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Production of Unintegrated Mouse Mammary Tumor Virus DNA in Infected Rat Hepatoma Cells Is a Secondary Action of Dexamethasone

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Dexamethasone, a synthetic glucocorticoid, selectively increased the rate of synthesis of mouse mammary tumor virus (MTV) RNA in clonal isolates of chronically infected rat hepatoma tissue culture cells. This hormonal effect occurred extremely rapidly and appeared to be mediated directly by the glucocorticoid-specific receptor protein. In addition to the viral RNA, unintegrated MTV DNA was also detected in these cells. Several lines of evidence are consistent with the idea that the unintegrated viral DNA is synthesized by reverse transcription of MTV RNA. (i) Unintegrated viral DNA accumulated only in the presence of dexamethasone and was produced with a time course that closely paralleled the increased accumulation of viral RNA. (ii) Density labeling of the viral DNA revealed that both strands were newly synthesized, implying a nonsemiconservative mode of replication. (iii) Inhibitors of viral RNA synthesis prevented the appearance of unintegrated viral DNA. These data suggest that the production of unintegrated MTV DNA after dexamethasone treatment occurs as a secondary consequence of the hormonal induction of synthesis of viral RNA. In contrast to infected rat hepatoma cells, no unintegrated MTV DNA was detected in mouse mammary tumor cells or mouse lymphoma cells, despite the presence of high levels of viral RNA.

Retroviruses are thought to replicate via a DNA intermediate that becomes covalently linked to the host cell genome (2, 26, 31). Unintegrated viral DNA is present after acute infection with retroviruses (5-7, 9, 28), and, under certain circumstances, chronically infected cells also contain viral DNA molecules that are not integrated into the host DNA (10, 19, 31). In a line of cultured rat hepatoma cells (HTC) infected with mouse mammary tumor virus (MTV), unintegrated MTV DNA can be detected many months after infection (19), suggesting that viral DNA synthesis can occur continuously in the infected cells. The predominant form of the unintegrated MTV DNA is a linear molecule found in the cytoplasm; it is composed of a genome-length strand complementary to the viral RNA (minus strand) and plus strands of subgenomic length (19). In the nucleus, both linear and covalently closed circular molecules (form I) of viral DNA are detected. The structure of the linear molecules implies that they may be the products of RNA-directed DNA synthesis, presumably catalyzed by the viral "reverse transcriptase"; subsequent ligation and circularization might then produce the form I molecules. Such a pathway has been proposed for the synthesis of unintegrated avian sarcoma virus DNA (23, 31).

Synthesis of MTV RNA is stimulated by glucocorticoid hormones (e.g., dexamethasone) in mouse mammary tumor cells and infected heterologous cells, including HTC cells (15, 18, 20, 36). In this report, we demonstrate that dexamethasone also stimulated the production of unintegrated MTV DNA in HTC cells, most likely as a secondary consequence of stimulating the intracellular accumulation of viral RNA, the presumptive template for viral DNA synthesis. The unintegrated viral DNA did not appear to serve as a major source of template for viral RNA synthesis. In contrast to the infected HTC cells, unintegrated MTV DNA was not detected in mouse mammary tumor cells or mouse lymphoma cells, despite the presence of large amounts of viral RNA.

MATERIALS AND METHODS

Cells and virus. GR3A and BALB/cfC3H (CFZ) mouse mammary tumor cells (17), S49 mouse lymphoma cells (17), and MTV-infected rat HTC cells (16) have been described. M1.19 and M1.20 are clones isolated from a population of infected HTC cells (HTC-M1) (34). All cells were grown in Dulbecco-

modified Eagle medium supplemented with 10% horse serum, except the infected HTC cells in suspension culture, which were grown in Swim 77 medium.

Mouse MTV was collected from the medium of GR3A cell cultures. Cellular debris was removed by centrifugation at 8,500 rpm for 10 min in a Sorvall GSA rotor. The virus was then pelleted in a Spinco SW27 rotor (25,000 rpm, 45 min) or L19 rotor (19,000 rpm, 90 min). MTV purified from Mm5mt/c_1 cells, used for synthesis of virus-specific DNA, was kindly provided by the Frederick Cancer Research Center.

Hybridization reagents. MTV 70S [^{32}P]RNA was prepared to a specific activity of 1×10^7 to 2×10^7 cpm/μg by labeling 2×10^7 to 4×10^7 GR3A cells with 2 mCi of $^{32}P_i$ (New England Nuclear Corp.) per ml; the medium was collected after each of three 12-h labeling periods, and the virus was concentrated by centrifugation at 25,000 rpm for 60 min in a Spinco SW27 rotor. RNA was purified as described previously (17) and then centrifuged in a Spinco SW65 rotor at 64,000 rpm for 70 min through a 15 to 30% (wt/vol) sucrose gradient. RNA sedimenting in a broad peak at 50 to 70S was collected and used for hybridizations.

DNA complementary to the genome of MTV (cDNA) was synthesized by the virion-associated polymerase and was labeled to specific activities of 5×10^7 cpm/μg with [^{32}P]dTTP (New England Nuclear Corp.) or to 2×10^7 cpm/μg with [^{3}H]dTTP (New England Nuclear Corp.) as previously described (17, 30).

For hybridizations to DNA transferred from agarose gels onto nitrocellulose filters (25), cDNA representative of the entire viral genome was prepared by a modification of the procedure of Taylor et al. (26), with purified viral RNA, avian myeloblastosis virus polymerase (kindly provided by J. Beard), and calf thymus DNA primers (8; primers provided by S. Hughes). The viral DNA was labeled to a specific activity of approximately 10^8 cpm/μg with [^{32}P]dCTP (Amersham/Searle). Before use, RNA was removed by treatment with 0.3 N NaOH for 12 h at 37°C. This probe has been shown to be representative of the entire viral genome by its ability to detect all restriction endonuclease fragments of viral DNA with an efficiency equal to that of iodinated viral RNA (P. R. Shank, J. C. Cohen, H. E. Varmus, K. R. Yamamoto, and G. M. Ringold, Proc. Natl. Acad. Sci. U.S.A., in press). Alternatively, MTV 70S RNA was iodinated by the procedure of Commerford (3) to a specific activity of 1×10^8 to 2×10^8 cpm/μg with [^{125}I]sodium (Amersham/Searle) and purified by equilibrium sedimentation in Cs_2SO_4 (P. R. Shank, H. J. Kung, S. H. Hughes, R. V. Gutanka, J. M. Bishop, and H. E. Varmus, in preparation).

Cell fractionation. Nuclear and cytoplasmic fractions were prepared as described previously (19). Briefly, cells were resuspended in a buffer containing 10 mM Tris-hydrochloride (pH 8.1), 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.1% Nonidet P-40 at a concentration of 0.5×10^7 to 1×10^7 cells per ml. After 5 min at 0°C, the cells were broken in a Dounce homogenizer, and the nuclei were pelleted by centrifugation at $1,000 \times g$ for 5 min.

Hirt fractionation. High-molecular-weight DNA was separated from unintegrated viral DNA by the method of Hirt (12). Either nuclei or whole cells (0.5×10^7 to 1×10^7/ml) were suspended in a solution containing 10 mM Tris-hydrochloride (pH 7.4) and 10 mM EDTA, lysed by the addition of sodium dodecyl sulfate to 1.2%, and incubated for 10 to 20 min at 37°C. Next, 5 M NaCl was added with gentle mixing to a final concentration of 1 M. The mixture was then gently poured into centrifuge bottles and placed at 4°C for 8 to 16 h. The coagulum of protein and high-molecular-weight DNA was then pelleted by centrifugation at $15,000 \times g$ for 60 min.

DNA extraction and preparation for hybridization. DNA was extracted from whole cells, cytoplasmic fractions, or Hirt supernatant fractions by previously described procedures (19). In brief, cells or cell extracts were incubated with sodium dodecyl sulfate and Pronase, and the nucleic acids were phenol extracted and ethanol precipitated. The precipitate was dissolved in Tris-EDTA, and RNA was degraded either by pancreatic RNase or by incubation for 12 h at 37°C in 0.3 N NaOH; Pronase digestion, phenol extraction, and ethanol precipitation was repeated. All DNA samples, including fractions from gradients, were finally incubated at 80°C in 0.3 N NaOH for 2 h to denature and reduce the size of the DNA to 200 to 300 nucleotides; approximately 50 μg of calf thymus DNA was added during the alkali treatment to all samples except whole-cell DNA. The final preparation was neutralized, ethanol precipitated, and suspended in small volumes of Tris-EDTA buffer.

Determination of intracellular viral RNA concentrations. Cellular RNAs were prepared as described previously (20) and hybridized with 600 to 1,000 cpm of [^{3}H]cDNA. The extent of annealing to varying amounts of RNA was assayed by determining the resistance of the cDNA to the single-strand-specific nuclease S1 (1). The concentration of virus-specific RNA in the sample was determined by comparing the $C_rt_{1/2}$ for the cellular RNA with the $C_rt_{1/2}$ for pure MTV RNA ($C_rt_{1/2}$, 1×10^{-2} to 2×10^{-2} mol·s/liter; [17]). All hybridizations were carried out at 68°C in 0.6 M NaCl, 5 mM Tris-hydrochloride (pH 7.4), and 2 mM EDTA.

CsCl density gradients. Separation of bromodeoxyuridine (BUdR)-substituted DNA was performed by CsCl equilibrium centrifugation as described by Varmus and Shank (31). For subsequent analysis of the DNA in agarose gels, 150 μg of yeast RNA was added, and CsCl was removed by extensive dialysis against Tris-EDTA buffer.

Gel electrophoresis, transfer procedure, and filter hybridizations. Slab gels of 0.8% agarose (Sea-Kem) were run by the procedure of Helling et al. (11) with Tris-acetate buffer containing 18 mM NaCl. Electrophoresis lasted 12 h at 2.5 V/cm. DNA fragments within the agarose gel were denatured in situ with 0.5 M NaOH–1.5 M NaCl, neutralized with 0.5 M Tris-hydrochloride (pH 7.4)–3 M NaCl, and transferred onto nitrocellulose membranes (type HAWP; Millipore) by the method of Southern (25), with 6× SSC (SSC = 0.15 *M* NaCl plus 0.015 M sodium citrate) mediating the transfer. The filters were then baked at 80°C for 2 h in a vacuum oven. Before hybridization, the filters were pre-annealed at 42°C for ≥6 h in 50% formamide–3× SSC–0.02% bovine serum albumin–

0.02% polyvinyl pyrrolidone-0.02% Ficoll (Denhardt buffer) (4). Hybridization was carried out in the same buffer containing 2×10^6 cpm of [^{32}P]cDNA ("representative") or MTV [^{125}I]RNA (see above) per ml, with a volume just sufficient to wet the filter completely. The filters were wrapped in plastic film (Saran Wrap), incubated at 42°C for 24 to 48 h, washed twice at 25°C with Denhardt buffer in 2× SSC, and then given several washes with 6× SSC containing 0.5% sodium dodecyl sulfate at 68°C over a period of 18 to 24 h. Finally, the filters were rinsed in 2× SSC, air dried, and exposed to X-ray film (R-P Royal X-omat; Kodak) with Lightning-Plus intensifying screens (Dupont) at −70°C (R. I. Swanstrom and P. R. Shank, Anal. Biochem., in press).

RESULTS

Kinetics of induction and deinduction of MTV RNA in infected HTC cells. The concentration and rate of synthesis of MTV RNA is increased in MTV-infected HTC cells by treatment with dexamethasone (16, 18); the extent of induction is 50- to 1,000-fold, depending upon the individual clone examined (34). To measure the concentration of MTV RNA as a function of time after the addition or removal of dexamethasone, cultures of M1.20 cells (a clone of infected HTC cells) were grown in the absence or presence of 10^{-6} M dexamethasone for 48 to 60 h, washed with fresh medium, and given medium with or without dexamethasone, respectively. At various times thereafter, RNA was extracted from the cells, and the concentration of MTV RNA was measured by hybridization with MTV [^{3}H]cDNA. Induction and deinduction occurred at about the same rate, exhibiting half-times of 10 h and nearing completion by 24 h (Fig. 1). In contrast, dexamethasone stimulated MTV RNA accumulation 10- to 20-fold in GR cells, with a half-time of 2 to 2.5 h and nearing completion by 6 h (20). It is significant that the onset of viral RNA induction occurred with no detectable lag in both GR and M1.20 cells; the rate of synthesis of viral RNA was stimulated to its maximal extent within 15 to 20 min after addition of the hormone (18). Taken together, these results imply that MTV RNA may be degraded more rapidly in GR than in M1.20 cells, thus accounting for the difference in the time required to reach the induced steady-state concentration of viral RNA in each cell type.

Effect of dexamethasone on the concentration of unintegrated and integrated MTV DNA. MTV-infected HTC cells acquire several copies of MTV DNA; some are integrated into the cellular genome, whereas others are detected in the unintegrated state, even months after infection, when the cells are propagated in the presence of dexamethasone (19). To determine whether dexamethasone affects

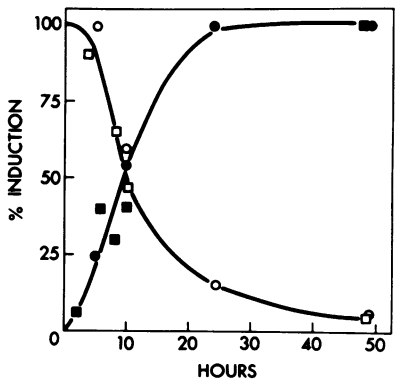

FIG. 1. *Time course of induction and deinduction of MTV RNA. M1.20 cells (1×10^7 to 2×10^7/100-mm dish) were grown in the absence (●, ■) or presence (○, □; 10^{-6} M, 60 h) of dexamethasone. At zero time, medium was replaced by fresh medium with or without 10^{-6} M dexamethasone, respectively. RNA was extracted at the times indicated, and MTV RNA was estimated as described in the text. Squares and circles represent data from two independent experiments; 100% induction represents a level ~200-fold above the basal.*

the production of unintegrated viral DNA, M1.20 cells were grown in the presence or absence of dexamethasone (10^{-6} M) for 48 h; cytoplasmic and nuclear fractions were prepared, and nuclear DNA was fractionated by the procedure of Hirt (12) to separate high- from low-molecular-weight DNA. DNA was prepared, and viral sequences were quantitated by hybridization with MTV 70-S [^{32}P]RNA. The cytoplasm from the hormone-treated cells contained more than 20 times as much MTV DNA as that from untreated cells (Fig. 2). The small amount of annealing in the control sample represents less than one molecule of viral DNA per cell and may reflect contamination of the cytoplasm with nuclear DNA (i.e., integrated viral DNA). Assays of the nuclear DNA showed that dexamethasone also stimulated the production of unintegrated viral DNA in the nucleus (Fig. 2), whereas the amount of integrated viral DNA was not detectably altered by hormone treatment (Fig. 2). Thus it is clear that the accumulation of detectable levels of unintegrated viral DNA in both the cytoplasm and the nucleus of M1.20 cells was dependent on treatment of the cells with dexamethasone.

In contrast, we were unable to detect unintegrated MTV DNA in the cytoplasm of two mouse mammary tumor lines (GR or CFZ cells), irrespective of the hormonal treatment (Table 1). The small amounts of viral DNA detected in the cytoplasm of GR cells can be accounted for by a 5% contamination with nuclear DNA, since

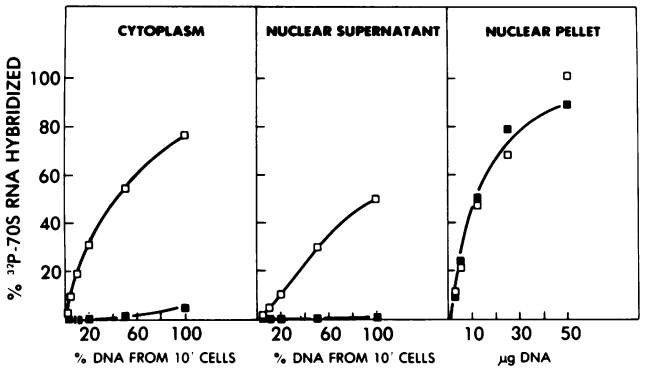

FIG. 2. *Dexamethasone-mediated induction of unintegrated MTV DNA. Approximately 3 × 10^7 M1.20 cells were grown in the absence (■) or presence (□; 10^{-6} M, 60 h) of dexamethasone. Nuclear and cytoplasmic fractions were prepared, and DNA from the nuclei was fractionated by the sodium dodecyl sulfate-NaCl procedure of Hirt (12). DNAs from the cytoplasm, nuclear pellet, and nuclear supernatant were prepared as described in the text and hybridized with MTV 70S [^{32}P]RNA (~1,000 cpm) for 48 h in 0.6 M NaCl at 68°C. The amount of hybridization was determined by the extent to which the [^{32}P]RNA resisted digestion by pancreatic RNase in 2× SSC at 37°C. The amounts of unintegrated DNA from the cytoplasm and nuclear supernatant are plotted as a function of the number of cells from which the DNA was derived; relative concentrations of viral DNA are estimated from the relative initial slopes of the hybridization curves (29).*

TABLE 1. *Unintegrated MTV DNA in rat and mouse cells*[a]

Cell line	Integrated copies of MTV DNA per diploid genome	Unintegrated copies of MTV DNA per cell	
		−Dex	+Dex
HTC-M1	10–20	<1	20–30
HTC-M2	10–20	<1	20–30
HTC-M1.19	10–20	<1	20–30
HTC-M1.20	10–20	<1	20–30
GR[b]	50–70	<3	<3
BALB/cfC3H	15–20	<1	<1
S49	10–15	<1	<1
Uninfected HTC cells	0	0	0

[a] Integrated copies of MTV DNA were quantitated by hybridization of MTV [^{3}H]cDNA to varying amounts of total cellular DNA extracted from cultures grown in the absence of dexamethasone (Dex). These hybridizations were calibrated against parallel reactions with RIII mouse DNA, which contains seven copies of MTV DNA per diploid genome (14). Unintegrated MTV DNA was prepared from the cytoplasm of each cell line, grown in the presence or absence of 10^{-6} M dexamethasone for 48 h, and measured as described in the legend to Fig. 2; RIII mouse DNA was used as the calibration standard.

[b] The number of copies of MTV DNA integrated in GR cells was determined in collaboration with V. Morris; C_0t analysis as described by Morris et al. (14) was used.

these cells contain 50 to 70 copies of MTV DNA per diploid genome. Similarly, S49 mouse lymphoma cells, which contain high concentrations of MTV RNA (17), produced no detectable unintegrated MTV DNA. The factors that govern the production of unintegrated viral DNA are not understood.

Time course and extent of induction of plus and minus strands of cytoplasmic MTV DNA. The time course of appearance of unintegrated MTV DNA was determined by preparing cytoplasmic DNA at various times after the addition of 10^{-6} M dexamethasone to M1.20 cells. Increasing amounts of the cytoplasmic DNAs were hybridized with either MTV [^{32}P]RNA or [^{3}H]cDNA to measure minus and plus strand viral DNA, respectively. Minus and plus strand viral DNAs were induced concurrently (Fig. 3); approximately 30% of the maximal level was present by 10 h after hormone addition, and maximal induction was reached between 15 and 24 h. A comparison of these data with those shown in Fig. 1 indicate that induction of unintegrated viral DNA occurred with a time course similar to the induction of viral RNA.

To estimate the amount of each strand of viral DNA present in hormone-treated cells, we compared the hybridization of strand-specific reagents (MTV [^{32}P]RNA or [^{3}H]cDNA) to either unintegrated DNA from M1.20 cells or RIII mouse DNA, which contains about seven copies of MTV DNA per cell (14). Taken at face value, the results (Fig. 4) indicate that there are approximately 20 to 25 copies of unintegrated minus strand viral DNA and 10 to 15 copies of plus strand viral DNA in M1.20 cells. However, unequal concentrations of the two strands of viral DNA resulted in different hybridization efficiencies for each probe, thus increasing the apparent

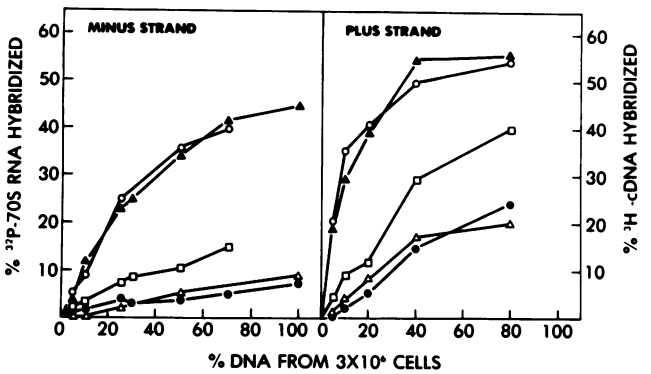

FIG. 3. *Time course of induction of cytoplasmic MTV DNA. M1.20 cells (1 × 10⁷ to 2 × 10⁷/75-cm² flask) were treated with 10⁻⁶ M dexamethasone. At the indicated times, cells were fractionated, and cytoplasmic DNA was prepared for hybridization. Increasing amounts of DNA from each time point were hybridized with either MTV 70-S [³²P]RNA or [³H]cDNA to measure the concentration of minus and plus strand viral DNA, respectively. Symbols: ●, 0 h; △, 6 h + dexamethasone; □, 10 h + dexamethasone; ▲, 15 h + dexamethasone; ○, 24 h + dexamethasone.*

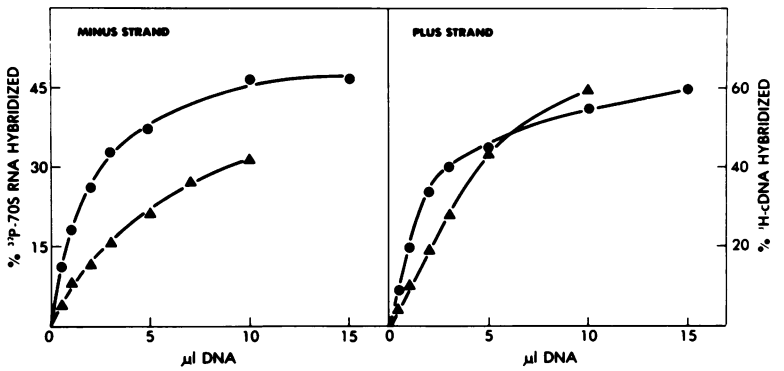

FIG. 4. *Unintegrated plus and minus strand MTV DNA in infected HTC cells. M1.20 cells were grown in the presence of 10⁻⁶ M dexamethasone for 24 h; DNA was fractionated by the Hirt procedure as described in the text and was purified from the supernatant. The indicated volumes of the final DNA preparation were then hybridized with either 70S [³²P]RNA to measure minus strands or with [³H]cDNA to measure plus strands (●). In each case, parallel hybridizations were carried out with the indicated amounts of RIII mouse DNA (3.5 mg/ml), which contains approximately seven copies of MTV DNA per diploid genome (▲). Hybridizations were performed in 25 µl at 68°C in 0.6 M NaCl for 42 h.*

difference in their concentration (i.e., there was greater competition for [³H]cDNA annealing than for [³²P]RNA annealing to their respective complementary strands). Therefore, the apparent twofold difference in the concentration of plus and minus strands is a maximum value, and the number of copies of each strand can be considered only as approximations. Excess minus strands may be present as partially double-stranded molecules, similar to those reported in murine leukemia virus-infected cells (7).

Kinetics of appearance of form I and form III MTV DNAs. The experiments described in Fig. 3 measured the kinetics of appearance of cytoplasmic MTV DNA, which has been shown to be a linear (form III) molecule (Shank et al., in press); in contrast, the nucleus contained both form III and covalently closed circular (form I) viral DNA. To determine whether form I MTV DNA is also induced by dexamethasone, we used M1.19, a clone of infected HTC cells in which viral form I DNA constitutes approximately 20% of the total unintegrated MTV DNA. Approximately 2 × 10⁷ cells were subjected to Hirt fractionation at various times after the addition or removal of 10⁻⁶ M dexamethasone. DNA was prepared from the supernatant fraction, subjected to electrophoresis on an 0.8% agarose gel, and transferred to a nitrocellulose membrane (25). The viral DNA was detected by hybridi-

zation with MTV [^{125}I]RNA, followed by autoradiography as described in Materials and Methods; markers of cytoplasmic MTV DNA (form III) and form I viral DNA from nuclei were run in parallel lanes. The autoradiograph (Fig. 5) indicated that form I and III viral DNAs were both induced by dexamethasone and that form III could be detected as early as 4 h after hormone treatment. In some experiments, the appearance of closed circular viral DNA lagged slightly behind the induction of linear MTV DNA. It is conceivable that the cytoplasmic linear DNA serves as a precursor to the nuclear form I viral DNA; there is direct evidence for such a precursor-product relationship in the case of viral DNAs in avian sarcoma virus-infected cells (23).

Note that there were two discrete size classes of viral form I DNA (Fig. 5). The larger species (~5.9 × 10^6 daltons), which represents only 10 to 30% of the total form I DNA, has the same molecular weight as the cytoplasmic form III DNA, whereas the smaller species is ~5.1 × 10^6 daltons and probably lacks a specific region of the viral genome (Shank et al., in press). These two size classes of viral form I DNA were induced by dexamethasone with similar kinetics (Fig. 5). In addition, there was a heterogeneous population of smaller than full-length viral DNA molecules that was also induced by dexamethasone and disappeared rapidly upon removal of the steroid. The nature of these molecules is unknown; they could represent intermediates in viral DNA synthesis.

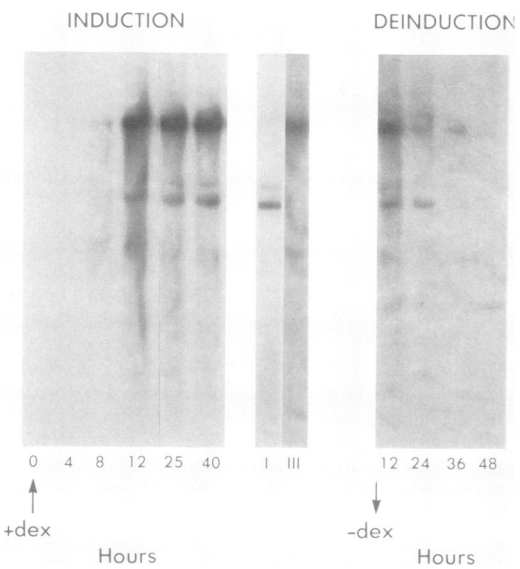

FIG. 5. *Time course of induction and deinduction of total unintegrated MTV DNA. M1.19 cells (~2 × 10^5 to 4 × 10^5 per ml) were treated with 10^{-6} M dexamethasone; after the indicated period of induction and deinduction, approximately 2 × 10^7 cells were removed, and unintegrated DNA was prepared by Hirt fractionation and subsequent purification (see text). Aliquots from each time point were subjected to electrophoresis in an 0.8% agarose gel, transferred onto a nitrocellulose membrane, and hybridized with MTV [^{125}I]RNA as described in the text. Markers of purified viral forms I and III DNAs were run in parallel lanes; form I DNA was purified as supercoiled viral DNA from a cesium chloride-propidium diiodide gradient; form III DNA was purified from the 18 to 20S region of a sucrose gradient containing cytoplasmic DNA (19).*

Density labeling of MTV DNA. To determine whether the unintegrated viral DNA in infected HTC cells was newly synthesized, M1.19 cells were labeled for 72 h with 0.2 μCi of [^{3}H]thymidine per ml and then incubated with BUdR (10 μg/ml) at the time of addition of dexamethasone (10^{-6} M); after 24 h, the cells were collected and fractionated by the procedure of Hirt (12). The unintegrated DNA was centrifuged to equilibrium in a cesium chloride gradient, and portions of each gradient fraction were analyzed by hybridization with MTV [^{32}P]cDNA. The results demonstrate that the bulk of the viral DNA banded in the position of heavy-heavy (HH) DNA (i.e., it was labeled in both strands with BUdR), whereas the cellular DNA was virtually all found in the heavy-light (HL; containing half-substituted DNA) and light-light (LL; containing unsubstituted DNA) regions of the CsCl gradient (Fig. 6). One interpretation of this result is that viral DNA is newly synthesized in a non-semiconservative fashion.

In addition to the HH MTV DNA, some hybridization was also detected in the region of HL and LL DNA (fractions 6 to 10 and 11 to 15, respectively, in Fig. 6). This may be due to integrated MTV DNA contaminating the supernatant from the sodium dodecyl sulfate-NaCl fractionation; alternatively, viral DNA might be excised from the cellular genome during the treatment with dexamethasone and BUdR. Contaminating nuclear DNA would be randomly sheared and therefore heterogeneous in molecular weight, whereas viral DNA specifically excised from the cellular genome would be of discrete molecular weights. To examine these possibilities, HL and LL DNA were subjected to electrophoresis in an 0.8% agarose gel and transferred to a nitrocellulose membrane; viral DNA was detected by hybridization with MTV [^{32}P]-cDNA followed by autoradiography. The viral DNA was present in a heterogeneous population of molecules ranging in size from 3 × 10^6 to 30 × 10^6 daltons (Fig. 7); the bulk of the MTV DNA was in molecules greater than 5.9 × 10^6 daltons (i.e., the size of unintegrated viral DNA).

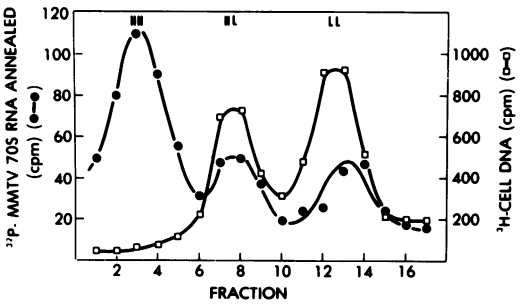

FIG. 6. *Density labeling of unintegrated MTV DNA. M1.19 cells were grown in the presence of 0.2 μCi of [^{3}H]thymidine per ml for 3 days. BUdR (10 μg/ml) and dexamethasone (10^{-6} M) were added for 24 h; cells (~10^{8}) were harvested and fractionated according to the procedure of Hirt (12). DNA was prepared from the supernatant fraction and centrifuged to equilibrium in CsCl in a Spinco type 40 rotor at 20°C and 33,500 rpm for 60 h. The gradient was collected from the bottom, and a sample of each fraction was counted to determine the positions of unsubstituted (LL) and half-substituted (HL) DNA. A portion of each gradient fraction was processed for hybridization as described in the text and annealed with MTV [^{32}P]cDNA to determine the banding position of MTV DNA.*

There were no detectable discrete bands at the positions of the marker form I or form III viral DNAs (Fig. 7), implying that the MTV DNA in the HL and LL regions of the gradient reflected contamination of the unintegrated DNA with integrated viral DNA. In contrast, the viral DNA from the HH region of the CsCl gradient was composed primarily of form III molecules together with a small amount of form I DNA (Fig. 7).

Effects of metabolic inhibitors and progesterone on the induction of viral RNA and unintegrated viral DNA. To gain a preliminary view of the relationship of viral DNA induction to viral RNA induction, and to assess the role of the glucocorticoid receptor in these processes, several experiments were carried out with inhibitors. Cytosine arabinoside, an inhibitor of cellular DNA synthesis, blocked the appearance of viral DNA without affecting the induction of viral RNA (Table 2). In contrast, an inhibitor of RNA synthesis, actinomycin D, blocked the induction of both viral RNA and DNA. Thus, it appeared that the production of unintegrated viral DNA was dependent on RNA synthesis (presumably viral RNA), whereas the induction of viral RNA was not detectably dependent on the presence of unintegrated viral DNA.

To examine whether induction of viral DNA is mediated by the glucocorticoid receptor protein, we tested the effect of an antiglucocorticoid, progesterone. This steroid competitively inhibits the glucocorticoid-receptor interaction, and thereby inhibits the dexamethasone-mediated induction of tyrosine aminotransferase in HTC cells (21) and MTV RNA in mouse mammary tumor cells (20, 22, 24, 35). When progesterone (5×10^{-5} M) and dexamethasone (10^{-7} M) were added simultaneously to M1.20 cells, induction of both viral DNA and RNA was inhibited (Table 2).

DISCUSSION

The bulk of the unintegrated MTV DNA in infected HTC cells is composed of a genome-length strand complementary to viral RNA (minus strand) and segmented plus strands (19); this form of viral DNA is characteristic of the product of reverse transcription (31, 33), imply-

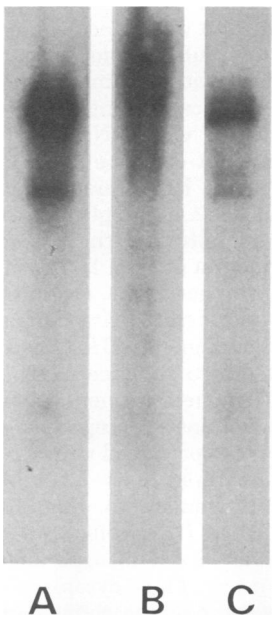

FIG. 7. *Analysis of labeled MTV DNA present in the supernatant fraction of a Hirt fractionation. Approximately 10^{8} M1.19 cells were grown in the presence of 0.2 μCi of [^{3}H]thymidine per ml for 60 h and then in the presence of BUdR (10 μg/ml) and dexamethasone (10^{-6} M) for 24 h. DNA was prepared by Hirt fractionation and centrifuged to equilibrium in a CsCl gradient (see text and legend to Fig. 6). DNA containing BUdR in both strands (HH) was separated from half and unsubstituted DNA (HL + LL), dialyzed against Tris-EDTA buffer, and run in an 0.8% agarose gel (in the dark) as described in the text. DNA was transferred onto a nitrocellulose membrane, and viral DNA was detected by hybridization with [^{32}P]cDNA followed by autoradiography. (A) Total unintegrated MTV DNA (not labeled with BUdR), showing the positions of authentic form I and III viral DNA; (B) HL + LL DNA; (C) HH DNA.*

TABLE 2. *Effects of inhibitors on dexamethasone-mediated induction of MTV RNA and DNA in M1.20 cells*[a]

Inhibitor	Relative incorporation (%)			Relative induction (%)	
	[^{3}H]TdR	[^{3}H]U	[^{3}H]Leu	Intracellular MTV RNA	Cytoplasmic MTV DNA
Dexamethasone	100	100	100	100	100
Dexamethasone + ara-C	2	55	100	>80	<5
Dexamethasone + actinomycin D	32	3	80	0	0
Dexamethasone + progesterone	NT	NT	NT	<10	<10

[a] Inhibitors were added 2 h before isotopic labeling (2 μCi/ml) or 30 min before a 20-h dexamethasone treatment. Concentrations of the inhibitors were: dexamethasone, 5×10^{-7} M; progesterone, 5×10^{-5} M; cytosine arabinoside (ara-C), 10^{-3} M; actinomycin D, 4 μg/ml. Isotope incorporation was determined as trichloroacetic acid-insoluble material. Intracellular MTV RNA concentration was measured as previously described (19), and cytoplasmic MTV DNA was measured as described in the legend to Fig. 2. TdR, Thymidine; U, uridine; Leu, leucine; NT, not tested.

ing that unintegrated MTV DNA is produced from viral RNA templates. The data presented here are consistent with that idea. First, unintegrated viral DNA was detected only after viral RNA began to accumulate in response to treatment of the cells with dexamethasone; the time course of appearance of unintegrated viral DNA paralleled that of viral RNA (Fig. 2 and 3). Second, viral DNA could be density labeled in both strands with BUdR under conditions in which cellular DNA had replicated at most one time. Third, inhibitors of the dexamethasone-mediated induction of viral RNA, such as actinomycin D or progesterone, also prevented the induction of unintegrated viral DNA (Table 2). Recently, clones of infected HTC cells have been isolated that fail to produce MTV RNA irrespective of hormone treatment. None that has been tested contains unintegrated viral DNA; only in clones that produced viral RNA have we detected unintegrated MTV DNA (J. Ring, M. Stallcup, and K. Yamamoto, unpublished data). Since the infected HTC cells produce little, if any, extracellular virus (16), superinfection probably did not occur. Thus, cytoplasmic, subviral particles may serve as the active complexes for synthesis of unintegrated viral DNA in dexamethasone-treated cells.

It is unlikely that the synthesis of unintegrated viral DNA plays a significant role in the replication of retroviruses in chronically infected cells. Mouse mammary tumor cells and mouse lymphoma cells contain no detectable unintegrated MTV DNA, even though they produce high levels of viral RNA (17). Similarly, several days after infection of chicken cells with avian sarcoma virus, no unintegrated viral DNA can be detected, although virus particles continue to be produced (13). Moreover, unintegrated DNA does not appear to serve as a template for viral RNA synthesis. Inhibitors of DNA synthesis (e.g. cytosine arabinoside) blocked the induction of unintegrated MTV DNA in infected HTC cells without interfering with the induction of viral RNA. This suggests that the bulk, if not all, of the viral RNA is synthesized from a template of integrated viral DNA, consistent with a requirement for viral DNA integration in the replication cycle of these viruses (2, 27); we do not exclude the possibility that a minor portion of viral RNA could be synthesized from a template of unintegrated viral DNA.

In addition to providing a useful source of material for restriction endonuclease analysis of the viral genome (Shank et al., in press), the induction of MTV DNA provides a unique system in which to study the process of retrovirus DNA synthesis in vivo. Hormonal triggering of this process circumvents the logistical problem of infecting large numbers of cells with high doses of virus. By exploiting the ability to induce viral DNA synthesis at will, it may be possible to elucidate the nature of viral and cellular components involved in the generation of linear and circular MTV DNA.

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