-specific binding of labelled transcripts. Transcription levels of sequences homologous to pAA8 and pLC7 were higher in 620-7 cells than in HEF. Transcription of pHD1-complementary RNA was almost undetectable, although on the original autoradiogram we could see a very faint signal from nuclei of 620-7 cells but not of control HEF. The control β-actin clone gave an equivalent signal in both HEF and 620-7 cells. Transcription of all three genes was sensitive to α-amanitin and was therefore presumably mediated by RNA polymerase II. This result suggests that the genes corresponding to pAA8 and pLC7 were both regulated, at least in part, at the level of transcription. The mRNA corresponding to pHD1 would seem to be synthesized at a lower rate and thus may have been more stable than that corresponding to the other two clones.

The results clearly indicate that the differential pattern of expression observed on Northern blots was due to altered transcription of the corresponding genes in HSV-2-transformed cells.

DISCUSSION

We have used a cDNA cloning strategy that permitted the isolation and analysis of induced cellular genes in HSV-2-transformed cell lines. This paper describes, for the first time, results showing that cellular genes might be involved in the establishment of the transformed phenotype by HSV-2. Transformation by DNA viruses has been documented for various systems, and the proteins involved have been identified. Examples are SV40 and polyoma virus (T antigens) and adenovirus (E1A) (Tooze, 1980). Such antigens have never been demonstrated in HSV-2-induced cell transformations and there is no evidence for the existence of a viral oncogene in the HSV-2 genome. Moreover, the efficiency of cellular transformation induced by HSV-2 is low when compared with other DNA or RNA tumour viruses. This strongly suggests that the mechanism of cellular transformation induced by HSV-2 is unusual and is the reason for some authors proposing the hypothesis of a 'hit and run' mechanism (Galloway & McDougall, 1983; Skinner, 1976).

The expression of cellular genes in HSV-2-transformed cells has been studied mainly by the analysis of proteins. Suh and co-workers (Suh et al., 1980; Suh, 1982) reported the presence of different polypeptides in HSV-2-transformed cell lines that were immunoprecipitated with a hyperimmune serum directed against HSV-2-infected cells. The expression of these proteins was specific to HSV-2-transformed cells.

Everett (1985) has described experiments in which infection with HSV-1 can stimulate stably integrated non-viral promoters in biochemically transformed cell lines and has shown that this effect is mediated by the immediate early gene products Vmw110 and Vmw175. Macnab et al. (1985) have suggested that cellular polypeptides accumulating as a result of HSV infection may be of importance in the initiation of transformation by HSV, i.e. at the level of immortalization of cells.

Pater et al. (1986) have reported the isolation of cDNA clones for a cellular protein which is up-regulated by HSV infection. HSV induces increased transcription of the gene encoding this protein, and viral protein must be synthesized in the infected cell for this to occur. The host proteins induced could be involved in defence against the virus, or the host gene products induced by the virus may take part in the lytic cycle. Alternatively, they may affect the ability of the virus to establish latency or may even be involved in the oncogenic potential of these viruses (Everett, 1985).
Scott et al. (1983) have described several cDNA clones of RNAs isolated from SV40-transformed cells, which are expressed at higher levels than in their normal parents. The expression of some of these RNAs was considered to be characteristic of fibroblastic cell transformation. It has also been reported that altered cellular gene expression in adenovirus-transformed cells is regulated by post-transcriptional control (Kao & Nevins, 1986).

Our results strongly suggest that the expression of the genes represented by cDNAs pAA8, pH1 and pLC7 are also related to the transformed phenotype. Their expression would not be implicated in immortalization, as indicated by the lack of activation in BHK, NIH 3T3 and C3H cell lines. Furthermore, and in contrast to the results of Scott et al. (1983), the expression of these genes seems to be specific to transformation by HSV-2 (see Fig. 1). The nature of the genes in question is unknown at present. Hybridization to a battery of known retroviral oncogenes (fes, r-fms, H-ras, K-ras, src, fos, myc and raf) did not reveal any detectable homology (data not shown). Thus, we seem to be dealing with sequences that are specific for certain HSV-2-transformed cells.

The cDNAs all showed a specific spectrum of expression as determined by Northern blot analysis. However, Southern blot analysis revealed identical patterns for all three probes. Several possible explanations can be put forward to explain this.

Perhaps these RNAs are members of a multigene family with complementary sequences in the cDNA. Alternatively, these RNAs could arise from one gene by differential initiation, termination or splicing. There are few examples where different transcripts of the same length are produced from the same gene. This is the case for the gene coding for the chromosomal protein HMG-17 gene which is transcribed to give different mRNAs of the same length (Landsman et al., 1986). Heteroduplex analysis of genomic clones and cDNA will enable us to determine the overall localization of exons and introns. Our cDNA inserts are rather small (average approximately 200 bp long). The isolation and sequencing of a complete cDNA clone and a genomic clone will certainly determine whether these transcripts are produced from a gene family or by a differential splicing mechanism. However, it seems that the mRNAs corresponding to each of these cDNAs are regulated by different mechanisms, since one (pAA8) is repressed when 620-7 cells reach a high level of confluence while the other two (pHD1 and pLC7) do not show any altered expression in these conditions. Moreover, each of these cDNAs detects cellular transcripts in a different spectrum of HSV-2-transformed cell lines (e.g. pLC7 is the only one expressed in AH cells). Run-off transcription experiments have also indicated that these genes are regulated by different mechanisms. The transcription rate of pHD1 seems to be much lower than that of the two others, since after 45 min of synthesis (the incubation time chosen for the run-off transcription experiments), only a faint signal is observed on the dot blots. This result indicates that this mRNA is very stable as the transcript detected by hybridization to this clone appears to be very abundant in HSV-2-transformed cells (see Fig. 1b, lanes 2 and 3).

We cannot conclude from our results whether the activation of the cellular genes represented by pAA8, pH1 and pLC7 is directly involved in initiation or maintenance of the transformed phenotype. It is clear, however, that this activation is a direct consequence of transformation by HSV-2 and may represent a step in a cascade of events leading at the end to the transformed state.

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