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Glucocorticoids Induce Focus Formation and Increase Sarcoma Viral Expression in a Mink Cell Line That Contains a Murine Sarcoma Viral Genome

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Dexamethasone (3×10^{-10} to 3×10^{-6} M) induced foci of morphologically transformed cells in a small proportion of a mink cell line that contains the Moloney murine sarcoma viral genome (S^+L^-). The induction was glucocorticoid specific, since other steroids with glucocorticoid activity (prednisolone, cortisol, and aldosterone) induced foci with an efficiency that paralled their glucocorticoid activity, and steroids lacking glucocorticoid activity (17B-estradiol, testosterone, and progesterone) failed to induce foci. Viral antigen, as measured by specific immunofluorescence, was localized to the foci. The induction of foci by dexamethasone (3×10^{-7} M) was accompanied by an approximately 10-fold increase in intracellular Moloney murine sarcoma virus-specific RNA and viral p30 antigen. Removal of dexamethasone was followed by the disappearance of foci and a decrease in viral RNA and p30. In this cell system, therefore, glucocorticoids can affect the intracellular levels of type C viral RNA and protein.

Glucocorticoids have been shown to enhance the expression of type B (14) and type C (12) viral genomes in tissue culture cell lines. For type B viruses, detailed studies of glucocorticoid induction of mouse mammary tumor virus (MMTV) have indicated that glucocorticoids stimulate the de novo synthesis of viral RNA (24), leading to increased intracellular levels of viral RNA and viral protein and to enhanced viral production (18, 19, 21). Glucocorticoids can also facilitate the expression of type C viruses, since they increase the titer in supernatant fluids of endogenous murine leukemia virus during chemically mediated viral induction. The mechanism of murine leukemia virus enhancement remains unclear; the one previously published biochemical study failed to detect an increase in viral RNA in dexamethasone-treated cells (23).

Recently, a morphologically untransformed mink cell line (S^+L^-) that contains the Moloney murine sarcoma viral genome in the absence of a helper virus has been described (16). Superinfection of the S^+L^- mink cells with a helper virus induces foci of morphologically transformed cells, associated with an increase in sarcoma-specific viral RNA (17). In this communication, we report that glucocorticoids can, in the absence of another chemical or a helper virus, induce similar foci of morphologically transformed cells in the S^+L^- mink cells. The foci contain increased amounts of viral antigen as determined by immunofluoresence, and the treated cultures have increased levels of both viral p30 antigen and sarcoma-specific viral RNA. The continued presence of glucocorticoid is required both for maintenance of the foci and for the enhanced expression of the sarcoma viral genome.

MATERIALS AND METHODS

Cells and virus. The S^+L^- mink cell line (S^+L^- $MiCl_1$) and the uninfected mink embryonic lung cell line (ATCC CCL-64) from which S^+L^- $MiCl_1$ was derived were obtained from P. T. Peebles (15). S^+L^- $MiCl_1$ had been cloned twice by limiting dilution in microtiter wells. R. H. Bassin provided the D56(S^+L^-) 3T3 mouse cell line (1) and the D56(S^+L^-) NRK rat cell line (2). CCC(S^+) (clone 81) cat cell line was obtained from S. Nomura (8). The sarcoma viral genomes in the mink, cat, and rat cell lines were all derived from the sarcoma genome in the D56(S^+L^-) 3T3 line. Mink and rat cells were grown in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.); mouse and cat cells were grown in McCoy 5a medium (Grand Island Biological Co.). Media were supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.), penicillin, and streptomycin. Cells were grown at 37°C in a humidified incubator (Wedco) with 10% CO_2.

RD-114 virus (11) was propagated in mink cells. Virus focus assay on S^+L^- $MiCl_1$ was performed as previously described (15).

Isolation of subclones. Subclones of S^+L^- $MiCl_1$

were derived from a petri dish that had been treated with 3×10^{-7} M dexamethasone for 1 week. The cells were lightly trypsinized in an attempt to select for transformed cells, counted, and plated at limiting dilution in microtiter wells (Falcon Plastics, Oxnard, Calif.). The dishes were inspected visually to determine that wells received no more than one cell and that growth was apparently from a single cell. Cells from 9 of 190 wells fulfilled these criteria. A 10th well had cells growing from two points, and was discarded. There was no growth in the remaining 180 wells. The cells were used within two passages of growth to a confluent 75-cm^2 tissue culture flask.

Steroid hormones. Hormones were purchased from Sigma Chemical Co., St. Louis, Mo., diluted to 3×10^{-2} M in absolute alcohol, and stored at −20°C. Control dishes with and without alcohol were run for each biological experiment; they were uniformly negative for foci, except as noted for subclone D8.

Immunofluorescence assay. Approximately 5,000 cells were seeded in 5-mm wells on Teflon-coated glass slides in petri dishes. Dexamethasone (3×10^{-7} M) was added to cultures on the day after seeding, and the cells were grown for 6 days in the continuous presence of dexamethasone. Media were changed every other day. The slides were then fixed in cold acetone and stored at −20°C. Fluorescein isothiocyanate-conjugated goat antiserum prepared against Moloney murine leukemia virus disrupted by Tween-ether (lot no. 5D10101) was obtained from R. E. Wilsnack through the Resources and Logistics Office of the National Cancer Institute. The fixed slides were incubated with a 1:20 dilution of the antiserum for 30 min at 37°C.

Radioimmunoassay for p30 protein. Double-antibody competition radioimmunoassay for murine viral p30 protein was carried out by W. P. Parks as previously described (13).

RNA extraction. Cells were grown in 150-cm^2 Costar flasks, trypsinized, centrifuged, and stored at −20°C. After thawing, the cell pellets were lysed with buffer containing 0.01 M Tris (pH 7.4), 0.1 M NaCl, 0.001 M EDTA, 0.5% sodium dodecyl sulfate, and 50 μg of proteinase K (EM Laboratories Inc., Elmsford, N.Y.) per ml. After 3 h of incubation at 37°C, the lysate was deproteinized by extraction with equal portions of phenol and chloroform isoamyl alcohol (24:1). The material was then ethanol-precipitated, and the nucleic acid was suspended in a solution of 0.01 M Tris (pH 7.4), 0.1 M NaCl, and 0.005 M magnesium acetate. DNase I (Sigma) was added to a final concentration of 30 μg/ml, and the mixture was incubated at 37°C for 1 h. Proteinase K was then added to a final concentration of 50 μg/ml, SDS was added to a final concentration of 0.5%, and the mixture was incubated an additional 1 h. After phenol and chloroform isoamyl alcohol extraction, the RNA was dialyzed, lyophilized, and suspended in 0.01 M Tris (pH 7.2).

DNA:RNA hybridization. A single-stranded fractionated [^{3}H]DNA probe specific for the sarcoma virus-specific portion of the Moloney murine sarcoma viral genome was prepared as previously described (20). Each 0.05-ml sample contained 0.02 M Tris (pH 7.2), 0.6 M NaCl, 0.17% sodium dodecyl sulfate, 5×10^{-5} M EDTA, 10 μg of yeast RNA, 5 to 250 μg of cell RNA, and approximately 1,400 trichloroacetic acid-insoluble counts of [^{3}H]complementary (c) DNA. After boiling, each sample was reacted under mineral oil at 65°C for 20 h. The samples were then digested with S_1 nuclease (3). In control tubes run with each assay, S_1 digested more than 95% of the input counts. D56(S^+L^-) NRK DNA was used as a positive control for each assay; at saturation, approximately 50% of the input counts were protected from S_1 degradation. For each assay, this value was normalized to 100% and C_0t was calculated (5) with correction to a 0.18 M monovalent salt concentration (6).

RESULTS

Focus induction by glucocorticoids. In the first experiment, 35-mm petri dishes seeded the previous day with 1.5×10^5 S^+L^- mink cells were treated continuously with various concentrations of dexamethasone. Parallel dishes were infected with RD-114 virus. Compared with the untreated controls, hormone-treated dishes achieved confluence more slowly, assumed a more epithelioid appearance, and grew in more organized cell colonies, as reported for other cells treated with glucocorticoids (4). These morphological effects were concentration dependent and were observed even at 3×10^{-10} M dexamethasone. Foci of morphologically transformed cells began to appear by day 4, before the cells became confluent; by day 6, foci had developed in dishes treated with 3×10^{-5} to 3×10^{-10} M dexamethasone. The dexamethasone-induced foci closely resembled the virus-induced foci (Fig. 1), except that some steroid-induced foci developed on a subconfluent monolayer and contained cells with large nuclei, whereas the virus-induced foci developed only on a confluent monolayer and the cells were of uniform size. Dexamethasone treatment of the mink cell line from which the S^+L^- mink was derived did not result in foci, although the treated mink cells grew more slowly and developed the other morphological features described above for the S^+L^- mink.

In the next experiment, the glucocorticoid specificity of the focus induction was investigated by treating cells with several classes of steroid hormones at concentrations ranging from 3×10^{-5} to 3×10^{-11} M. Testosterone, progesterone, and 17β-estradiol, which do not have glucocorticoid activity, failed to induce transformation (data not shown). By contrast, aldosterone, cortisol, prednisolone, and dexamethasone, which possess glucocorticoid activity, induced transformation with an efficiency that parallels their reported glucocorticoid activity (Fig. 2; 22). These results strongly suggest that the focus induction is glucocorticoid specific. Each of the three more potent glucocorticoids induced a similar maximum number of foci (about 50 foci per

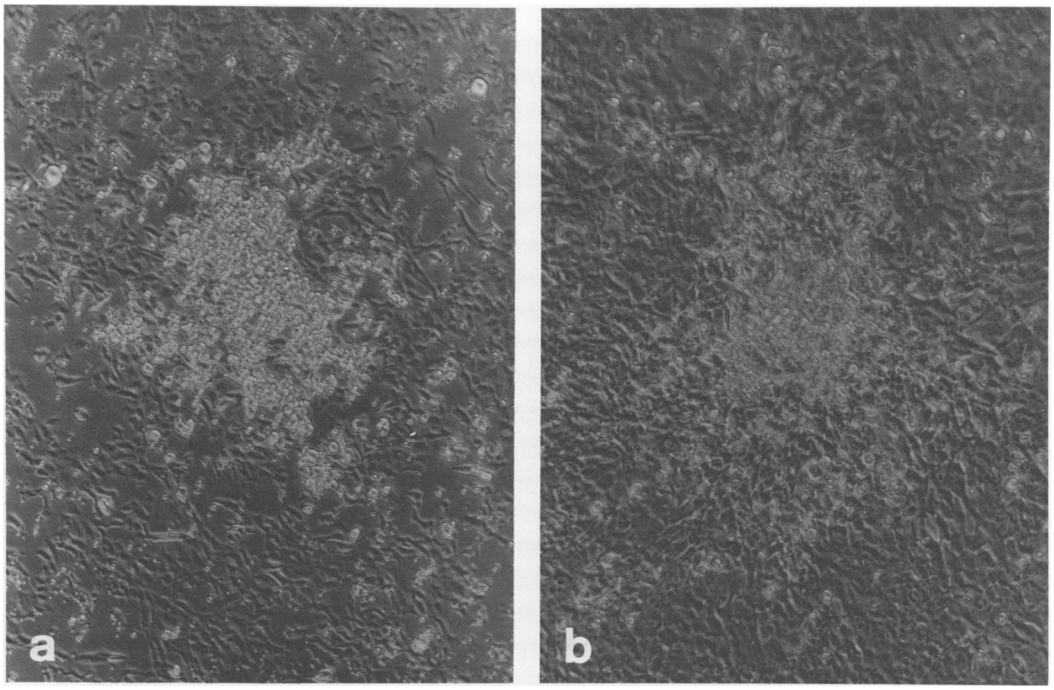

FIG. 1. *Phase-contrast photomicrographs of S^+L^- mink cells. Magnification, ×150. (a) Focus induced by 3 × 10^{-8} M dexamethasone 9 days after initiation of treatment. (b) Focus induced by RD-114 virus 9 days after infection.*

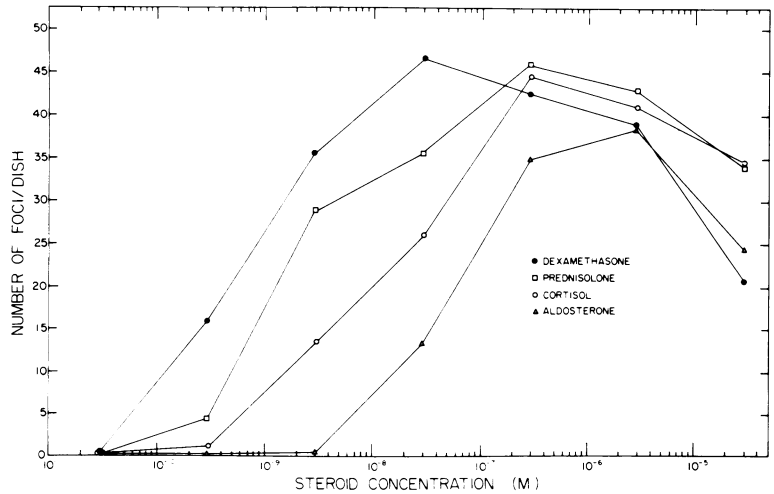

FIG. 2. *Mean number of foci induced in S^+L^- mink cells by steroid hormones at various concentrations. Dishes (35 mm) were seeded with 1.5 × 10^5 cells. On the following day, duplicate dishes were treated with 10-fold serial dilutions of steroid hormones, from 3 × 10^{-5} to 3 × 10^{-11} M. Hormone treatment was continued for the remainder of the experiment; media were changed every other day. Foci were counted 9 days after treatment was begun.*

plate), which implies that only a small proportion of the cells are susceptible to focus induction by glucocorticoids.

Maintenance of focus formation requires the continued presence of dexamethasone. The requirement for the continued presence of dexamethasone for the maintenance of focus formation was demonstrated in two ways. First, cells that had been treated with dexamethasone were subcultured for 1 week, either in the con-

tinued presence of dexamethasone (3×10^{-7} M) or in its absence. Although the dishes treated with dexamethasone continued to show many transformed cells, no foci were seen in the untreated dishes. Second, dishes with fresh cells were treated with 3×10^{-7} M dexamethasone for 48 h, washed twice, and observed for the development of foci. Those foci that developed consisted of only a few cells at their peak and disappeared within 7 days after removal of dexamethasone.

Focus formation is associated with increased sarcoma viral expression. Previous studies of sarcoma virus-containing cells have shown an excellent correlation between the degree of morphological transformation and the level of sarcoma viral expression. Therefore, the effect of dexamethasone on Moloney sarcoma viral expression in the S^+L^- mink cells was examined.

Since the Moloney sarcoma viral genome of the S^+L^- mink cell contains nucleic acid sequences that code for some of the virion proteins of the Moloney leukemia virus, it was possible to determine by immunofluorescence whether transformed cells contained higher levels of viral proteins than did nontransformed cells. Control cultures showed an occasional cell with slight fluorescence, but these cells were never grouped together. By contrast, the cells of transformed foci contained intense cytoplasmic fluorescence (Fig. 3). This reaction is viral specific, since premixing antiserum with Moloney leukemia virus prevented it.

The effect of dexamethasone on the expression of murine p30 antigen was then examined (Table 1). As reported by others, the S^+L^- mink cells contain low levels of p30, and this murine antigen cannot be detected in the parental mink lung cells (16). S^+L^- mink cells treated for 3 days with dexamethasone had levels of p30 approximately 10-fold higher than the untreated controls; those cells treated with dexamethasone for 3 days and then incubated for a further 4 days in the absence of dexamethasone had p30 levels only twice as high as the controls. By contrast, dexamethasone treatment of the parental mink cells failed to induce detectable p30 levels.

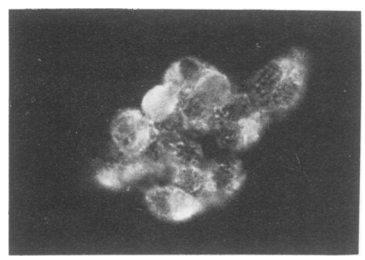

FIG. 3. *Direct immunofluorescence staining of dexamethasone-induced focus in S^+L^- mink cells. Magnification, ×300. Control slides showed no foci of stained cells.*

TABLE 1. *p30 Antigen levels of dexamethasone-treated mink cells*[a]

Cell line (treatment)	p30 antigen level (ng/mg of protein)
Mink (control)	<25
Mink (Dxm × 3 days)	<25
S^+L^- mink (control)	200
S^+L^- mink (Dxm × 3 days)	2,200
S^+L^- mink (off Dxm)	350

[a] 10^6 cells were seeded in 75-cm^2 flasks. Beginning the following day, cells were treated with 3×10^{-7} M dexamethasone (Dxm) for 3 days and then harvested for p30 antigen determination. Control cultures were also harvested at this time. One flask of S^+L^- $MiCl_1$ ("off Dxm") that had been treated for 3 days with dexamethasone was incubated for a further 3 days in the absence of dexamethasone and then harvested for p30. Levels of p30 are expressed as nanograms per milligram of total cellular protein.

Although the above studies show that dexamethasone treatment results in increased levels of viral antigen, they do not indicate whether there is a coordinate increase in the levels of sarcoma viral RNA. The expression of sarcoma viral RNA was therefore determined by molecular hybridization of cellular RNA to a complementary DNA probe specific for the sarcoma viral genome. Cells treated with dexamethasone had levels of sarcoma virus-specific RNA about 10 times higher than untreated controls (Fig. 4). After removal of dexamethasone, the levels of viral RNA returned to control values. Even with dexamethasone treatment, the parental mink cells did not have detectable levels of viral RNA (data not shown).

Since superinfection of the S^+L^- cell with a replication-competent helper virus leads to the development of transformed foci, it is possible that the glucocorticoids may be inducing foci through activation of a putative endogenous mink virus. However, media from dishes with dexamethasone-induced transformed cells did not contain reverse transcriptase activity, and electron micrographs of these cells did not show evidence of budding viral particles. Although these data do not support the hypothesis that focus induction is mediated through a mink virus, they do not rule out this possibility.

Analysis of subclones of S^+L^- mink cells. In an attempt to select for cells that would be uniformly sensitive to focus induction by glucocorticoids, subclones of the S^+L^- mink cells were

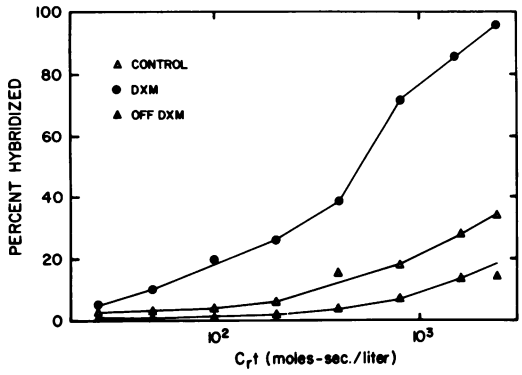

FIG. 4. *Hybridization of Moloney murine sarcoma virus [³H]DNA with total RNA from S⁺L⁻ MiCl₁. 2 × 10⁶ S⁺L⁻ MiCl₁ cells were seeded in 150-cm² flasks. Beginning the next day, cultures were treated for 4 days with 3 × 10⁻⁷ M dexamethasone; then cultures were trypsinized and the RNA was extracted* (●). *Control cultures were treated identically except for the omission of dexamethasone* (△). *After 4 days of treatment with 3 × 10⁻⁷ M dexamethasone and subsequent trypsinization, some cells were seeded at a 1:20 split ratio, incubated for 4 days in the absence of dexamethasone, and trypsinized again, and the RNA was extracted* (▲).

established by growing single cells obtained after brief trypsinization of a culture that had been treated for 1 week with dexamethasone. Since trypsin tends to remove the cells of the foci sooner than it removes the remaining cells, this procedure enriched for cells that responded to dexamethasone by focus formation. The initial characterization of these subclones has revealed several interesting features of the S⁺L⁻ mink cell line (Table 2). First, five of the nine subclones were not susceptible to focus induction, either by superinfection with a permissive helper virus or by dexamethasone treatment; sarcoma virus could not be rescued from them by superinfection, but they were not resistant to retransformation by the Moloney murine sarcoma virus. In addition, these five subclones were devoid of detectable p30 antigen, even after dexamethasone treatment. Second, the four subclones that were susceptible to focus induction upon superinfection contained a rescuable sarcoma viral genome and were sensitive to focus induction by dexamethasone. Interestingly, two (clones 2 and 3) of the four subclones were less susceptible to focus induction by dexamethasone than was the S⁺L⁻ mink line. Of the other two lines, subclone 8 contained many cells that were spontaneously transformed, whereas subclone 9 behaved similarly to the S⁺L⁻ mink cell line. For all subclones, there was an excellent correlation between the number of foci induced by dexamethasone and the p30 antigen levels in the culture.

TABLE 2. *Characterization of subclones of S⁺L⁻ mink cells*

Subclones	Rescuable sarcoma virus[a]	Foci induced by Dxm[b]	p30 Without Dxm[c]	p30 With Dxm[c]
D1	−	−	<25	<25
D2	+	2	100	200
D3	+	3	200	300
D4	−	−	<25	<25
D5	−	−	<25	<25
D6	−	−	<25	<25
D7	−	−	<25	<25
D8	+	>500[d]	1,200	3,500
D9	+	197	200	2,000

[a] Cultures positive (+) for rescuable sarcoma virus developed foci after infection with RD-114 virus; media from these infected cultures induced foci in mink cells. Cultures negative (−) for rescuable sarcoma virus did not develop foci after infection with RD-114; media from these infected cultures did not induce foci in mink cells. These cultures were, however, productively infected with RD-114, since media from all infected cultures induced foci in S⁺L⁻ MiCl₁.

[b] Cells (1.5 × 10⁵ seeded in duplicate 35-mm petri dishes) were treated for 9 days with 3 × 10⁻⁷ M dexamethasone (Dxm). Foci are expressed as number per dish.

[c] Cultures (10⁶ cells seeded in 75-cm² flasks) were treated for 5 days with 3 × 10⁻⁷ M dexamethasone and processed along with parallel untreated control cultures. Levels of p30 are expressed as nanograms per milligram of total cellular protein.

[d] There were more than 500 foci in control cultures.

The kinetics of sarcoma viral RNA expression during treatment with dexamethasone was examined for subclone 9 (Fig. 5). Compared with untreated controls, there was a small increase after 1 day, a substantial increase at 3 days, and a further small increase after 5 days of treatment, when foci were just beginning to be recognized.

Effect of dexamethasone on other S⁺L⁻ cells. To determine whether dexamethasone can regulate the expression of the Moloney sarcoma virus in other S⁺L⁻ cells, several other lines have been tested, so far with negative results. Dexamethasone treatment resulted in less than twofold differences in p30 and viral RNA levels of the CCC(S⁺) cat cell line, D56(S⁺L⁻) rat cell line, or D56(S⁺L⁻) 3T3 mouse cell line (Table 3).

DISCUSSION

These experiments indicate that a population of cells within the cloned S⁺L⁻ mink cell line

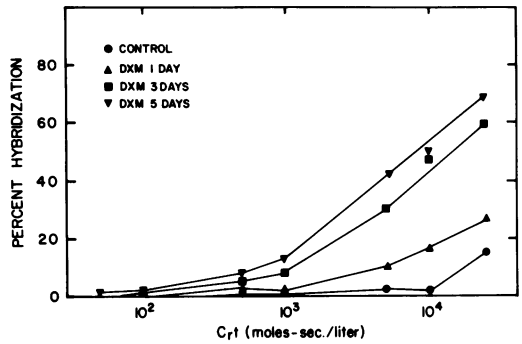

FIG. 5. *Hybridization of Moloney murine sarcoma virus [^{3}H]DNA with total RNA from subclone D9 of S^+L^- $MiCl_1$. 2×10^6 cells were seeded in 150-cm^2 flasks. Beginning the next day, cultures were treated continuously with 3×10^{-7} M dexamethasone. RNA was extracted from cells after 1, 3, and 5 days of treatment. Control cells were harvested for RNA extraction at the end of the experiment.*

TABLE 3. *Comparison of levels of Moloney murine sarcoma virus RNA and murine p30 antigen in S^+L^- cells*[a]

Cell line	Treatment	½ C_rt	p30
CCC(S^+)	Control	300	2,800
	Dexamethasone	150	2,900
D56(S^+L^-) NRK	Control	100	1,900
	Dexamethasone	100	1,800
D56(S^+L^-) 3T3	Control	270	2,300
	Dexamethasone	150	3,900

[a] Cultures were treated for 5 days with 3×10^{-7} M dexamethasone, then were harvested with untreated controls for hybridization and p30 determinations. All RNAs hybridized to the same final extent as S^+L^- $MiCl_1$. Levels of p30 are expressed as nanograms per milligram of total cellular protein.

are susceptible to focus induction by glucocorticoids. This induction is associated with a coordinate increase in sarcoma viral expression as measured by immunofluorescence, immunoreactive p30 antigen, and sarcoma virus-specific RNA. These studies establish for the first time that glucocorticoids can affect the intracellular levels of a type C viral genome.

Previous studies have found that focus induction by superinfection of S^+L^- mink cells is associated with a marked increase in sarcoma viral RNA (17). In addition, Rous sarcoma virus-containing hamster cells show an excellent correlation between the level of sarcoma viral RNA and the degree of cellular transformation (7). The data reported here complement these observations and further support the hypothesis that sarcoma viral expression mediates the phenotypic alterations.

In contrast to the results reported here, the only other biochemical study of glucocorticoid-mediated enhancement of type C viral expression failed to find changes in intracellular levels of sarcoma viral RNA, which were already high before treatment (23). In that system, glucocorticoid treatment of Kirsten sarcoma virus-transformed BALB cells during virus activation by halogenated pyrimidines increased the level of sarcoma virus in culture fluids. Whether the glucocorticoids in these two systems are enhancing virus expression by similar or different processes remains an open question. One unifying hypothesis might be that the effect of glucocorticoids on sarcoma viral expression represents a secondary effect. The primary event might be enhanced production of an endogenous type C viral genome.

There appear to be numerous parallels between the glucocorticoid-mediated stimulation of the S^+L^- mink cells and that of MMTV. In both systems, the increased expression of viral RNA and viral protein depend on the continued presence of glucocorticoid. In addition, dexamethasone stimulates MMTV only from cells already expressing viral information; the results with the S^+L^- mink cells are consistent with a similar enhancement rather than induction of viral expression in cells that were totally negative for virus expression before treatment.

However, the glucocorticoid-mediated changes are apparently detected later in the S^+L^- mink cells than in cells expressing MMTV. Dexamethasone stimulates MMTV viral RNA synthesis in all cells in a culture within minutes of beginning treatment, leading to significant increases in viral RNA levels in 1 h (21, 24). By contrast, only a limited proportion of the S^+L^- mink cells respond to dexamethasone with increased viral expression, and viral RNA and viral protein levels change more slowly than with MMTV. The reason for this difference in kinetics is not clear. As noted above, it is possible that the effect of dexamethasone on the Moloney murine sarcoma virus is less direct than on MMTV, such as through activation of a putative endogenous mink virus. Another possibility for the S^+L^- mink cells is that even a rapid rise in the intracellular levels of sarcoma viral RNA and p30 in a small proportion of cells might not be detected initially. The immunofluorescence results suggest that the rise in viral RNA and p30 occurs principally in the focus-forming cells. Since the focus-forming cells replicate faster than the other cells (Fig. 1a), the focus-forming cells represent a progressively greater proportion of the total cell population as the cells remain on dexamethasone treatment. Therefore, the apparent lag in detection of viral expression and the progressive rise in viral expression with con-

tinued dexamethasone treatment might be due to this increase in the proportion of focus-forming cells.

Why such a small proportion of the cells are sensitive to focus induction by glucocorticoids is an important but still incompletely resolved question. High-frequency reversion with apparent loss of the sarcoma viral genome has been reported for this strain of Moloney sarcoma virus in D56(S^+L^-) 3T3 (9) and CCC(S^+) cells (10), and the data from the S^+L^- mink subclones indicate that many of the S^+L^- mink cells do not contain a rescuable sarcoma viral genome. However, dexamethasone apparently does not induce foci from all cells that contain a rescuable sarcoma viral genome, since superinfection with a high titer of helper virus will induce more than 500 foci, whereas in a parallel dish only one or two foci may be induced by dexamethasone (unpublished data). Further examination of this cellular heterogeneity is necessary.

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