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Detection and Localization of Virus-Specific DNA by *In Situ* Hybridization of Cells During Infection and Rapid Transformation by the Murine Sarcoma-Leukemia Virus

(RNA-directed DNA polymerase/transformed cell)

MARIA CARLA LONI* AND MAURICE GREEN

Institute for Molecular Virology, St. Louis University School of Medicine, 3681 Park Avenue, St. Louis, Missouri 63110

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ABSTRACT **Cytological preparations of interphase nuclei and chromosomes from mouse 3T6 cells prepared at various times after infection with the murine sarcoma-leukemia virus complex were hybridized with the [^{3}H]DNA product of the viral RNA-directed DNA polymerase. While uninfected nuclei had an average of 4 autoradiographic grains, infected nuclei had 30 grains at 5 hr after infection and 63–65 grains at 11 and 25 hr. Virus-specific grains were localized in the chromocenters of interphase nuclei and were found also in the centromeric heterochromatin region of metaphase chromosomes. These findings provide evidence that the viral RNA-directed DNA polymerase functions to synthesize virus-specific DNA early after infection and that newly synthesized viral DNA rapidly becomes associated with or integrated into specific intranuclear sites.**

The RNA-directed DNA polymerase of RNA tumor viruses (oncornaviruses) has been actively studied since 1970, but direct evidence for its intracellular function in the synthesis of virus-specific DNA has been obtained only recently (reviewed in ref. 1). Inhibitor studies beginning in 1964 showed that the replication of and cell transformation by oncornaviruses requires DNA synthesis during the first 6–12 hr of infection (see ref. 2), but the nature of this essential DNA was difficult to establish. Studies attempting to demonstrate an increased content of virus-specific DNA sequences in virus-transformed cells did not agree, for some workers found no differences between normal and transformed murine and avian cells (3–7), while others found a higher content of virus-specific DNA in transformed cells (8–11). These discrepancies probably reflect the presence of DNA sequences from the endogenous oncornavirus in normal mouse (12, 13) and chicken (14) cells and differences in the ability of the molecular probes to detect the endogenous DNA sequences and those introduced by exogenous virus.

In studies reported here, we have utilized *in situ* hybridization of cytological preparations (15, 16) in order to detect and localize virus-specific DNA sequences that are formed early during oncornavirus infection and cell transformation. The ability to prepare [^{3}H]thymidine-labeled virus-specific DNA of high specific activity by the endogenous RNA-directed DNA polymerase of detergent-activated virus particles provides the sensitivity needed to detect small numbers of viral gene sequences in cells. With this viral DNA probe, we have analyzed a clonal line of mouse 3T6 cells rapidly infected by and transformed by the Harvey strain of the murine sarcoma-leukemia virus [H-MSV(MLV)] (17). We find that virus-specific DNA is synthesized early after infection and by 5 hr becomes associated with the chromocenters of interphase nuclei and with the centromeric heterochromatin regions of some chromosomes.

Abbreviations: H-MSV(MLV), the Harvey strain of the murine sarcoma-leukemia virus; SSC, standard saline–citrate, 0.15 M NaCl–0.015 M Na citrate, pH 7.4; 2 × SSC means that the concentration of the solution used is twice that of the standard, etc.; Na dodecyl SO_4, sodium dodecyl sulfate.

* On leave from the University of Louvain, Faculty of Medicine, 4, Avenue Chapelle aux Champs, 1200 Brussels, Belgium.

MATERIALS AND METHODS

Cells and Virus. The preparation of virus stocks and experimental infection were performed as described (17). Mouse 3T6 cells (clone 91) isolated by Dr. N. Takemori and virus-producing mouse MEH cells (18) transformed by H-MSV-(MLV) were grown in Eagle's minimal essential medium containing 10% calf serum. Uninfected 3T6 cells and 3T6 cells at 5, 11, and 25 hr after infection were harvested by trypsin treatment, fixed on slides with 3:1 alcohol:acetic acid, and air-dried (15).

Preparation of Virus [^{3}H]DNA Product. Single-stranded [^{3}H]DNA was prepared by incorporation of [^{3}H]dTTP into 0.02% Nonidet P-40-disrupted H-MSV(MLV). The reaction mixture consisted of 0.1 mM each of dATP, dGTP and dCTP, 30 mM NaCl, 5 mM dithiothreitol, 2.5 mM $MgCl_2$, 40 mM Tris·HCl (pH 8.3), 100 μCi/ml of [^{3}H]dTTP (40.7 Ci/mmol), and 20 μg/ml of actinomycin D. After 6 hr of incubation at 37°, [^{3}H]DNA was purified by Pronase digestion, phenol–chloroform (1:1) extraction, treatment with 0.1 M NaOH at 80° for 20 min, followed by neutralization (18), dialysis against 0.1 × standard saline–citrate (SSC), and passage through a Sephadex G-100 column. The [^{3}H]DNA product hybridized 70–80% with MSV(MLV) 60–70S RNA when annealed in 2 X SSC at 66° for 22 hr, showing that most of the nucleotide sequences are virus-specific negative strands. Even with 200 μg/ml of actinomycin D in the incubation mixture no increase in hybridization efficiency of the DNA product was found (Green, unpublished data).

Cellular DNA. DNA for competition experiments was prepared from human KB cells and 3T6 cells infected for 25 hr. Cells were lysed with Na dodecyl SO_4, digested with Pronase (200 μg/ml), and extracted with chloroform:isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase with 2 volumes of cold ethanol and banded to equilibrium in CsCl

by centrifugation for 50 hr at 35,000 rpm in the Spinco Ti 50 rotor. The DNA-containing fractions were pooled, dialyzed against 0.1 × SSC, precipitated with alcohol, and dissolved in 0.1 × SSC. The concentration of DNA was determined by the diphenylamine reaction (19). Cellular DNA was fragmented by sonication and denatured by heating in a boiling water bath for 10 min.

In Situ Hybridization. The hybridization solution (100–150 μl) containing the [^{3}H]DNA product (2.8 × 10^6 dpm/ml, 6.4 × 10^7 dpm/μg) in 2 × SSC was placed over fixed cells on slides, incubated at 66° for 18 hr, and processed as described (15, 20). In some experiments, unlabeled cellular DNA (100 μg/2.2 × 10^4 cpm) was included for competition analysis. Slides were dipped in NTB-2 Kodak emulsion (diluted 1:1 with distilled water) and stored for 13 weeks in the dark at 4°. Autoradiographs were developed in Kodak D19 for 4 min, fixed for 2 min, and stained with Giemsa. Some slides were incubated with 10 mM Tris·HCl (pH 7.3)–1 mM $MgCl_2$ containing 0.2 mg/ml of DNase (Worthington, RNase-free) for 2 hr at 37° prior to annealing with [^{3}H]DNA.

RESULTS

In situ hybridization with viral [^{3}H]DNA was performed with uninfected 3T6 cells, with 3T6 cells harvested 5, 11, and 25 hr after efficient infection with H-MSV(MLV) (17), and with MEH cells. An average of five grains was detected over interphase nuclei of uninfected 3T6 cells (Fig. 1 and Table 1). The number of grains increased sharply after infection. At 5 hr after infection, an average of 31 grains was found over interphase nuclei (Table 1) and some chromosomes were clearly labeled (Fig. 2). At 11 hr, the average number of grains per nucleus had doubled and numerous chromosomes were labeled (Table 1 and Fig. 3). The number of grains per nucleus remained constant from 11 to 25 hr after infection (Fig. 4, Table 1). The established MEH cell line had an average of 24 grains per nucleus (Table 1). The background density over areas with no cellular structures was 1.0 ± 0.08 and was randomly distributed. The calculated χ^2 is 2.81, less than the tabulated value of $\chi^2 = 7.81$ at $P = 0.05$ with 3 degrees of freedom. The background level was constant for different sets of slides and was low compared to the number of grains over nuclei. Pretreatment of slides with DNase eliminated the grains over nuclei. Competition with DNA from H-MSV(MLV)-infected 3T6 cells reduced the grain count to the background value (Table 1), while competition with DNA

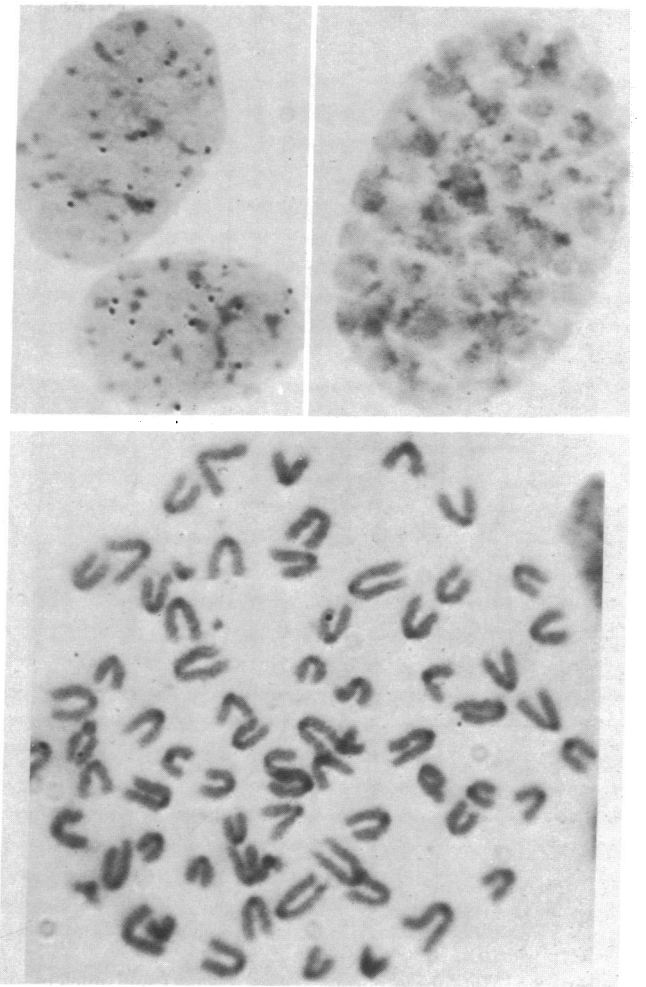

FIG. 1. Autoradiographs of nuclei (*top*) and metaphase chromosomes (*bottom*) of uninfected 3T6, clone 91 cells, after *in situ* hybridization with viral [^{3}H]DNA (specific activity, 6.4 × 10^7 dpm/μg). Exposure 13 weeks. Giemsa-stained (×1600).

TABLE 1. *Hybridization of viral [^{3}H]DNA with interphase nuclei of H-MSV(MLV)-infected and transformed mouse cells*

Cell preparation	No. of nuclei scored		Grain density per nucleus[a] (mean ± SEM)	
	Experimental	Experimental plus competing DNA[b]	Experimental	Experimental plus competing DNA
Uninfected 3T6 cells	80	120	5 ± 0.26	1 ± 0.10
5 hr after infection	40	120	31 ± 1.55	2 ± 0.13
11 hr after infection	40	120	66 ± 2.95	1 ± 0.12
25 hr after infection	40	120	64 ± 2.44	<1
Established H-MSV-transformed mouse cell line (MEH)	40	—	25 ± 1.09	—
	40	—	23 ± 1.12	—

[a] Background of 1.0 ± 0.08 grains, estimated by counting vacant portions on the slide, was not subtracted. Grain counts were performed on photographs at a magnification of 2080. The average nucleus surface (2700 mm^2) was estimated by a surface integration and comparable areas with no cellular structure were located on prints. Experiments were run in duplicate and three to five slides were processed for each time period.

[b] DNA from 3T6 cells infected with H-MSV(MLV) for 25 hr used at a concentration of 100 μg/2.2 × 10^4 cpm.

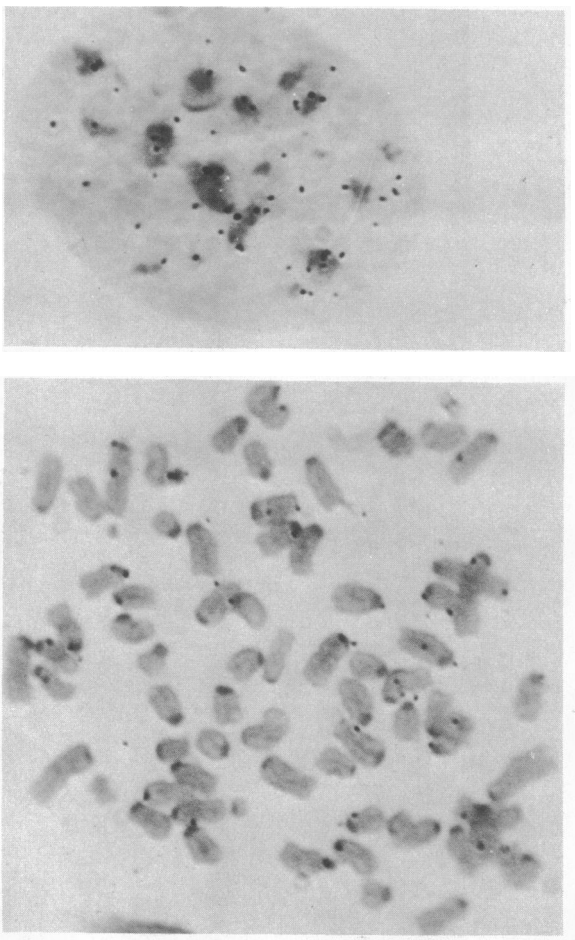

FIG. 2. Metaphase chromosomes (*bottom*) and nucleus (*top*) of 3T6, clone 91 cells, 5 hr after infection with the Harvey strain of murine sarcoma–leukemia virus. Autoradiographs show the hybridization of the viral [^{3}H]DNA product (specific activity 6.4×10^7 dpm/μg) to some chromosomes and the dense Giemsa-stained regions of the interphase nucleus. Exposure 13 weeks (×1600).

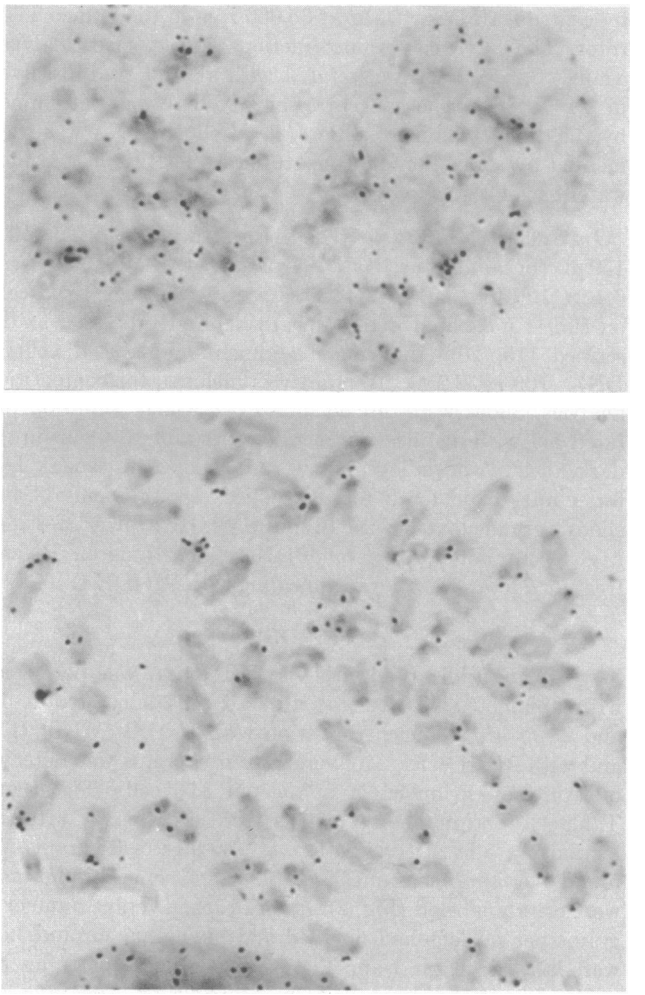

FIG. 3. Autoradiographs of metaphase (*bottom*) and interphase (*top*) nuclei of 3T6, clone 91 cells, 11 hr after infection. Conditions are the same as described in Fig. 2 (×1600).

from uninfected human KB cells had no effect (unpublished data).

The mean grain counts of 5-, 11-, and 25-hr-infected 3T6 cells and MEH cells were compared by placing the values in sequential order and testing the differences at $P = 0.05$ (21). The mean count over nuclei at 11 and 25 hr after infection did not differ significantly. The number of grains over MEH cells differed from that at 5, 11, and 25 hr after infection of 3T6 cells.

The distribution of label showed a distinct concentration of grains over the Giemsa-staining dense areas of interphase nuclei (Figs. 2, 3, and 4), and over the centromeric heterochromatin area of some metaphase chromosomes (Fig. 5). Grains were found also in intermediate and terminal regions of chromosomes (Fig. 5).

DISCUSSION

Cytological hybridization combines the specificity of molecular hybridization with the spatial localization of tritium autoradiography (15, 16). The use of a viral DNA product with a specific activity of 6.4×10^7 dpm/μg, together with 13 weeks of exposure to the photographic emulsion, provides the sensitivity to detect viral gene sequences during virus replication and cell transformation. Uninfected 3T6 cells showed an average of 4 autoradiographic grains above an average background of 1 grain. These grains could represent DNA sequences from the endogenous murine C-type oncornavirus of normal mouse cells (12, 13), as implied by the oncogene hypothesis (22) and by molecular hybridization studies (6). The increase in grain count to 30 at 5 hr, and 63–65 at 11 and 25 hr after infection indicates that virus-specific DNA is rapidly synthesized after infection and becomes associated with the cell nucleus. The absence of significant grains after pretreatment with RNase-free DNase, or upon competition with DNA from MSV(MLV)-infected 3T6 cells but not with DNA from uninfected human KB cells, demonstrates the specificity of the cytological DNA·DNA hybridizations. Moreover, considerable reduction in grain count was obtained upon competition with 60–70S RNA (unpublished data). Finally, *in situ* hybridization with viral 60–70S [^{3}H]-RNA gave similar results with a somewhat lower grain count, because of the lower specific activity of viral RNA (unpublished data), ruling out the possibility that cell DNA sequences in the DNA product were responsible for autoradiographic grains.

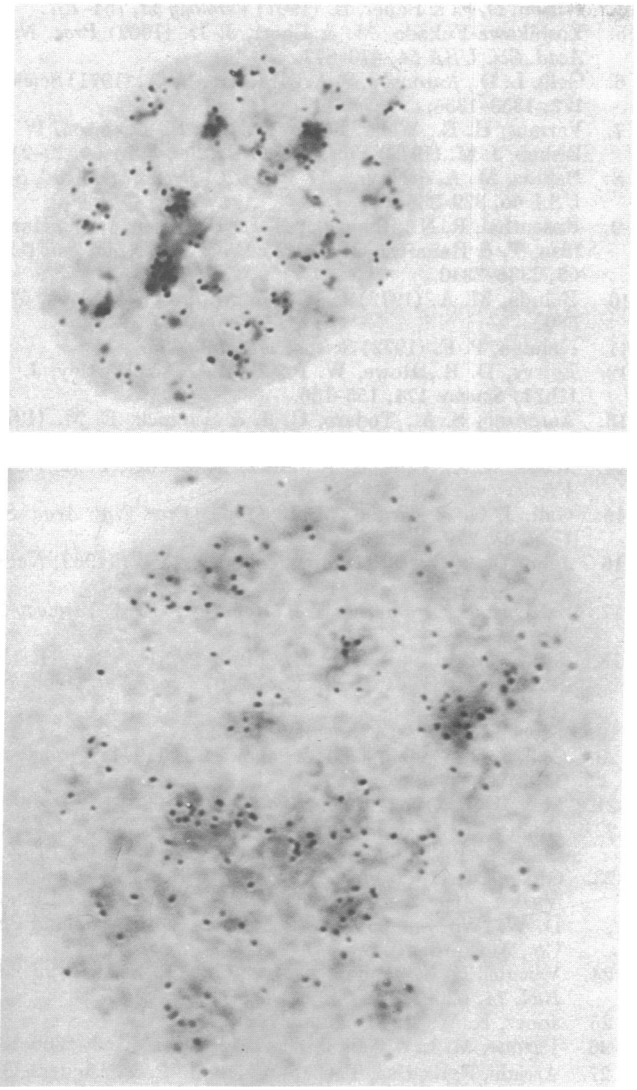

FIG. 4. Autoradiographs of interphase nuclei of 3T6, clone 91 cells, 25 hr after infection. An occasional giant cell is shown (on the *bottom*) with the same pattern of grain distribution over the Giemsa-staining dense areas (×1600).

We show here that viral DNA sequences are synthesized and become associated with interphase nuclei and chromosomes by 5 hr after infection. These results support but do not prove that the viral RNA-directed DNA polymerase functions to synthesize virus-specific DNA. In addition, some intracellular virus-specific DNA sequences could have been synthesized by noninfectious virus particles. However, MEH cells established in culture for many generations yielded an average of 24 autoradiographic grains per cell when annealed with the viral DNA product. The possibility that most autoradiographic grains are unrelated to functional viral genomes thus seems unlikely.

We report here that cytological hybridization with the single-stranded H-MSV(MLV) product, representing most of the viral genome (23), detected a large increase in viral DNA sequences in 3T6 cells after infection with H-MSV-(MLV). Another study utilizing reassociation kinetic measurements with a double-stranded murine leukemia virus (MLV)-DNA product measured a similar content of viral DNA sequences in uninfected mouse cells, MLV-infected mouse cells, and nonvirus-producing mouse cells transformed by the Kirsten murine sarcoma virus (6). The inability to detect an increased viral DNA content in infected cells was most likely due to the fact that the MLV DNA product utilized in the latter study represented only a small portion of the viral genome (6). Evidence for an increased content of viral DNA sequences in both avian (8–10) and murine (24) cells transformed by avian oncornaviruses has been obtained recently.

The relationship between grain count and the number of complementary DNA sequences in interphase nuclei is difficult to quantitatively evaluate. If we assume (*i*) a 10% efficiency for autoradiographic detection (25), (*ii*) a 10% hybridization efficiency, and (*iii*) the presence of complete copies of the viral genome in the cell nucleus, then a viral DNA probe with a specific activity of 6.4×10^7 dpm/μg would give 1.39 grains per viral genome after an exposure of 13 weeks. Dividing the observed grain counts by 1.39 gives 2–3 viral genome equivalents per uninfected cell and 18 and 44 newly synthesized viral genomes at 5–25 hr after infection. For MEH cells, 10.8 copies of the viral genome per cell were calculated based on the same exposure time. It must be emphasized, however, that these values are rough estimations, and that no information is available on the size, genome content, and possible tandem arrangement of the virus-specific DNA sequences present in the infected cell.

Of special interest is the association of autoradiographic grains with the chromocenters of interphase nuclei of infected

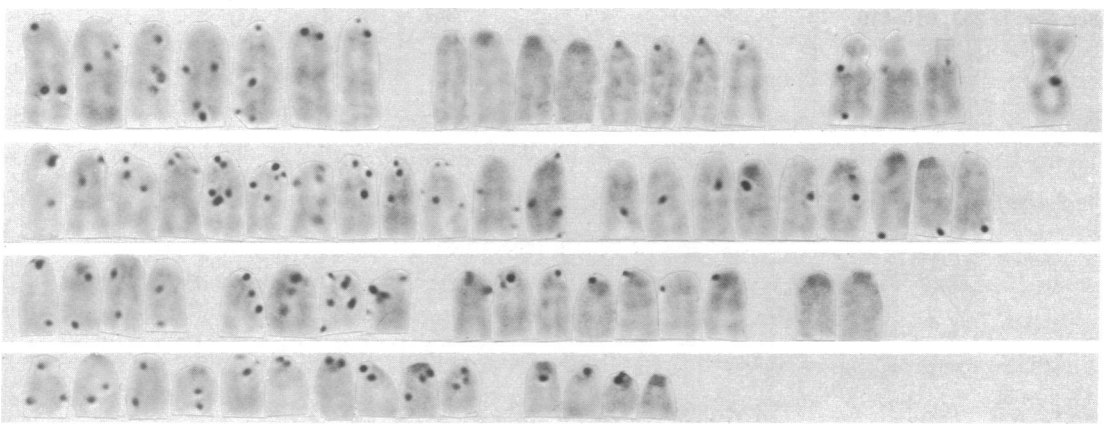

FIG. 5. Karyotype of a 3T6, clone 91 cell, at 25 hr after infection. Chromosomes are arranged roughly in order of size. Groups with similar pattern of grain distribution are evident. Conditions are the same as described in Fig. 2 (×2080).

3T6 cells that stain darkly with Giemsa, and with the centromeric heterochromatin regions of several chromosomes. Pardue and Gall (26) demonstrated the association of mouse satellite DNA with the centromeric portions of metaphase chromosomes. Highly repeated DNA sequences have also been detected in other species, including humans, by *in situ* hybridization (27–31), although the significance of the pattern of distribution of repetitive DNA sequences in eukaryotic cells is not understood.

Britten and Davidson (32) have suggested that repetitive sequences may play a role in genetic regulation. The high efficiency of transformation of clonal 3T6 mouse cells by H-MSV(MLV) might possibly be related to the specific binding of viral DNA to some repetitive sequences in the heterochromatin region of chromosomes. This could explain, in part, the rapid association of virus-specific DNA with the heterochromatic regions as revealed by the Giemsa-staining patterns. The results with MEH cells, an established MSV transformed cell line, also show association between virus-specific DNA sequences and the centromeric heterochromatin regions of chromosomes (unpublished data).

The estimate of virus-specific DNA sequences in 3T6 cells at 11 and 25 hr after infection (63–65 grains) differs from those in MEH cells (23 grains) and could be accounted for, at least in part, by numerical or structural variations in their karyotype. In the 3T6 cell line, the great majority of cells had variations around a modal number in the seventies (Fig. 5). In such heteroploid cells, certain chromosomes occur in higher and lower proportion than in normal mouse cells. The MEH cell line displays a chromosome pattern in the range of a normal diploid mouse cell (unpublished data). Alternately, it is also possible that the higher mean grain count over the nuclei of infected 3T6 as compared to MEH cells reflects the synthesis of some viral DNA that does not become integrated into chromosomes.

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