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Use of a Glucocorticoid-Inducible Promoter for Expression of Herpes Simplex Virus Type 1 Glycoprotein gC1, a Cytotoxic Protein in Mammalian Cells

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Abundant expression of herpes simplex virus type 1 glycoprotein gC (gC1) in transfected mammalian cells has not previously been achieved, possibly because gC1 protein is toxic to cells. To approach this problem, the gC1 coding sequence was placed under the control of the weak but inducible glucocorticoid-responsive promoter from the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). As controls to evaluate for gC1 cytotoxicity, the MMTV LTR promoter was used to express glycoprotein gD1, and a strong, constitutive promoter from the Moloney murine sarcoma virus LTR was used to express gC1. L cells were transfected with these constructs, and a clone expressing gC1 from the inducible MMTV LTR promoter was analyzed. In the absence of glucocorticoid (dexamethasone) stimulation, only a low level of gC1 mRNA expression was detected; after overnight stimulation with dexamethasone, transcription increased approximately 200-fold. Abundant gC1 protein that was functionally active in that it bound complement component C3b, was produced. From passages 5 through 26 (70 cell population doublings), the gC1-producing clone became less responsive to overnight dexamethasone stimulation. The block to gC1 expression occurred at the level of transcription and was associated with hypermethylation of the MMTV LTR DNA. Treatment of the clone with 5-aza-2′-deoxycytidine partially reversed the block in gC1 protein production. Late-passage cells assumed a gC1-negative phenotype that appeared to offer a selective growth advantage, which suggested that gC1 was cytotoxic. Several findings support this view: (i) some cells expressing gC1 after overnight stimulation with dexamethasone assumed bizarre, syncytial shapes; (ii) continuous stimulation with dexamethasone for 5 weeks resulted in death of most cells; (iii) cells transfected with gC1 under the control of the strong Moloney murine sarcoma virus promoter assumed bizarre shapes, and stable gC1-expressing clones could not be established; and (iv) cells induced to express gD1 retained a normal appearance after overnight stimulation or 15 weeks of continuous stimulation with dexamethasone. The inducible MMTV LTR promoter is useful for expressing gC1 and may have applications for expressing other cytotoxic proteins.

Glycoproteins gC1 and gC2 of herpes simplex virus type 1 (HSV-1) and HSV-2, respectively, bind complement component C3b (13, 17, 40, 52). Differences between the C3b-binding activities of gC1 and gC2 have emerged. On the surfaces of HSV-infected cells, only gC1 binds C3b (17); however, as purified glycoproteins, gC1 and gC2 both bind C3b (13, 40). Transient transfection assays have been performed in NIH 3T3 cells with gC1 and gC2 under the control of the Rous sarcoma virus long terminal repeat (LTR) (52). In contrast to HSV-infected cells, transfected cells expressing gC1 or gC2 bind C3b. Detailed comparisons of gC1 and gC2 as C3b-binding proteins are difficult to perform because many fewer cells produce gC1 protein after transfection. The inability to express abundant amounts of gC1 in mammalian cells has also hampered attempts to compare gC1 with other C3b-binding proteins, such as complement receptor type 1, which is present on human leukocytes and shares several biologic properties with gC1 (19).

The gC1 gene, a gamma-regulated gene (reviewed in reference 58), has not been stably expressed in mammalian cells under the control of its own promoter. However, a permanent cell line has been developed in which gC1 is under the control of its own promoter and is expressed after superinfection with a gC-negative mutant of HSV-1 (2). Recently, limited amounts of gC1 have been produced in permanent cell lines by placing the gC1-coding sequence under the control of the glycoprotein gD (gD1) promoter, a beta-regulated gene, and transfecting this construct into cells containing the HSV ICP4 and ICP47 immediate-early genes (48). gB and gD have been stably expressed in mammalian cells under the control of strong, constitutive promoters (1, 3, 45, 48). To date, no such success has been reported for gC1, possibly because gC1 is cytotoxic; i.e., high levels of gC1 expression are lethal to cells. To address this problem, the gC1-coding sequence was placed under the control of a weak but inducible promoter derived from the mouse mammary tumor virus (MMTV) LTR.

MMTV DNA, expressed in transfected cells, has been shown to respond to glucocorticoid stimulation (5, 30). The sequence element required for this response is located in the viral LTR (15, 25, 28, 34). The addition of dexamethasone (Dex), a synthetic glucocorticoid, to the culture medium of

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transfected cells leads to an increase in the level of RNA polymerase II molecules, initiating transcription at the MMTV LTR promoter (16), and to an approximately 100-fold accumulation of the corresponding specific mRNA (6).

Reports describing the MMTV LTR as an inducible promoter can be considered in three categories. The first includes those designed to evaluate regulatory mechanisms of the MMTV LTR. The second uses the MMTV LTR to examine the function of genes placed under its control. In this category, the MMTV LTR promoter has been used to regulate expression of various oncogenes, including the middle T antigen of polyomavirus (47), v-*ras* (28), v-*mos* (46, 56), and v-*src* (31), and recently to express gG of HSV-2 (54). A third application, used in this study, is the induced expression of genes whose products are cytotoxic (33). The MMTV LTR promoter has certain advantages over other inducible promoters, such as heat shock or metallothionein. In the uninduced state, MMTV LTR is a weak promoter whereas base-line transcription from the metallothionein promoter is considerable (26, 39, 59), an obvious problem if the goal is to avoid cytotoxicity. The heat shock promoters require elevated temperatures for induction (60), which may be detrimental to cells or to the protein under study.

In this report, we demonstrate that abundant amounts of gC1 protein are made after Dex stimulation of Ltk$^-$ cells transfected with the gC1 gene under the control of the MMTV LTR promoter. The gC1 protein was cytotoxic in that L cells induced to express the protein assumed bizarre, syncytial shapes and did not survive continuous Dex stimulation. In contrast, induction of the gD1 gene did not have this effect. Cells transfected with the gC1 gene, when grown in the absence of Dex, remained responsive to hormonal stimulation for at least 100 population doublings.

MATERIALS AND METHODS

Preparation of gC1 constructs. Two constructs were made: pMMTV-gC, with gC1 under the control of the MMTV LTR-inducible promoter, and pMSV-gC, with gC1 under the control of the strong, constitutive Moloney murine sarcoma virus (Mo-MSV) LTR promoter (Fig. 1).

(i) Polyadenylation signal. A 530-base-pair (bp) *Sma*I-to-*Sac*I fragment was excised from the Mo-MSV LTR contained in plasmid pMLTR (57) and ligated into the corresponding sites of the M13 polylinker in pSP64. This fragment contains imperfect repeated simian virus 40 enhancerlike sequences located 3′ of the polyadenylation signal (35, 55). When positioned 3′ of the gene, the enhancer activity is diminished (42); however, a transcriptionally active Dex-responsive region has been detected in enhancer elements (42) that are similar to those present in this construct.

(ii) Promoter for plasmid pMMTV-gC. The MMTV LTR promoter was excised with *Pst*I and *Pvu*II from a plasmid containing almost the entire MMTV LTR (15), which was derived from a complete clone of exogenous MMTV DNA (5). The resulting 1.35-kbp fragment was ligated into the *Pst*I and *Hin*cII sites of the M13 polylinker of pSP64. This fragment contains the TATA box and the complete glucocorticoid regulatory element extending 5′ to approximately position −200 bp (6, 29, 36).

(iii) Promoter for pMSV-gC. The Mo-MSV LTR promoter was excised from pMLTR with *Hin*dIII and *Sma*I and inserted into *Hin*dIII and *Hin*cII sites of the M13 polylinker in pSP64.

(iv) gC1 gene. The gC1 DNA was obtained from the *Bam*HI fragment (20) of HSV-1 NS cloned into pBR322.

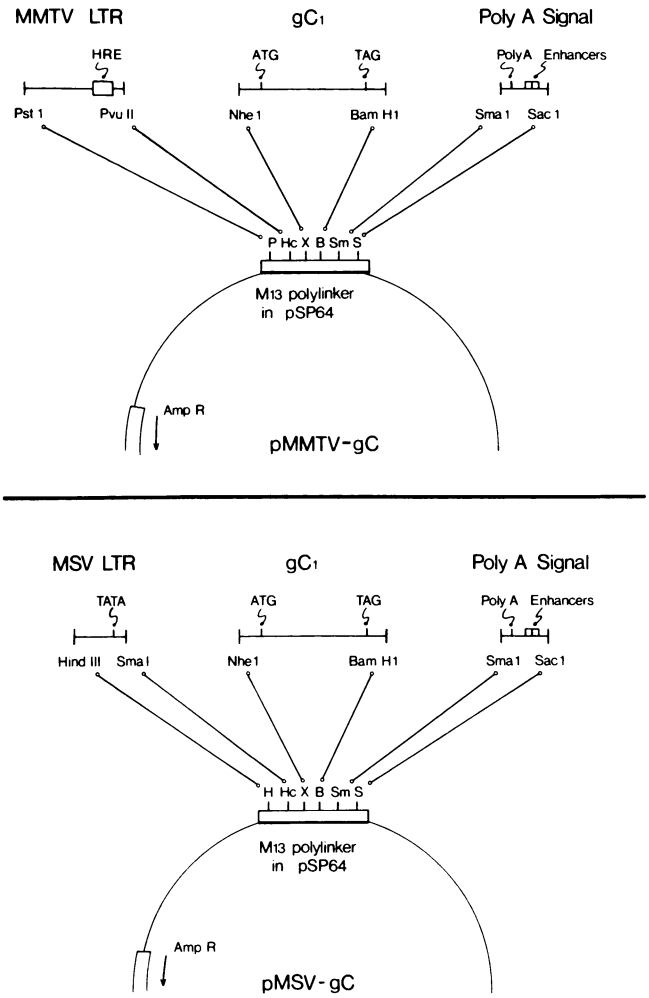

FIG. 1. DNA fragments comprising the pMMTV-gC (glucocorticoid-inducible) or pMSV-gC (strong, constitutive) gC1 expression plasmid. Origins of the DNA inserts are described in Materials and Methods. All fragments were cloned into the M13 polylinker site of pSP64. Abbreviations: HRE, hormone regulatory element of MMTV LTR; P, *Pst*I; H, *Hin*dIII; Hc, *Hin*cII; X, *Xba*I; B, *Bam*I; Sm, *Sma*I; S, *Sac*I.

This plasmid was digested with *Nhe*I and *Bam*HI; the former removes the gC1 promoter by cutting between the TATA box and the gC1 translation start site (52). The 1.97-kbp *Nhe*I-*Bam*HI fragment was ligated into the *Xba*I and *Bam*HI sites of the M13 polylinker in pSP64.

Preparation of gD1 construct. The gD1 gene from HSV-1 Patton was excised from plasmid pRE4 (10) by using *Hin*dIII. This removes the gD1 promoter, leaving the gD1-coding sequence intact. The excised fragment was blunt ended, *Bam*HI linkers were attached, and the fragment was cloned into the *Bam*HI site of the M13 polylinker.

Cell cultures, transfections, and gC1 or gD1 induction. Ltk$^-$ cells were cotransfected (24) with a gC1- or gD1-containing construct and with plasmid pX343, which confers resistance to hygromycin B (4). Surviving cells were selected in the presence of 200 μg of hygromycin B per ml, pooled, and cloned to obtain colonies derived from a single cell. Cultures were maintained in the absence of Dex and were grown in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum. Cells were passaged at a 1:10

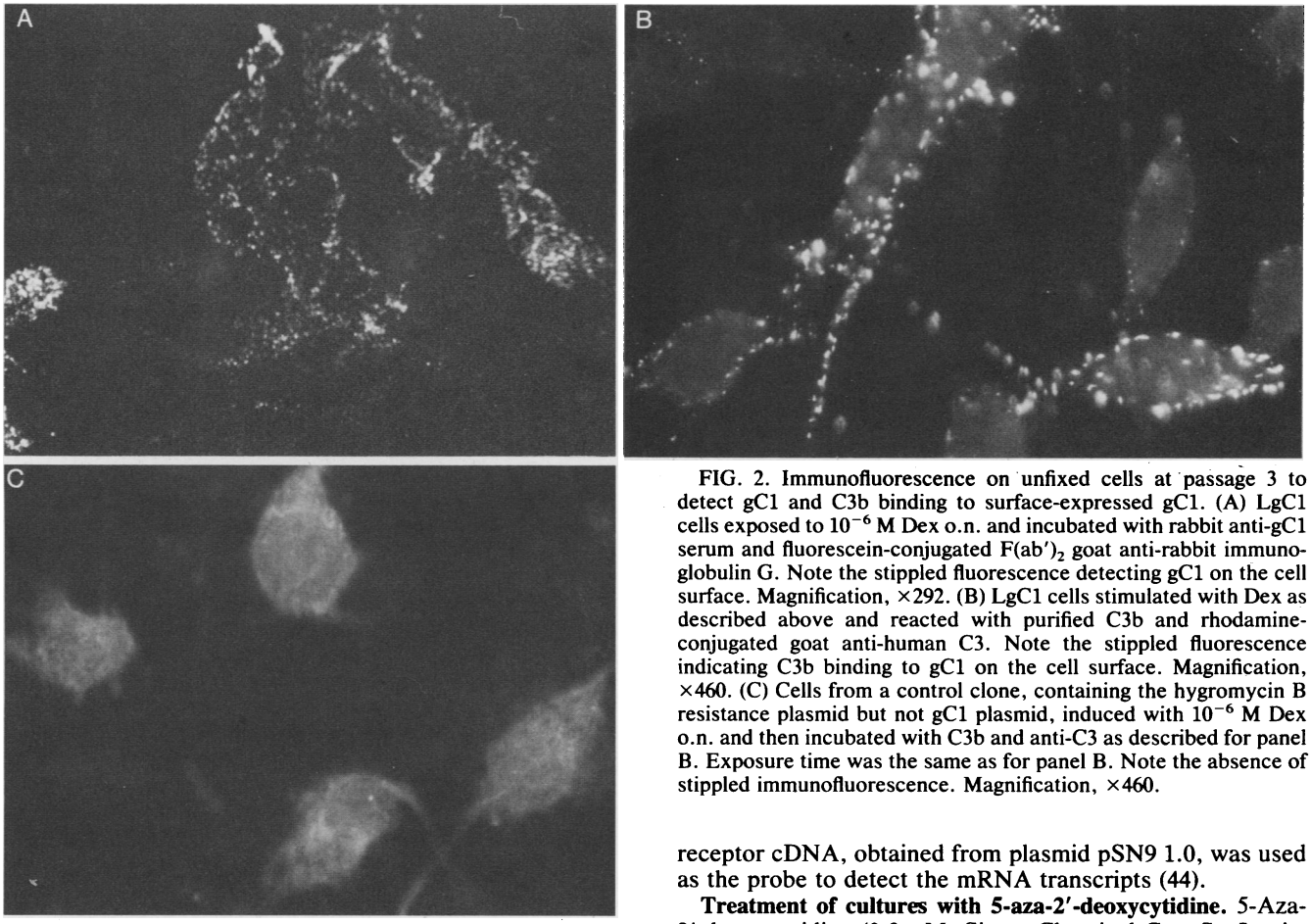

FIG. 2. Immunofluorescence on unfixed cells at passage 3 to detect gC1 and C3b binding to surface-expressed gC1. (A) LgC1 cells exposed to 10^{-6} M Dex o.n. and incubated with rabbit anti-gC1 serum and fluorescein-conjugated $F(ab')_2$ goat anti-rabbit immunoglobulin G. Note the stippled fluorescence detecting gC1 on the cell surface. Magnification, ×292. (B) LgC1 cells stimulated with Dex as described above and reacted with purified C3b and rhodamine-conjugated goat anti-human C3. Note the stippled fluorescence indicating C3b binding to gC1 on the cell surface. Magnification, ×460. (C) Cells from a control clone, containing the hygromycin B resistance plasmid but not gC1 plasmid, induced with 10^{-6} M Dex o.n. and then incubated with C3b and anti-C3 as described for panel B. Exposure time was the same as for panel B. Note the absence of stippled immunofluorescence. Magnification, ×460.

ratio weekly. To induce the MMTV LTR promoter, cultures were exposed overnight (o.n.; 15 h) to 10^{-6} M Dex.

Assays for gC1 expression. (i) Southern and Northern (RNA) blotting. Nuclear DNA or cytoplasmic RNA was extracted, and poly(A)$^+$ RNA was prepared by oligo(dT) chromatography (37). Southern transfers were performed by using nitrocellulose (37); Northern transfers were performed by using GeneScreen (Dupont, NEN Research Products, Boston, Mass.) as recommended by the manufacturer.

(ii) Western blotting (immunoblotting). Cytoplasmic extracts were prepared, electrophoresed on 10% polyacrylamide gels, and transferred to nitrocellulose as previously described (53). gC1 was detected by using polyclonal rabbit anti-gC1 serum (13).

(iii) Dot blot immunoassay. Cultures were harvested at confluence. A 2-μl amount of a serial twofold dilution of cytoplasmic extracts was blotted onto nitrocellulose, and gC1 was detected by using rabbit anti-gC1 serum and ^{125}I-protein A (9).

(iv) Immunofluorescence. gC1 was detected by indirect immunofluorescence, using either polyclonal rabbit anti-gC1 serum (rabbit serum 46) or purified complement component C3b (13, 17).

Assays for gD1 expression. Dot blot immunoassays and immunofluorescence at various passage levels were performed on the gD1-expressing cell clone as described above, using polyclonal rabbit anti-gD1 serum (rabbit serum 2).

Assays for glucocorticoid receptor mRNA. Northern blots were performed as described above. Murine glucocorticoid receptor cDNA, obtained from plasmid pSN9 1.0, was used as the probe to detect the mRNA transcripts (44).

Treatment of cultures with 5-aza-2′-deoxycytidine. 5-Aza-2′-deoxycytidine (0.3 μM; Sigma Chemical Co., St. Louis, Mo.) was added to subconfluent cultures for 24 h (41). Cells were then washed, allowed to grow for an additional 48 h, and harvested for analysis of gC1 protein.

RESULTS

Expression of gC1 in mammalian cells. Ltk$^-$ cells were transfected with a plasmid conferring resistance to hygromycin B and with either pMMTV-gC (inducible promoter) or pMSV-gC (strong, constitutive promoter). Cells surviving hygromycin B selection were pooled and examined for gC expression by immunofluorescence. In the absence of Dex induction, only a rare cell containing pMMTV-gC was positive for gC1 antigen; however, after o.n. stimulation with 10^{-6} M Dex, 1 to 5% of cells became gC1 antigen positive. Similarly, 1 to 5% of pMSV-gC cells were antigen positive.

Single-cell clones were prepared from cultures transfected with pMMTV-gC. Of the 100 clones analyzed, 6 were positive for gC1 by immunofluorescence after o.n. Dex stimulation. One clone, designated LgC1, expressed gC1 in almost 100% of cells after Dex stimulation and in <1% of cells when unstimulated. This clone was chosen for further study. Despite repeated attempts, no such clone could be established from the pMSV-gC construct. When gC1 was expressed under the control of this strong, constitutive promoter, cells assumed a bizarre, elongated shape and were unable to grow into a clone. The uncloned parental cell line lost all gC1-expressing cells after approximately 3 months (43 cell population doublings).

The time course of appearance of gC1 protein was examined by exposing LgC1 cells to 10^{-6} M Dex for 0, 3, 6, 12, 18, 24, or 48 h. Cytoplasmic extracts were evaluated by dot blot immunoassay. Inducible expression of gC1 was first detected at 6 h and reached maximum levels at 12 h. Glycoprotein gC1 levels remained constant through 24 h and decreased slightly at 48 h. For subsequent experiments, cells were stimulated o.n. (15 h) with 10^{-6} M Dex.

LgC1 cells were examined by immunofluorescence to determine the distribution of gC1 and its ability to bind C3b. When cells were stimulated o.n. with 10^{-6} M Dex, gC1 antigen was detected on the cell surface (Fig. 2A) and the expressed gC1 bound C3b (Fig. 2B). In contrast, a control clone, which contained the hygromycin B resistance gene only, failed to bind C3b after o.n. Dex stimulation (Fig. 2C). The ability of gC1 in LgC1 cells to bind C3b indicated that the expressed protein was biologically active, with properties similar to those of the protein in HSV-1-infected cells (18).

Long-term stability of clone LgC1. To examine the long-term stability of gC1 expression, clone LgC1 was maintained in the absence of Dex and stimulated o.n. with Dex at various passage levels. At passage 3, close to 100% of Dex-induced cells were gC1 antigen positive, as judged by immunofluorescence. By passage 14, 10% of cells were antigen positive; this proportion decreased to 1% by passage 18. This decline in inducible gC1 expression was reproduced on five occasions when early-passage LgC1 cells were revived from liquid nitrogen and monitored over time. Increasing the concentration of Dex for o.n. stimulation did not increase gC1 expression in late-passage cells.

To further quantitate the decrease in gC1 induction, cytoplasmic extracts were prepared at every second passage of LgC1 cells from passages 5 to 23, which represents a span of 60 cell population doublings. Abundant gC1 (3 $\mu g/10^7$ cells) was detected by dot blot immunoassay at passage 5. This yield decreased to approximately 750 ng at passage 11, to 375 ng at passage 17, and to undetectable levels at passage 23 (Fig. 3). These results indicated that clone LgC1 did not remain responsive indefinitely to Dex stimulation.

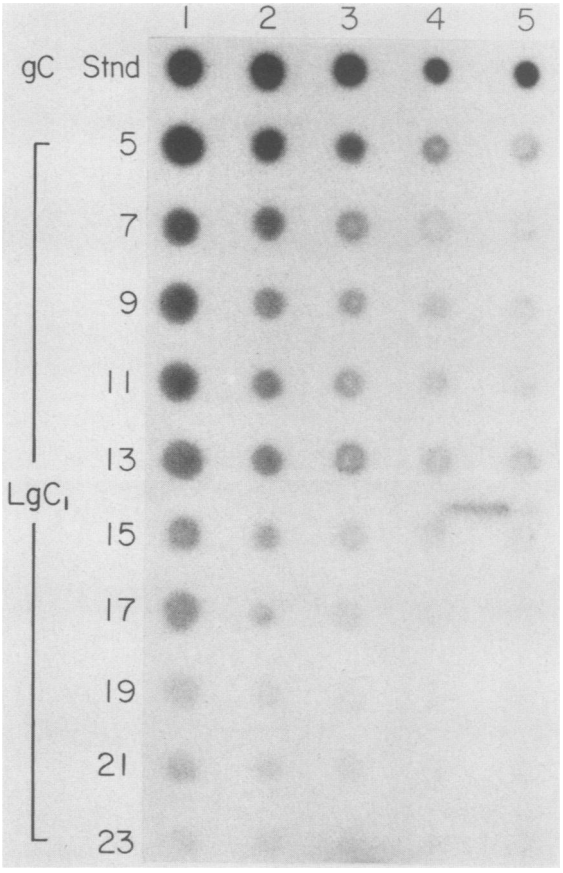

FIG. 3. Dot blot immunoassay for gC1 protein in clone LgC1. At every other passage between passages 5 and 23, cells were stimulated o.n. with 10^{-6} M Dex, and cytoplasmic extracts were prepared. Lanes: 1, undiluted extract; 2 to 5, serial twofold dilutions (1:2 to 1:16). Stnd, Purified gC1 standard containing serial twofold dilutions of gC1, ranging from 6 ng to 375 pg in lanes 1 to 5.

Evaluation of gC1 DNA, mRNA, and protein at early and late passages of clone LgC1. To define the mechanism for loss of gC1 inducibility, nuclear DNA, poly(A)$^+$ RNA, and gC1 protein were analyzed at passages 5 and 26 of LgC1 cells. Passage 26 represents approximately 100 population doublings (assuming that it took approximately 16 population doublings to progress from a single cell to a confluent monolayer). Figure 4 shows a Southern blot probing for gC1 DNA in clone LgC1 at passages 5 and 26 and in a control clone that contained the plasmid conferring resistance to hygromycin B but not the pMMTV-gC construct. Nuclear DNA was digested for 18 h with *Bam*HI and *Hin*dIII. These enzymes cut at the 5′ end of the MMTV LTR and at the 3′ end of the gC1 gene, separating plasmid sequences from cellular DNA. The blot was probed with the *Nhe*I and *Bam*HI fragments of gC1 DNA. gC1 DNA was stable from passages 5 to 26 (Fig. 4, lanes 5 to 8) and was present in approximately 100 copies per cell (lane 2). As expected, o.n. stimulation with Dex had no effect on the amount of gC1 DNA detected (compare lanes 5 and 6 with lanes 7 and 8), and gC1 DNA was not detected in the control clone (lanes 9 to 11). These results demonstrate that no loss in gC1 DNA had occurred during passage of clone LgC1. Therefore, some other mechanism must account for the decrease in gC1 protein production at passage 26.

Figure 5 shows a Northern blot probing for gC1 poly(A)$^+$ RNA at early and late passage of clone LgC1 and of the control clone. A 5-μg sample of poly(A)$^+$ RNA was loaded in each of lanes 3 to 7, and the blot was probed with nick-translated DNA comprising the gC1-coding sequence. Abundant gC1 mRNA was detected at passage 5 (lane 3) when cells were induced o.n. with Dex. In contrast, much less gC1 mRNA was noted in Dex-stimulated cells at passage 26 (lane 4). Densitometry analysis of a light exposure of the gel shown in Fig. 5 indicated a 40-fold difference between passages 5 and 26, which suggested that the block to gC1 expression was occurring at the level of transcription. Faintly discernible was the gC1 mRNA made at passage 5 in the absence of o.n. Dex stimulation (lane 5). Comparison of LgC1 at passage 5 with and without Dex stimulation showed an approximately 200-fold difference in the amount of gC1 mRNA (lane 3 versus lane 5). This result indicated that a low level of constitutive expression of gC1 mRNA occurred from the uninduced promoter and that Dex markedly increased transcription. No gC1 mRNA was detected at passage 26 in the absence of Dex (lane 6) or in the control clone (lane 7). From these results, we conclude that the number of cells transcribing gC1, the amount of gC1 transcribed per cell, or the stability of the gC1 mRNA had declined between passages 5 and 26.

Figure 6 shows a Western blot to detect gC1 protein.

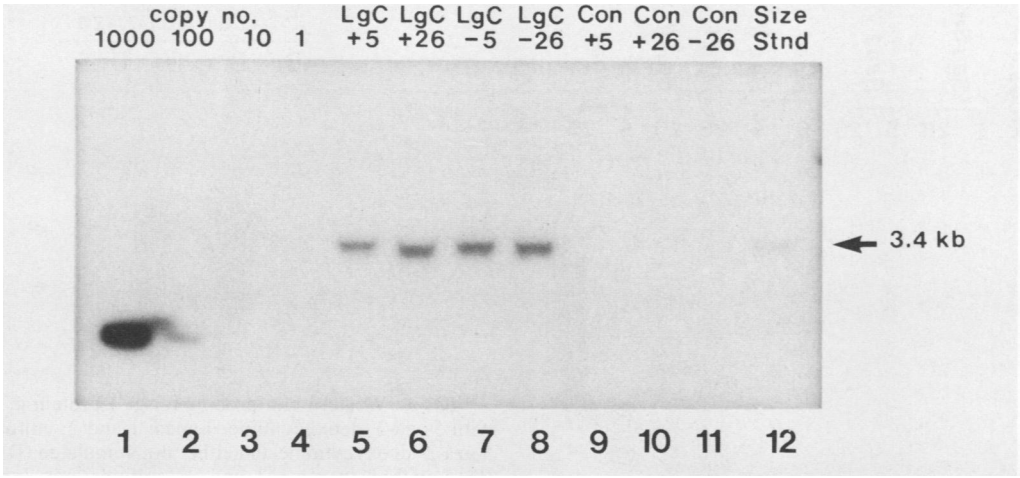

FIG. 4. Southern blot probing for gC1 DNA in clone LgC1 and in a control clone containing the hygromycin resistance gene. Lanes: 1 to 4, gC1 standards with 1,000, 100, 10, and 1 copy of pMMTV-gC per cell; 5 to 8, clone LgC1 at passage 5 or 26 after o.n. stimulation with (+) or without (−) Dex; 9 to 11, control clone at passage 5 or 26 in the presence (+) or absence (−) of o.n. Dex stimulation. Size Stnd, Standard for the expected size of the pMMTV-gC plasmid after digestion with *Hin*dIII and *Bam*HI. The gel was developed for 18 h, which was optimal for calculating DNA copy number.

Abundant amounts of gC1 were made in induced passage 5 cells (lane 5). Both precursor and fully processed forms of gC1 were present (asterisks). In contrast, induced passage 26 cells expressed faintly detectable amounts of gC1 (lane 6). In the absence of o.n. Dex stimulation, gC1 production was barely detectable at passage 5 (lanes 2 and 3; lane 3 contained twice as much protein as did lane 2) and was undetectable at passage 26 (lane 4). These results agree with those of the immunofluorescence, dot blot, and Northern blot assays, indicating a low level of constitutive expression of gC1 from uninduced cells, a marked increase in gC1 protein production after Dex stimulation at early passage, but a decrease in Dex inducibility at later passage.

Changes in clone LgC1 that may account for the decrease in gC1 transcription at late passages. gC1-containing cells assume a gC1-negative phenotype over time in culture. Since the presence of a functional receptor for Dex is required to obtain a response to hormone stimulation (reviewed in reference 22), we asked whether down-modulation of the glucocorticoid receptors of LgC1 cells occurred with passage. At passages 5 and 26, the LgC1 cells and the control clone (containing the hygromycin resistance gene only) were probed for glucocorticoid receptor mRNA. Cells were maintained in the absence of Dex and stimulated o.n. with 10^{-6} M Dex before extraction of RNA and isolation of poly(A)$^+$ RNA. In both control and gC1-containing cells, 5- and 7-kbp glucocorticoid receptor transcripts (44) were present. No decrease in glucocorticoid receptor mRNA was noted in

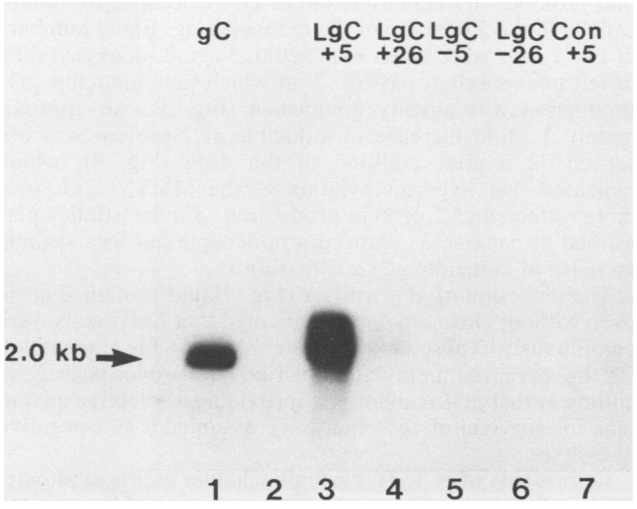

FIG. 5. Northern blot of poly(A)$^+$ RNA prepared from clone LgC1 and from a control clone. Lanes: 1, size of the *Nhe*I-*Bam*HI fragment comprising the gC1-coding sequence; 3 to 6, gC1 poly(A)$^+$ RNA from LgC1 cells at passage 5 or 26 in the presence (+) or absence (−) of o.n. Dex stimulation; 7, control clone containing the hygromycin resistance gene at passage 5 after o.n. Dex stimulation.

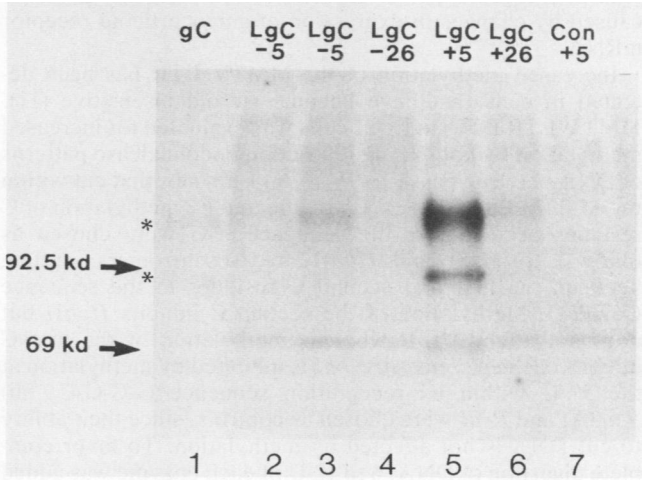

FIG. 6. Western blot probing for gC1 protein, using polyclonal rabbit anti-gC1 serum (rabbit serum 46). Lanes: 1, 5 ng of purified gC1 as a standard (*, positions of precursor and fully glycosylated forms of gC1); 2 to 6, cytoplasmic extracts from clone LgC1 at passage 5 or 26 with (+) or without (−) o.n. Dex stimulation; 3, twice the amount of protein added as in lane 2 (both lanes contained extracts from LgC1 at passage 5 without o.n. Dex stimulation); 7, control clone (hygromycin resistance gene only) at passage 5 after o.n. Dex stimulation. Arrows indicate positions of molecular size markers (in kilodaltons).

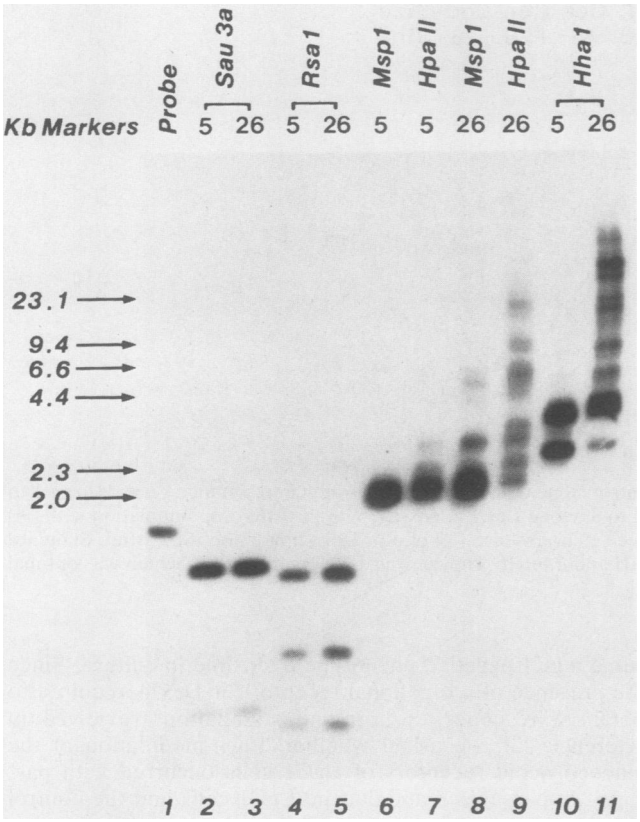

FIG. 7. Southern blot probing for MMTV LTR DNA in LgC1 at passages 5 and 26. DNA at passages 5 and 26 was digested with the indicated restriction enzymes. The blot was probed with MMTV LTR DNA cut with *Pst*I and *Xma*III, which spans almost the entire LTR (lane 1). Arrows indicate size standards.

LgC1 cells between passages 5 and 26, which indicated that diminished gC1 expression with passage in culture was not caused by changes in expression of glucocorticoid receptor mRNA.

Increased methylation of the MMTV LTR has been detected in cells that have become steroid insensitive (11). MMTV LTR DNA in LgC1 cells was evaluated for increased methylation by comparing restriction endonuclease patterns of DNA between passages 5 and 26. Enzymes that cut within the MMTV LTR DNA and are affected by methylation of C residues occurring in the sequence C-G were chosen as follows. (i) *Msp*I and *Hpa*II are isoschizomers, cutting between the first and second C residues in the sequence C-C-G-G. Methylation of the second C inhibits *Hpa*II but does not affect *Msp*I, whereas methylation of the first C inhibits each enzyme. (ii) *Hha*I is inhibited by methylation at the 5′ C within its recognition sequence G-C-G-C. (iii) *Sau*3AI and *Rsa*I were chosen as controls, since their ability to cut DNA is not affected by methylation. To favor complete digestion of DNA, 6 to 12 U of each enzyme was added per μg of DNA for 16 h, and then 3 to 6 U was added per μg of DNA for an additional 2 h.

Figure 7 shows a Southern blot in which 2 μg of DNA was loaded per lane and probed with a 1.3-kbp *Pst*I-*Xma*III fragment spanning almost the entire MMTV LTR (lane 1). Evidence for increased methylation of the MMTV LTR over passage in culture is as follows: (i) minor differences in the sizes of DNA digests prepared with *Msp*I versus *Hpa*II were present at passage 5, which indicated methylation of the internal C of the sequence C-C-G-G (lanes 6 and 7); (ii) comparison of *Msp*I and *Hpa*II showed major differences at passage 26, which indicated further methylation of the internal C (lanes 8 and 9); (iii) major differences were detected with *Hha*I, which indicated methylation at the 5′ C in the sequence GCGC by passage 26 (lanes 10 and 11); and (iv) in contrast, both control enzymes (*Sau*3AI and *Rsa*I) showed identical restriction patterns at passages 5 and 26 (lanes 2 versus 3 and lanes 4 versus 5). These control enzymes cut at more than one site within the MMTV LTR, which accounts for the detection of fragments smaller than the probe. The patterns of *Msp*I digestion were different at passages 5 and 26 (lanes 6 and 8), probably because increased methylation had also occurred at the first C in the recognition sequence. The conclusion from these studies is that hypermethylation of the MMTV LTR is detected in comparison of early- with late-passage LgC1 cells.

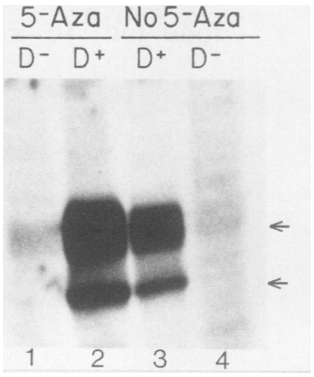

FIG. 8. Western blot probing for gC1 protein in cultures treated with 5-aza-2′-deoxycytidine. Lanes: 1 and 2, cultures treated with 5-aza-2′-deoxycytidine and either not stimulated (D−) or stimulated (D+) with Dex o.n.; 3 and 4, cultures not exposed to 5-aza-2′-deoxycytidine. Arrows show positions of high-mannose precursor and fully glycosylated gC1.

As further evidence for hypermethylation, LgC1 cells were exposed to 5-aza-2′-deoxycytidine, a cytidine analog that promotes hypomethylation of DNA because of modifications in the 5 position of the cytosine ring. Equal numbers of LgC1 cells were either exposed to 5-aza-2′-deoxycytidine or left unexposed at passage 7, at which time inducible gC1 production was already diminished (Fig. 3). An approximately 2.5-fold increase in inducible gC1 protein was observed 72 h after addition of the drug (Fig. 8), which indicated that hypermethylation of the MMTV LTR promoter affected gC1 protein production. Similar studies performed at passage 35 showed a noticeable but less striking increase in inducible gC1 expression.

The detection of gC1 mRNA (Fig. 5) and protein (Fig. 6) even without Dex stimulation indicated that LgC1 cells were continuously exposed to this protein. A possible explanation for the increased methylation of LgC1 cells over passage in culture is that gC1 is cytotoxic, providing a selective advantage for survival of cells that have assumed a gC1-negative phenotype.

Cytotoxicity of gC1. To evaluate whether gC1 is cytotoxic, the basal level of gC1 production was increased by continuous exposure of clone LgC1 to Dex. Cells were first stimulated o.n. with various Dex concentrations to determine the dose response to induction. Exposure of cells o.n. to 10^{-6}, 10^{-7}, and 10^{-8} M Dex induced gC1 production (Fig. 9A, rows 2 to 4), whereas exposure to 10^{-9} M Dex did not (row

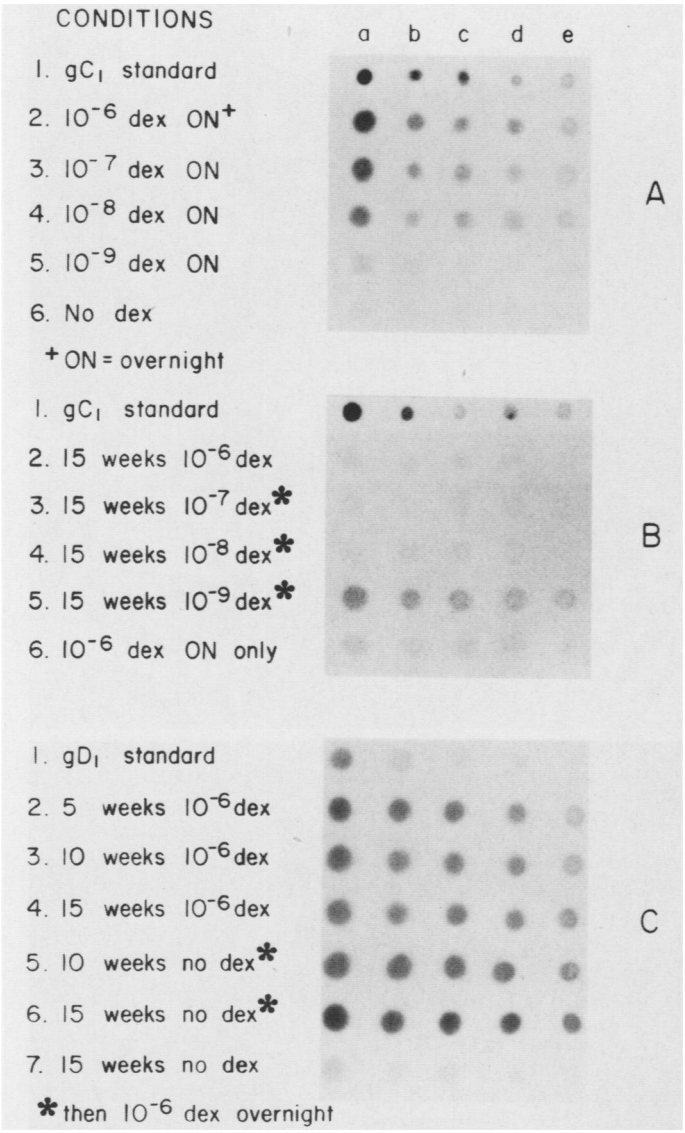

FIG. 9. Dot blot immunoassay. (A) Response of clone LgC1 at passage 6 to o.n. Dex stimulation. Rows: 1, gC1 standard containing 6 ng, 3 ng, 1.5 ng, 750 pg, and 375 pg in columns a to e, respectively; 2 to 5, cells stimulated overnight with 10^{-6} to 10^{-9} M Dex; 6, cells not stimulated with Dex; columns a to e contain serial twofold dilutions of cell extracts. (B) Effects of prolonged Dex stimulation on gC1 expression. Rows: 1, gC1 standard, diluted as in panel A; 2 to 5, gC1-expressing clone exposed continuously for 15 weeks to 10^{-6} to 10^{-9} M Dex, after which Dex was withdrawn and surviving cells were stimulated o.n. with 10^{-6} M Dex; 6, gC1-expressing clone not stimulated with Dex and exposed after 15 weeks to 10^{-6} M Dex o.n. (C) Results for gD1-expressing cells exposed continuously for 5 to 15 weeks to Dex. Rows: 1, gD1 standard containing 20, 10, 5, 2.5, and 1.25 ng in columns a to e, respectively; 2 to 4, cells stimulated continuously with 10^{-6} M Dex for 5, 10, or 15 weeks (passages), respectively; 5 and 6, cells grown in the absence of Dex, passaged at weekly intervals, and, at passage 10 (row 5) or 15 (row 6), stimulated o.n. with 10^{-6} M Dex; 7, cells at passage 15 with no Dex stimulation.

5). Cells were maintained in 10^{-6}, 10^{-7}, 10^{-8}, or 10^{-9} M Dex continuously for 15 weeks, and cultures were observed for cytopathic effects. The experiment using continuous Dex stimulation was performed at passage 5, at which point, as determined by immunofluorescence, more than 99% of the cells expressed gC1 when stimulated o.n. with 10^{-6} M Dex. Continuous stimulation with 10^{-6}, 10^{-7}, or 10^{-8} M Dex resulted in the appearance of bizarre, large cells with abundant cytoplasm and some multinucleated cells (Fig. 10A). These cells were first noted after 1 week of Dex stimulation. Over a 5-week period, almost every cell died in cultures exposed to 10^{-6}, 10^{-7}, or 10^{-8} M Dex. The rare survivors had a normal cell morphology. These normal cells eventually repopulated the culture flask but did not express gC1 when stimulated o.n. with 10^{-6} M Dex (Fig. 9B, rows 2 to 4). In contrast, LgC1 cells continuously exposed to 10^{-9} M Dex did not assume bizarre shapes or die. These latter cells remained responsive to o.n. stimulation with 10^{-6} M Dex (Fig. 9B, row 5). These results indicated that when Dex was used at concentrations which induced gC1 production, most cells in the culture died; the rare survivors were those which were Dex nonresponsive. This finding supports the concept that gC1 is cytotoxic and indicates that the surviving LgC1 cells had assumed a gC-negative phenotype.

A similar experiment was performed on LgC1 cells at passage 12, at which point only 10% of cells induced with Dex o.n. expressed gC1, as assessed by immunofluorescence. At this passage, continuous exposure to 10^{-6} M Dex resulted in many fewer cells assuming bizarre and syncytial shapes than at passage 5 (Fig. 10B). No bizarre or syncytial cells were detected in passage 12 cells not continuously exposed to Dex (Fig. 10C). Immunofluorescence was performed to examine the distribution of gC1 in the passage 12 cultures continuously stimulated with Dex. gC1 was detected only in bizarre, syncytial cells and not in normal-appearing surrounding cells (Fig. 10D and E). These results support the hypothesis that gC1 is cytotoxic, since the bizarre, syncytial cells were the ones expressing gC1.

Several control experiments were performed to evaluate the effects of continuous Dex stimulation on cell morphology and survival. A control clone, containing the hygromycin resistance gene but not the gC1 gene, was grown in the presence of Dex. Cell morphology appeared normal. Most important, another HSV-1 glycoprotein, gD1, was placed under the control of the MMTV LTR promoter. Clones derived from single cells were obtained, one of which (LgD1) was chosen for further study. At passage 5, 100% of LgD1 cells were positive for gD1 by immunofluorescence after o.n. stimulation with 10^{-6} M Dex but were negative in the absence of Dex. Most gD1-expressing cells maintained normal cell morphology after continuous stimulation with 10^{-6} Dex, although an occasional syncytium was seen (Fig. 10F, arrow). The gD1-expressing clone showed no decrease in gD1 production, as assessed by dot blot immunoassay, despite continuous stimulation for 5 to 15 weeks with 10^{-6} M Dex. The amount of gD1 produced after continuous Dex stimulation (Fig. 9C, rows 2 to 4) was similar to that found after o.n. stimulation (rows 5 and 6), in sharp contrast to the marked decrease in gC1 production that occurred under similar conditions of continuous Dex stimulation (compare gD1 production [Fig. 9B, row 4] with gC1 production [Fig. 9B, row 2]). These results indicate that the bizarre morphology of the LgC1 clone after continuous Dex stimulation was specific for gC1-containing cells. In addition, the decrease in gC1 inducibility over time, which occurred even in cultures grown in the absence of Dex, was also specific for gC1, since no such decrease was noted with gD1 (compare gC1 in Fig. 3, passage 15, with gD1 in Fig. 9C, row 4).

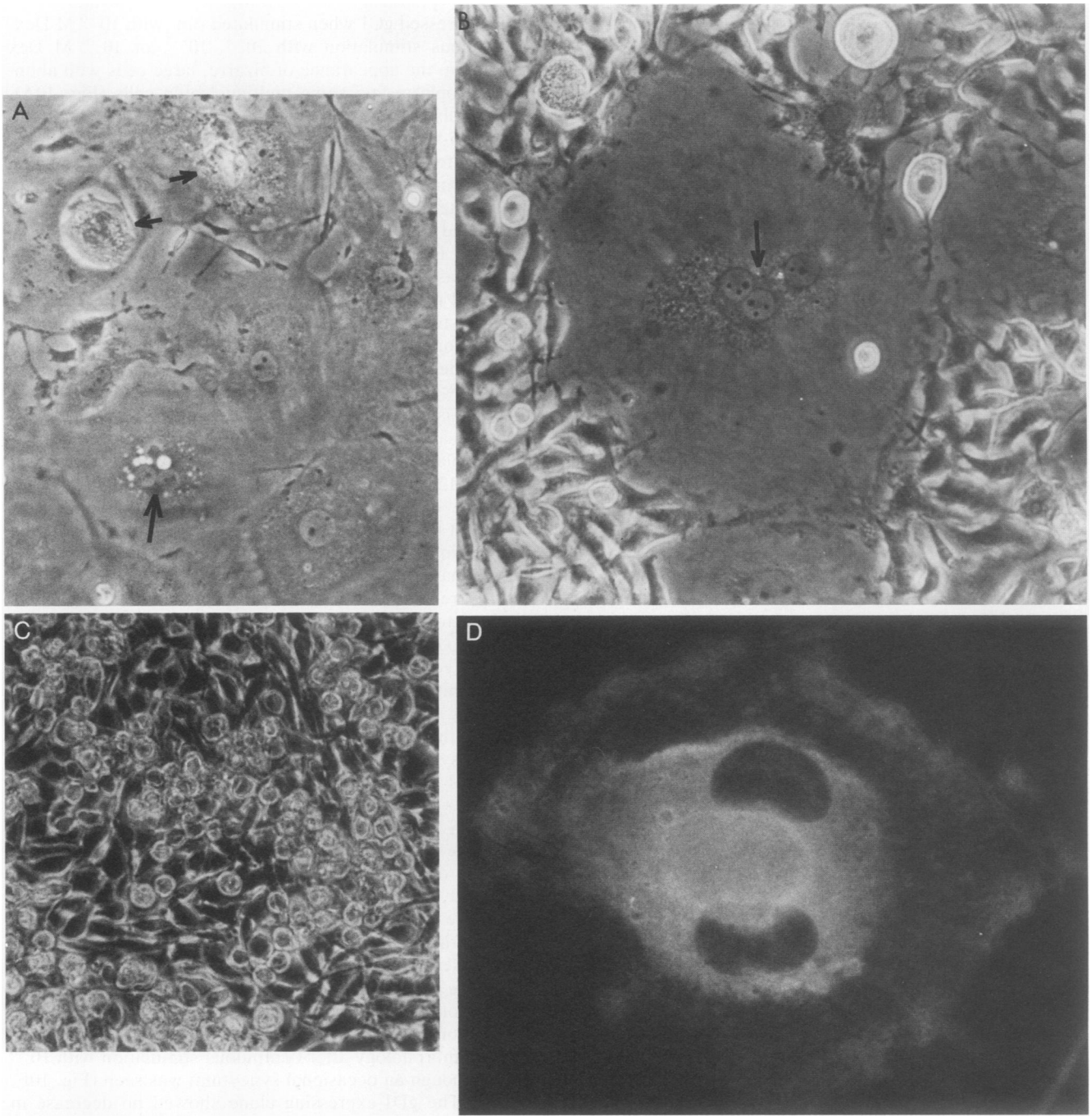

FIG. 10. (A) Phase-contrast micrograph of clone LgC1 at passage 5 exposed to 10^{-6} M Dex continuously for 3 weeks (magnification, ×340). Virtually every cell became markedly abnormal, with swollen cytoplasm and multinucleated (large arrow) or degenerating (small arrows) nuclei. (B) Phase-contrast micrograph of clone LgC1 at passage 12 after 5 weeks of 10^{-6} M Dex stimulation (magnification, ×340). Syncytia (arrow) as well as many normal-appearing cells can be seen. (C) Passage 12 LgC1 cells prepared as for panel B but without Dex stimulation (magnification, ×340). No syncytia were present. (D and E) Immunofluorescence of cells shown in panel B, acetone fixed and stained for gC1 (magnification, 536). A syncytium (D) and a bizarre, syncytial cell with long dendritic processes (E) were positive for gC1. (F) LgD1 cells stimulated continuously for 3 weeks with 10^{-6} M Dex (magnification, ×340). In contrast to the cells shown in panel A, morphology remained virtually normal, with only an occasional enlarged cell seen (arrows).

DISCUSSION

We have studied the properties of a cell clone containing the gC1 gene under the control of the glucocorticoid-responsive MMTV LTR promoter. At early passage of this clone, gC1 expression from the uninduced promoter was low, whereas o.n. Dex stimulation produced a 200-fold increase in gC1 mRNA. This marked stimulation has several possible explanations. First, the high copy number of plasmids in the clone may result in neighboring LTRs exerting a positive

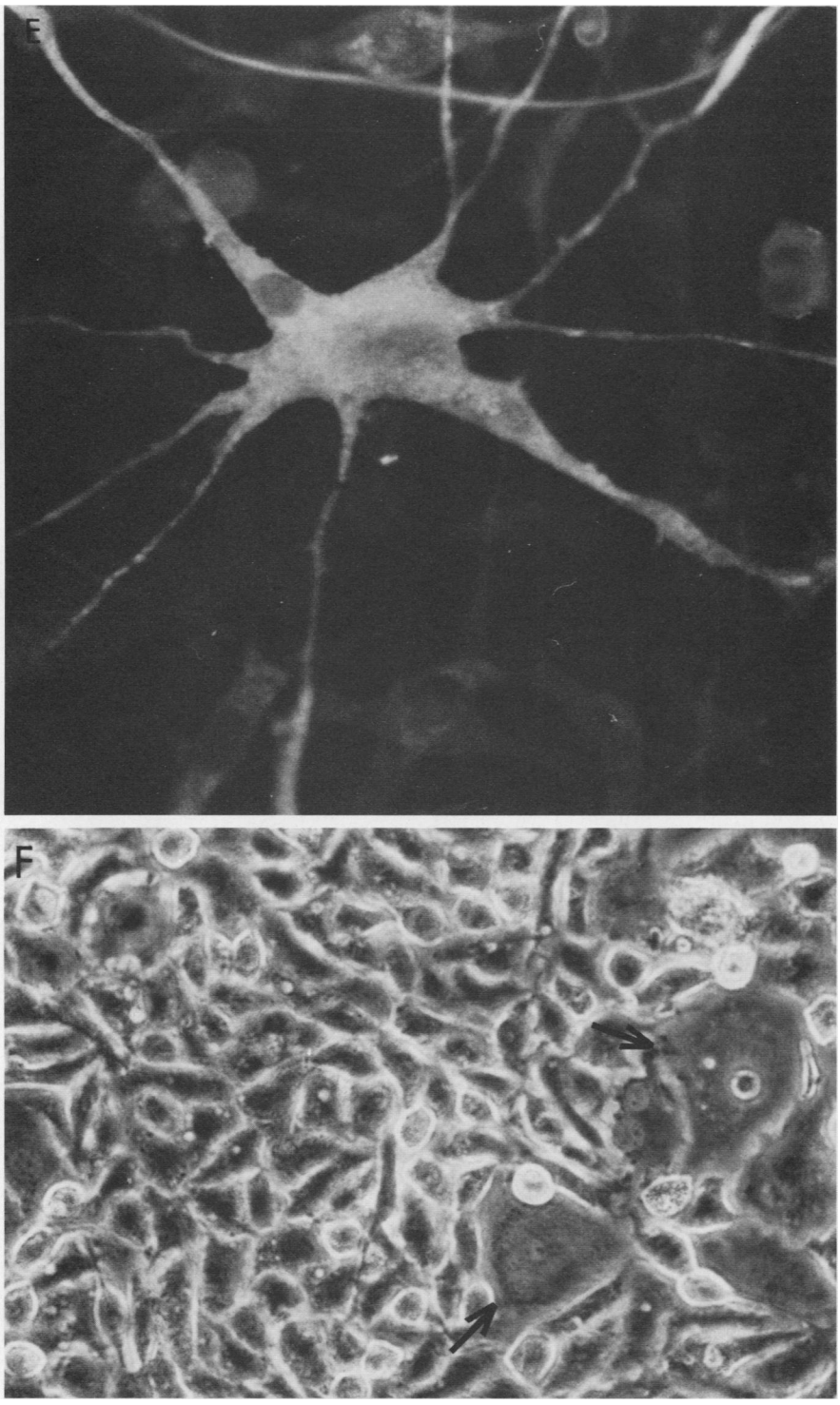

FIG. 10—*Continued*

effect on one another (7). Second, the enhancer within the polyadenylation fragment used in the gC1 construct is similar to one shown to contain a Dex-responsive element, which may produce a further increase in Dex-inducible transcription (42). gC1 responsiveness to o.n. Dex stimulation declined over time despite maintenance of the clone in the absence of Dex. Low levels of gC1 expression from uninduced cells apparently represented a sufficiently toxic stimulus such that surviving cells assumed a gC-negative phenotype.

Several lines of evidence support the conclusion that gC1 is cytotoxic. (i) Transfected cells expressing gC1 from the strong, constitutive Mo-MSV promoter assumed a bizarre, elongated, dendritic appearance. After several passages, these cells were no longer seen; concomitantly, no cells were expressing gC1, as judged by immunofluorescence. Despite

repeated efforts, no gC1-expressing clone could be established. (ii) In contrast, when gC1 was placed under the control of the weak but inducible MMTV LTR promoter, cell morphology remained normal and clonal cell lines could be established when cells were maintained in the uninduced state. When clone LgC1 was stimulated o.n. with Dex, many of the cells expressing gC1 became elongated and had dendritelike projections. (iii) Cytotoxic effects of gC1 were further studied by continuously exposing different passages of a gC1-expressing clone to Dex (passages 5 and 12). Examination of two different passages permitted the passage 12 cells to serve as controls for effects seen at passage 5. At passage 5, more than 99% of cells expressed gC1 when stimulated o.n. with Dex, whereas 10% responded at passage 12. Continuous exposure of passage 5 cells to Dex resulted in marked alterations in cell morphology. Almost all cells died; the rare surviving cells eventually grew into confluent monolayers, and these cells no longer expressed gC1 after Dex stimulation. In contrast, at passage 12 few cells became bizarre after continuous Dex stimulation. By immunofluorescence, gC1 expression was detected only in the bizarre-appearing cells. (iv) For comparison with gC1, the gD1 gene was placed under the control of the MMTV LTR promoter. Cells grown in the absence of Dex remained responsive to o.n. Dex stimulation. Continuous stimulation with Dex for 15 weeks had minimal effects on cell morphology and no effect on cell survival or on the inducibility of gD1. Similarly, continuous Dex stimulation did not affect the morphology or survival of a control clone containing the hygromycin resistance gene. Taken together, these results support the conclusion that gC1 is cytotoxic.

The finding that gC1 is cytotoxic in transfected L cells does not necessarily indicate that this protein is cytotoxic during HSV-1 infection in vivo. gC1 is not essential for HSV-1 replication in vitro; as a result, a variety of gC1-deficient mutants are available for study (27, 49). These mutants produce cytopathic changes in vitro, and the changes in many gC1 mutants resemble those caused by wild-type HSV-1. This fact implies that gC1 is not the only HSV-1 gene product capable of causing cell damage.

The finding of syncytia in continuously stimulated LgC1 cells was unexpected. gB, gD, and gH all have properties related to virus fusion with cells (8, 21, 23, 38, 43, 50), although gC1 has not previously been implicated in fusion. The syncytia were likely the result of cell fusion rather than inhibition of cell division, as judged by the sizes of the syncytia (Fig. 10B). gC1-induced syncytia were detected after 1 week of continuous Dex stimulation. It is possible that cell fusion is an artifact of the expression system used in this study, requiring both gC1 and Dex for its occurrence. However, relatively few, small syncytia were detected in clone LgC1 or in the control clone containing the hygromycin resistance gene, which indicates that the syncytium-inducing properties of gC1 are not related solely to Dex stimulation.

Increased methylation of promoters has been implicated as a mechanism of gene regulation (reviewed in reference 12). The observation of increased methylation of LgC1 cells at passage 26 raises the possibility that hypermethylation of the MMTV LTR altered gC1 gene expression, offering LgC1 cells a survival advantage. Reactivation of several genes has been accomplished by using cytidine analogs (14, 32, 51). Increased expression of gC1 was established in this study by using 5-aza-2′-deoxycytidine. Whether hypermethylation occurred randomly or as a specific event to regulate gC1 production cannot be determined from our results. However, our findings support the concept that increased methylation modified gC1 expression.

In conclusion, the MMTV LTR-inducible promoter construct used in this study permits high yields of gC1 to be obtained at early passage while minimizing glycoprotein-induced cytotoxicity. By freezing cells at an early passage, a sufficient supply can be made available for detailed evaluation of this glycoprotein. Alternatively, periodic treatment of LgC1 cells with a cytidine analog may prevent the decrease in gC1 protein production noted in late-passage cells. The MMTV LTR promoter will be useful for examining functions of gC1 in mammalian cells and may have broad applications for studying other cytotoxic proteins.

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