Dimethyl Sulfoxide Affects the Amount of Extrachromosomal Spleen Focus-Forming Virus DNA in Murine Erythroleukemia Cells

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We used Southern blot hybridization to titrate and map restriction enzyme cleavage sites of a 6.3-kilobase-pair species of extrachromosomal viral DNA found in derivatives of the 745A line of murine erythroleukemia cells, which vary in their ability to be induced to differentiate by dimethyl sulfoxide (DMSO). Greater than an eightfold variation was observed in the amount of this DNA, with the largest amounts being found in cells that were resistant to the induction of differentiation by DMSO. This increase in the level of extrachromosomal viral DNA was found to be dependent upon the continued presence of DMSO in the culture medium. The increase was shown not to be due to an immediate stimulatory effect of this agent on the synthesis or maintenance of this DNA, since cell lines sensitive to the differentiation-inducing effects of DMSO were shown to undergo a transient reduction in the amount of extrachromosomal viral DNA after the addition of DMSO to the culture medium. In addition to the 6.3-kilobase-pair linear form found in the cytoplasm, in some preparations two hybridizing bands were observed that migrated in agarose gels in the position expected of covalently closed circular species of viral DNA. Restriction enzyme mapping of the cytoplasmic linear form indicated a close relationship of this DNA to two polycythemic strains of spleen focus-forming virus that have been molecularly cloned by other workers. No obvious change in the number or arrangement of chromosomal viral sequences could be detected after treating cells with DMSO. Thus, the exposure of murine erythroleukemia cells to DMSO caused an obvious change in the amount of extrachromosomal spleen focus-forming virus DNA but no obvious change in the integration of the provirus.

The Friend virus complex induces in susceptible mice a disease characterized by an acute initial erythroblastosis and splenomegaly (12). Later in the course of the disease, there emerges a transformed population of erythroid cells that can be established *in vitro* (13, 21, 30) and induced to terminally differentiate along the erythroid pathway by adding a variety of chemical agents to the culture medium (14, 34). Recent work utilizing *in vitro*-constructed insertion-deletion mutants of a molecularly cloned DNA copy of the spleen focus-forming virus (SFFV) component indicates that information encoded within the viral *env* gene region is required for inducing at least the initial erythroblastosis (25). However, it is unknown whether or not a function of the virus complex is involved either in the emergence of transformed cells or in maintaining the cells that can be established *in vitro* in an undifferentiated transformed state.

Previous studies addressing this question of

virus involvement typically have utilized various biological and biochemical assays to examine the Friend virus complex in murine erythroleukemia cells (MELC) that have been exposed to the differentiation-inducing agent dimethyl sulfoxide (DMSO). An early finding was an increase in the number of budding virus particles on the surface of DMSO-treated cells as detected by electron microscopy (37). It could not be determined by these studies whether this increased number of particles represented the increased synthesis of virus or an inhibition of virus release. However, additional work indicates that a commonly observed phenomenon that occurs in many separately established cell lines is a transient increase in virus synthesis after exposure to DMSO (9, 10, 22, 31, 32). Increases in SFFV activity (5- to 200-fold) have been reported during the first 2 days of DMSO treatment. Coupled with this increase is a change in the tropism of the helper virus, suggesting that DMSO treat-

ment also causes the induction of an endogenous virus in MELC (9, 31, 32). Increased reverse transcriptase activity after DMSO treatment has also been reported in some MELC lines (22, 32, 42), as have increases in the amount of polysomal viral RNA (7, 31, 32, 33). In addition, changes in the genome composition and the ratios of SFFV to helper virus have been observed in the virus complex that is released by DMSO-treated MELC. This is detected by changes in the distribution of buoyant densities of virus particles in the virus complex and by changes in the composition and amount of the different RNA subunits contained within these released virus particles (31, 32).

Variant cell lines exist that do not show the increase in virus synthesis and yet still can be stimulated to differentiate. Cell lines resistant to the differentiation-inducing effects of DMSO that nevertheless show an increase in virus release upon exposure to DMSO have also been described. Therefore, it has been suggested that the augmentation of virus gene expression and the induction of differentiation are independent events (9, 22, 38, 42). It has also been suggested that this increase in virus synthesis may be related to the effects of DMSO on the cellular growth cycle of MELC (38).

An extrachromosomal form of SFFV DNA has been observed in one DMSO-resistant MELC line (23). We asked whether this form of DNA is present in other cell lines and what changes occur in this DNA after MELC are exposed to DMSO. We found that the amount of extrachromosomal SFFV DNA is different in cell lines that differ in their ability to be induced by DMSO and that the treatment of cells with DMSO can affect the amount of extrachromosomal viral DNA.

MATERIALS AND METHODS

Cell lines and cell cultures. All cell lines are derivatives of C. Friend's 745A. 10HK7, 10HK10, 10HK23, and 6A11A are cell lines with various sensitivities to DMSO. They were isolated without previous exposure to DMSO or mutagens by sib-selection and repeated subcloning as previously described (1). Cells were maintained in Dulbecco modified Eagle medium with pyruvate supplemented with 10% fetal calf serum (DME 10). Cells were incubated at 35°C in 7.5% (vol/vol) CO₂ in a humidified incubator. Cells used in isolating extrachromosomal DNA were maintained in DME 10 or in DME 10 containing 1.5 or 1.8% DMSO at concentrations of less than 10⁶ cells per ml. 27E6 is a cell line resistant to the induction of terminal differentiation by DMSO. It was isolated as follows. A portion of a population that had been enriched for growth in DME 10 containing 0.5% DMSO was subsequently placed in DME 10 containing 1.5% DMSO and maintained by serial dilution in this medium. They were cloned in microtiter dishes at one cell per five wells in DME 10 containing 1.5% DMSO. 27E6 repre-

sented such a clonal isolate and was continuously maintained in DME 10 containing 1.5% DMSO.

Isolation of DNA. Cells were pelleted by centrifugation, washed, and suspended in phosphate-buffered saline without calcium or magnesium. The cell concentration was determined with a Coulter counter model ZB, and a fixed number of cells was centrifuged. In initial experiments, the cell pellet was suspended in 0.01 M Tris-hydrochloride–0.01 M EDTA (pH 8.0) at a concentration of 3.0×10^6 cells per ml, and cell lysis occurred after the addition of Sarkosyl to 1.0%. The lysate was digested with 20 µg of proteinase K per ml at 60°C for 0.5 h, and the chromosomal DNA was then removed by centrifugation at $30,000 \times g$ for 0.5 h. The supernatant was removed and adjusted to 0.5 M NaCl, and 2 volumes of ethanol was added. The precipitate was suspended in 1 volume of 0.01 M Tris-hydrochloride–0.001 M EDTA (pH 8.0), and the ethanol precipitation step was repeated, followed by resuspension in 1 volume of 0.01 M Tris-hydrochloride–0.001 M EDTA buffer that was suitable for electrophoresis.

In subsequent experiments, a modification of the Hirt DNA extraction procedure was used (19). Lysis with 1.0% sodium dodecyl sulfate (SDS) was performed at 60°C for 0.5 h followed by the addition of 5 M NaCl to give a final concentration of 1 M. After incubation overnight at 0°C, the lysate was centrifuged at $17,000 \times g$ for 1 h, and the Hirt supernatant was removed and treated with 50 µg of proteinase K per ml for 1 h, followed by one chloroform-isoamyl alcohol (24:1) extraction. The aqueous supernatant was removed, precipitated with ethanol, and suspended in 0.01 M Tris-hydrochloride–0.001 M EDTA buffer, pH 8.0. Before electrophoresis, heat-treated RNase A (95°C for 10 min) was added to 100 µg/ml, followed by incubation at 37°C for 0.5 h. In some experiments, the same amount of a dilution of agarose gel-purified 8.5-kilobase (kb) Friend murine leukemia virus (F-MuLV) DNA plasmid insert was added to all samples after cell lysis to serve as an internal control for the extraction and subsequent hybridization procedures.

High-molecular-weight chromosomal DNA was isolated from DBA/2J tissues that previously had been frozen at –70°C. The tissues were homogenized in 25 ml of 0.02 Tris-hydrochloride–0.1 M NaCl–0.01 M EDTA, pH 8.0. SDS and proteinase K were then added to 1.0% and 100 µg/ml, respectively, and incubated at 37°C overnight. For the isolation of chromosomal DNA from MELC, cell cultures were centrifuged for 5 min at $1,000 \times g$, and the cell pellets were washed once in phosphate-buffered saline without calcium or magnesium, centrifuged, and suspended in 0.01 M Tris-hydrochloride–0.1 M NaCl–0.001 M EDTA, pH 8.0. Cells were lysed with the SDS and proteinase K treatment described above for DBA/2J tissues. Both MELC and DBA/2J lysates were extracted once with 0.01 M Tris-hydrochloride (pH 8.0)-saturated phenol and once with chloroform-isoamyl alcohol (24:1). Alternatively, DNA was isolated from the Hirt pellets obtained from MELC after suspension in 0.01 M Tris-hydrochloride–0.001 M EDTA, proteinase K treatment, and extraction with phenol-chloroform-isoamyl alcohol. The aqueous phase was precipitated with 2 volumes of ethanol, and the DNA was spooled on a glass rod, dried, and suspended in 0.01 M Tris-hydrochloride–0.001 M EDTA, pH 8.0. RNase A was added to 100 µg/ml, and the concentration of NaCl

was adjusted to 0.1 M. Incubation at 37°C for 1 h was followed by treatment with 100 µg of proteinase K per ml at 37°C for 1 h. The DNA solutions were then extracted with organic solvents, dialyzed against Tris-EDTA buffer, and again precipitated with ethanol. The DNA was then recovered by spooling, drying, and suspension in Tris-EDTA buffer. DNA concentrations were determined by absorbance at 260 nm. DNA prepared in this manner had optical density ratios at 260 and 280 nm of 1.8 to 1.9, and the majority were larger than 49 kb, as determined by migration in agarose gels with phage lambda DNA as a molecular weight marker.

Agarose gel electrophoresis and Southern blot hybridization. Electrophoresis was performed in a 0.04 M Tris-0.02 M sodium acetate-0.002 M EDTA buffer (pH 7.8) containing 0.5 µg of ethidium bromide per ml in both the gel and buffer with constant buffer recirculation. Transfer to nitrocellulose and hybridization were carried out by the procedure of Wahl et al. (45) and included partial acid depurination before transfer. Hybridization was performed in 50% formamide-3× SSC-0.01 M phosphate buffer (pH 6.8)-100 µg of sheared and sonicated salmon testes DNA per ml (Sigma Chemical Co., St. Louis, Mo.)-0.3% SDS-10% dextran sulfate (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) at 42°C overnight.

Gel-eluted (6) 8.5-kb F-MuLV (29) or 5.7-kb SFFV (26) DNA plasmid inserts (recombinant plasmids generously provided by A. Oliff and D. Linemeyer, respectively) were nick translated to a specific activity of 1×10^8 to 4×10^8 cpm/µg, essentially by the method of Rigby et al. (35). A 0.7-kb *Bam*HI-*Eco*RI double-digestion restriction fragment that maps within the *env* gene region of SFFV, isolated from a polyacrylamide gel by the procedure of Maxam and Gilbert (28), was nick translated to a specific activity of 3×10^7 cpm/µg and used as a hybridization probe with filters containing transferred genomic *Hind*III restriction fragments. High-molecular-weight DNA (10 µg) was digested with 20 U of restriction enzyme *Hind*III (Bethesda Research Laboratories, Gaithersburg, Md.) in a buffer consisting of 0.02 M Tris-hydrochloride (pH 7.4), 0.005 M MgCl₂, 0.001 M dithiothreitol, 0.05 M NaCl, and 100 µg of autoclaved gelatin per ml. Lambda phage DNA was used to monitor the extent of the digestion.

Hybridization probes were used at a concentration of 1 to 5 ng/ml. After hybridization, the filters were washed twice in 2× SSC-0.2% SDS at 45°C for 15 min each, followed by two washes in 0.1× SSC-0.2% SDS at 45°C, and exposed to preflashed Kodak XR-1 X-ray film at -70°C for 1 to 5 days with DuPont Lightning-Plus intensifying screens (24).

Quantification of 6.3-kb extrachromosomal SFFV DNA. A Zeineh soft-laser scanning densitometer was used to scan the autoradiographs, and the area under the peaks and above background was determined with an Apple II microcomputer and Apple Graphics Tablet. Control experiments included dilutions of F-MuLV 8.5-kb DNA to determine that film response was a linear function of the amount of DNA applied to the gel in the range in which comparisons were being made. In several experiments, an equal amount of a dilution of 8.5-kb F-MuLV DNA was added to the lysate immediately after the addition of SDS to serve as an internal control. However, comparisons based

on the ratios of the amounts of 6.3-kb DNA to the amounts of 8.5-kb DNA generally yielded results comparable to those based on the amount of 6.3-kb DNA alone.

Restriction enzyme mapping of 6.3-kb unintegrated DNA. Cytoplasmic DNA from the DMSO-resistant variant 27E6 cultured continuously in DME 10 containing 1.5% DMSO was used for restriction enzyme mapping of the 6.3-kb linear form. Cells (1.8×10^9) were washed in phosphate-buffered saline without calcium or magnesium and suspended in 0.05 M Tris-hydrochloride-0.025 M NaCl-0.002 M EDTA, pH 8.0. Nonidet P-40 (Bethesda Research Laboratories) was added to 0.5%, and the cells were placed on ice for 5 min. The nuclei were removed by centrifugation at $1,000 \times g$ for 5 min. The supernatant was treated with 50 µg of proteinase K per ml at 37°C for 0.5 h, followed by phenol extraction and ethanol precipitation. The precipitate was suspended in 0.01 M Tris-hydrochloride-0.005 M EDTA (pH 8.0) and incubated for 2 h at 37°C with RNase A at 100 µg/ml. This was followed by proteinase K treatment at 50 µg/ml for 1 h at 37°C, two extractions with chloroform-isoamyl alcohol (24:1), and ethanol precipitation. Low-molecular-weight molecules were removed by gel filtration on an A-50m (Bio-Rad Laboratories) column (2.0 by 45 cm) equilibrated with 0.01 M Tris-hydrochloride-0.5 M NaCl-0.001 M EDTA, pH 8.0. Fractions containing a 6.3-kb species hybridizing to SFFV [³²P]DNA were pooled and ethanol precipitated. The extent of restriction enzyme digestion was monitored by including either phage lambda DNA (Bethesda Research Laboratories) or T7 DNA (generously provided by W. T. McAllister) in the reaction mixture. Digestions with restriction endonucleases (Bethesda Research Laboratories) were carried out in the buffer recommended by the supplier containing the appropriate concentration of NaCl for the particular enzyme being used.

RESULTS

Variation in amount of 6.3-kb extrachromosomal virus DNA in MELC. Previous studies have indicated the presence of a 6.3-kb species of linear extrachromosomal DNA with sequence homology to the SFFV in Hirt supernatants of an MELC line resistant to the induction of differentiation by DMSO (23). This line was derived from the infection of DDD mice with the polycythemic strain of F-MuLV and produces large amounts of SFFV (23). Since little or no SFFV activity has been detected in cell lines derived from the infection of DBA/2J mice with the anemic strain of virus (10, 15, 17, 38), we were interested in determining whether derivatives of the 745A line also contained this extrachromosomal DNA and whether or not this DNA was specific to cell lines resistant to DMSO.

Extrachromosomal DNA was isolated from an equal number of cells, from populations having various degrees of sensitivities to DMSO, by pelleting the chromosome by high-speed centrifugation. This extrachromosomal DNA was then analyzed by the method of Southern (39) with a ³²P-labeled genomic F-MuLV probe. In addition

to a diffuse band of hybridizing DNA due to chromosomal contamination of these preparations, an additional discrete band of hybridizing DNA with a size of approximately 6.3 kb was observed (Fig. 1). No hybridization was observed in the region of 9.1 kb, the size that would be expected for a linear extrachromosomal DNA copy of the F-MuLV helper virus (29).

The quantification of the amount of hybridizing 6.3-kb DNA by densitometry indicated that there was more than an eightfold variation in the amount of this extrachromosomal 6.3-kb DNA in the cell lines examined (Table 1). A similar degree of variation was observed when the Hirt DNA extraction procedure (19) was used (data not shown). Moreover, the amount of this DNA appeared to be inversely related to the ability of these cell lines to be induced to differentiate by DMSO. The largest amount of extrachromosomal virus DNA was observed in 27E6, a noninducible DMSO-resistant cell line that was maintained in DME 10 containing 1.5% DMSO. In

TABLE 1. Variation in amount of extrachromosomal virus DNA in MELC

Cell line	Relative amt of 6.3-kb virus DNA ^a	% Benzidine-positive cells ^b
10HK7	1.0	85.3
10HK23	2.3	13.6
27E6	8.4	4.6 ^c

^a Determined by densitometry tracing of the autoradiograph (Fig. 1) and quantitated as described in the text.

^b Determined after 5 days in DME 10 containing 1.5% DMSO.

^c Determined with a population of cells that had been removed from DMSO-containing medium and cultured in DME 10 for 5 days before being replaced in DME 10 containing 1.5% DMSO for 5 days.

FIG. 1. Extrachromosomal virus DNA present in murine erythroleukemia cells. Extrachromosomal DNA was prepared by high-speed centrifugation of a cellular lysate from 4.6×10^6 cells as described in the text and electrophoresed in a 0.8% agarose gel at 50 V for 18 h. The DNA was denatured, transferred to nitrocellulose, and hybridized to a ^{32}P -labeled F-MuLV probe. Lane A, DNA prepared from 10HK7, a high-inducible cell line, cultured in DME 10. Lane B, DNA prepared from 10HK23, a low-inducible cell line, cultured in DME 10. Lane C, DNA prepared from 27E6, a noninducible cell line, cultured continuously in DME 10 containing 1.5% DMSO.

this experiment, 10HK23, a low-inducible cell line (13.6% benzidine-positive cells after 5 days in 1.5% DMSO), had more than twice the amount of extrachromosomal 6.3-kb virus DNA observed in a high-inducible cell line, 10HK7 (greater than 80% benzidine positive). These high- and low-inducible cell lines were isolated from the same starting population of cells by a process of sib-selection and three rounds of subcloning (1).

To determine whether the amount of extrachromosomal virus DNA was related to DMSO inducibility, we examined the grandparents and high- and low-inducible sibs of the grandparents that were also isolated by single-cell cloning. Both pairs showed a similar inverse correlation between the amount of extrachromosomal virus DNA and DMSO inducibility, e.g., both the low-inducible grandparent and low-inducible sib of the grandparent had an amount of extrachromosomal virus DNA two to three times that observed in the high-inducible grandparent and high-inducible sib of the grandparent. However, an apparent exception to this correlation was observed in one low-inducible cell line isolated in a similar manner from a different starting population of cells. This cell line did not show an elevated amount of extrachromosomal virus DNA (data not shown). These results suggest that the amount of extrachromosomal virus DNA may be one of the factors related to differences in DMSO inducibility of cell differentiation, but it is not the only factor.

Restriction enzyme mapping of the 6.3-kb extrachromosomal viral DNA. Restriction enzyme cleavage sites have been mapped for two polycythemic strains of molecularly cloned SFFV DNA (25, 26, 46). To determine what relationship the extrachromosomal DNA present in MELC had to these strains, we isolated cytoplasmic DNA from the DMSO-resistant variant

cell line 27E6 and performed single or double digests with the restriction enzymes (Fig. 2). The results of this experiment indicated two restriction enzyme cleavage sites for enzymes *Hind*III, *Eco*RI, and *Kpn*I and three cleavage sites for *Bam*HI. *Xba*I and *Sal*I failed to cleave this DNA. Fragments smaller than approximately 0.4 kb would not be detected by this procedure. Based on double digests, we were able to predict the location of these cleavage sites (Fig. 3). The 5' to 3' orientation of this map is based upon the partial *Bam*HI and *Kpn*I double digest and on the location of *Kpn*I sites within the large terminal repeat of MuLV DNA (3). Since partial *Kpn*I digestion of a 2.8-kb *Bam*HI fragment yielded fragments of approximately 2.8 and 2.3 kb in size and since the *Kpn*I site is located approximately 0.5 kb from the 5' end of the 5' long terminal repeat in MuLV DNA, this places the 2.8-kb *Bam*HI fragment at the 5' end of the genome. The similarity between the map shown in Fig. 3 and published restriction maps for two polycythemic strains of SFFV DNA (25, 26, 46) indicates that the extrachromosomal cy-

toplasmic virus DNA in MELC is a linear full-length DNA copy of a form of SFFV.

Effect of DMSO on amount of extrachromosomal SFFV DNA. The cell line resistant to the induction of differentiation by DMSO exhibited eightfold-higher levels of extrachromosomal SFFV DNA than did a cell line highly sensitive to this agent. This resistant cell line was continuously maintained by serial dilution in DME 10 containing 1.5% DMSO, whereas the sensitive cell lines assayed for extrachromosomal SFFV DNA were maintained in DME 10 without DMSO. To determine whether the elevated amounts of extrachromosomal SFFV DNA in the resistant variant were related to the treatment with DMSO, we isolated another such variant. A population of cells that was initially highly sensitive to DMSO was repeatedly subcultured in DME 10 containing 1.5% DMSO. This procedure, therefore, enriched for that subpopulation of cells that was resistant to the terminal differentiation inducing effects of DMSO.

We then examined the amount of extrachro-

FIG. 2. Restriction enzyme mapping of cytoplasmic virus DNA. Cytoplasmic DNA was isolated as described in the text, digested with the indicated restriction enzymes, electrophoresed, denatured, and transferred to nitrocellulose. Hybridization was with a 32 P-labeled 5.7-kb SFFV DNA insert isolated from a recombinant plasmid. (A) Electrophoresis was in a 1.2% agarose gel at 45 V for 19 h. *Kpn*I digestion was incomplete in the *Kpn*I plus *Eco*RI (E + K) and the *Kpn*I plus *Bam*HI (K + B) double digests. E + B, *Eco*RI plus *Bam*HI double digest. K + H, *Kpn*I plus *Hind*III double digest. (B) Results of *Hind*III plus *Bam*HI (H + B) and *Hind*III plus *Eco*RI (H + E) double digests with a longer exposure of the same autoradiograph juxtaposed to allow the visualization of a 0.4-kb fragment. Electrophoresis was in a 1.4% agarose gel at 50 V for 18 h.

FIG. 3. Location of restriction enzyme cleavage sites in cytoplasmic virus DNA isolated from MELC. The alignment of fragments was based on an analysis of the size of the restriction fragments that hybridized to the SFFV [32 P]DNA probe shown in Fig. 2. A 5' to 3' orientation was deduced from partial *KpnI* cleavage in double digests as described in the text. The numbers refer to the approximate distance in kb pairs from the 5' end.

mosomal SFFV DNA present in the Hirt supernatant extracted from an equal number of cells of both the parental and DMSO-resistant populations. The results of this analysis (Fig. 4, lanes A and B) indicate that selection for DMSO resistance resulted in the emergence of a population of cells that had an amount of extrachromosomal SFFV DNA approximately fourfold that observed in the DMSO-sensitive parental population. In this particular experiment, we took advantage of the sequence homology that exists between the F-MuLV helper and the SFFV. This homology allowed us to add an equal amount of a dilution of an 8.5-kb species of molecularly cloned F-MuLV DNA to each extract immediately after cell lysis by SDS for use as an internal control on subsequent extraction, transfer, and hybridization steps. The equal intensities of this 8.5-kb band in the lanes containing the Hirt supernatants of DMSO-resistant or -sensitive cells indicated that the fourfold variation observed in the amount of 6.3-kb SFFV DNA was not due to any experimentally induced variations that may occur during these steps (Fig. 4).

When a similar analysis was performed on another population of DMSO-resistant cells grown in the absence of DMSO for a period of approximately 9 months, a twofold reduction in the amount was observed, and the cells remained resistant to the differentiation-inducing effects of DMSO. The amount of extrachromosomal SFFV DNA was again increased by adding DMSO back to the growth medium for a period of approximately 5 weeks (Fig. 4, lanes C through E).

These results indicate that the elevated amounts of extrachromosomal SFFV DNA observed in two different populations of DMSO-resistant cells are related to DMSO treatment. They fail, however, to determine whether DMSO selects for a subpopulation of cells pre-existing within the initial DMSO-sensitive population that has elevated amounts of extrachromosomal SFFV DNA or whether DMSO

actually induces the synthesis of extrachromosomal SFFV DNA. We therefore examined whether DMSO had an immediate effect on the levels of extrachromosomal SFFV DNA in cells that were highly sensitive to this agent.

FIG. 4. Effect of long-term DMSO treatment on the amount of extrachromosomal SFFV DNA in MELC. Hirt supernatants from 5.0×10^6 cells (lanes A and B) or 4.5×10^6 cells (lanes C through E) were electrophoresed in 0.8% agarose gels, denatured, transferred to nitrocellulose, and hybridized to 32 P-labeled F-MuLV DNA (lanes A and B) or 32 P-labeled SFFV DNA (lanes C through E). Lane A, Hirt supernatant from high-inducible cell line 6A11A cultured in DME 10. Lane B, Hirt supernatant from cell line 6A11A after selection for resistance to the induction of terminal differentiation by DMSO by continued serial dilution into DME 10 containing 1.5% DMSO for approximately 7 weeks. Lane C, Hirt supernatant from DMSO-resistant variant 27E6 cultured continuously in DME 10 containing 1.5% DMSO. Lane D, Hirt supernatant from cell line 27E6 removed from DMSO-containing medium and cultured in DME 10 for approximately 9 months. Lane E, Hirt supernatant from cell line 27E6 that had been cultured in DME 10 for 8 months and replaced in DME 10 containing 1.5% DMSO for approximately 5 weeks. An equal amount of a dilution of an 8.5-kb F-MuLV plasmid insert (29) was added to the cell lysates in lanes A and B to serve as an internal control for subsequent extraction, transfer, and hybridization procedures. The marker fragments, expressed in kb, were lambda DNA digested with *HindIII*.

Hirt DNA extractions were performed on a cell line that is inducible by DMSO to the extent that approximately 85% of the cells stained positive for hemoglobin at 96 h after the addition of this agent. Hirt supernatants from an equal number of cells incubated in DME 10 containing 1.8% DMSO for 0, 6, 18, and 24 h were analyzed by the method of Southern for the presence of extrachromosomal SFFV DNA with ^{32}P -labeled cloned genomic SFFV DNA probe. The amount of this DNA was quantitated by densitometry.

The results of this and similar experiments are shown in Fig. 5 and 6. They demonstrate that a reduction in the amount of extrachromosomal linear 6.3-kb SFFV DNA could be observed within 6 h after the addition of DMSO. By 18 to 24 h post-DMSO treatment, the amount of this DNA was reduced to approximately one half of the amount present in untreated cells (Fig. 6). In the particular experiment shown in Fig. 5, two bands migrating ahead of the 6.3-kb linear SFFV band that also hybridize to the ^{32}P -labeled SFFV probe were also observed. These bands were located where the two forms of covalently closed circular virus DNA would be expected to migrate. The amount of the slower-migrating form also appeared to be reduced in cells treated with DMSO. However, in light of the susceptibility of covalently closed circular species to nucleases, the significance of this finding is difficult to assess.

These results indicate that the increased amount of extrachromosomal SFFV DNA observed in cells maintained in DME 10 containing 1.5% DMSO was not due to an immediate increase in the synthesis or maintenance of this DNA when DMSO was added to the medium. Two possibilities, therefore, remain that could account for this increase: (i) there is a continual selection for a preexisting subpopulation of cells that contain increased amounts of extrachromosomal SFFV DNA, or (ii) there is an increased synthesis or maintenance of this DNA associated with the prolonged exposure to DMSO required for the transition from DMSO sensitivity to DMSO resistance.

Integration of SFFV in MELC variants. The results described above indicate a greater than eightfold difference in the amount of extrachromosomal SFFV DNA in a DMSO-resistant variant cell line compared with that in a cell line that was highly inducible by this agent. To determine whether this increase in the amount of extrachromosomal SFFV DNA is also reflected in an increased number of integrated copies in DMSO-resistant cell lines, we used Southern blotting to analyze the integration of SFFV in these MELC variant cell lines. High-molecular-weight DNA was isolated, and a portion was cleaved with restriction enzyme *Hind*III. The

FIG. 5. Effect of short-term DMSO treatment on the amount of extrachromosomal SFFV DNA in MELC. Hirt supernatants from 1×10^7 cells of a high-inducible cell line that had been cultured in DME 10 or in DME 10 containing 1.8% DMSO for 6, 18, and 24 h before Hirt extraction were electrophoresed in a 0.8% agarose gel at 40 V for 17 h, denatured, transferred to nitrocellulose, and hybridized to an SFFV [^{32}P]DNA probe. On the left are dilutions of an 8.5-kb F-MuLV DNA plasmid insert (29) that had been electrophoresed together with 1.0 μg of sheared sonicated salmon testes DNA as a control for subsequent transfer, hybridization, and autoradiographic procedures. The amount of F-MuLV, expressed in picograms, is the best approximation based on an estimation of the concentration of an undiluted F-MuLV solution by a comparison of the ethidium bromide fluorescence produced to that produced by different amounts of salmon testes DNA. The marker fragments, expressed in kb, were lambda DNA digested with *Hind*III. I and III refer to the location of the covalently closed circular and linear forms of virus extrachromosomal DNA, respectively.

resulting fragments were separated by size and transferred to nitrocellulose. We used the 0.7-kb *Bam*HI-*Eco*RI double-digest restriction fragment isolated from a 5.7-kb insert of SFFV DNA that had been molecularly cloned in pBR322 (Fig. 7). This 0.7-kb fragment was gel eluted and

FIG. 6. Quantification of the effect of short-term DMSO treatment on the amount of extrachromosomal SFFV DNA in MELC. Densitometric tracings of the autoradiograph shown in Fig. 5 and of those produced in similar experiments were made with a Zeineh soft-laser scanning densitometer, and the areas under the 6.3-kb peak and above the background were quantified as described in the text. Values are expressed as the percentage of the amount of extrachromosomal 6.3-kb SFFV DNA that was observed in a Hirt supernatant taken from a culture that was not treated with DMSO during the same particular experiment. Whenever possible, the values were based upon a comparison of the ratios of SFFV to 8.5-kb F-MuLV that was added as an internal control, as shown in the left panel of Fig. 4. However, a similar pattern was observed when the values were based solely on the amount of 6.3-kb SFFV DNA.

nick translated for use as a hybridization probe. This probe contains mink cytopathic focus-forming virus determinants and maps within the *env* gene region (3, 4). Since proviral integration is colinear with the unintegrated viral DNA (20), using this combination of enzyme and probe, one can distinguish cell-virus junction fragments that presumably represent the 3' end of the virus.

The results of this analysis are shown in Fig. 7. Two hybridizing bands present in MELC DNA that are not found in DNA extracted from either DBA/2J kidney or brain tissues were observed; these correspond to fragments of 3.3 and 3.0 kb. A similar analysis using a 0.5% agarose gel and a full-length genomic viral probe failed to distinguish any cell-virus junction fragments larger than 3.3 kb. No cell-virus junction fragments were observed in the lane containing DNA from the noninducible DMSO variant that

were not also observed in the lane containing DNA from the highly inducible cell line.

Figure 7 shows that these presumed 3' cell-virus junction fragments were also observed in

FIG. 7. Hybridization of a 0.7-kb *Bam*HI-*Eco*RI SFFV *env* probe to *Hind*III-digested MELC and DBA/2J DNA. A 5- μ g amount of DNA was digested with restriction enzyme *Hind*III, electrophoresed in a 1.2% agarose gel at 50 V for 40 h, denatured, transferred to nitrocellulose, and hybridized to a 0.7-kb *Bam*HI-*Eco*RI restriction fragment that had been gel eluted and nick translated. Lane A, DNA prepared from DBA/2J brain tissue. Lane B, DNA prepared from the Hirt pellet of 10HK7, a high-inducible cell line, cultured in DME 10. Lanes C and D, DNA prepared from the Hirt pellet of the same high-inducible cell line cultured in DME 10 containing 1.5% DMSO for 16 and 120 h, respectively. Lane E, DNA prepared from the DMSO-resistant variant 27E6 cultured continuously in DME 10 containing 1.5% DMSO. Lane F, DNA prepared from 10HK23, a low-inducible cell line, cultured in DME 10. Lane G, DNA prepared from DBA/2J kidney tissue. The numbers on the left denote the position of lambda *Hind*III restriction fragments expressed in kb pairs. The markers on the right denote the position of hybridizing fragments observed in lanes containing MELC DNA that were not observed in lanes containing DBA/2J DNA.

DNA taken from a fully induced culture 5 days after the addition of DMSO to the medium, a time at which greater than 85% of the cells stained positive for hemoglobin with benzidine. In addition, we found that the intensities of the internal 2.3-kb *Hind*III and 1.9-kb *Eco*RI viral fragments were the same when DNA taken from uninduced, induced, and noninducible MELC was used in combination with those enzymes and a full-length genomic viral probe in a similar analysis (data not shown).

These results appear to indicate that the increase in the number of extrachromosomal copies of SFFV DNA observed in a DMSO-resistant variant was not accompanied by an increase in the number of integrated copies of the provirus and that the integration of SFFV proviral DNA in MELC was stable upon the induction of differentiation.

DISCUSSION

In this report, we have demonstrated that derivatives of the 745A MELC line possess extrachromosomal forms of SFFV DNA. The amounts of this DNA were found to vary in cell lines that varied in their ability to be induced to differentiate by DMSO. In experiments designed to determine the effect of DMSO on extrachromosomal SFFV DNA, the results indicated that DMSO can affect the amount of extrachromosomal SFFV DNA present in chronically infected MELC in either of two ways. Over the long term, continuous DMSO treatment of a population originally sensitive to the differentiation-inducing effects of this agent resulted in a population with an increased amount of SFFV DNA. In contrast, the short-term effect of DMSO treatment was an immediate transient reduction in the amount of extrachromosomal SFFV DNA that could first be detected within 6 h after the addition of DMSO to the culture medium.

An extrachromosomal linear form of SFFV DNA exists in a DMSO-resistant variant derived from a tumor induced in DDD mice by the polycythemic strain of Friend virus. This cell line produces large amounts of SFFV (23). A defective virus component has also been isolated from the anemia-inducing strain. Upon rescue by a suitable helper virus, this defective virus induces the erythroleukemia and subsequent anemia characteristic of the disease initially described by Friend and consequently is referred to as the anemic strain of SFFV (27, 41). However, cell lines derived from mice infected with the anemic strain of the Friend virus complex constitutively produce little or no virus with spleen focus-forming activity (10, 15, 17, 38). It is therefore interesting to note that linear and covalently closed circular forms of SFFV extrachromosomal DNA are present in DMSO-

sensitive, as well as DMSO-resistant, cell lines derived from the anemic strain of Friend virus.

The linear extrachromosomal SFFV DNA present in a DMSO-resistant 745A derivative was shown here to be similar in size to and to have restriction enzyme cleavage sites in common with two polycythemic strains of SFFV that have previously been molecularly cloned by other workers (25, 26, 46). However, there exist restriction enzyme cleavage site polymorphisms among these previously mapped polycythemic strains of SFFV and the extrachromosomal SFFV DNA present in FV-A-derived MELC. A restriction map of the anemic strains of SFFV has yet to be published. It is therefore unknown whether the restriction enzyme cleavage site polymorphisms reflect a difference in pathogenic potential or are related to the different passage histories and hosts used to propagate these strains.

Although some pairs of high- and low-inducible subclones showed different amounts of extrachromosomal SFFV DNA and two DMSO-resistant variants had elevated amounts of this DNA, generalizations based on these observations must be made with caution. In addition to the low-inducible cell line that did not have increased levels of extrachromosomal SFFV DNA compared with its high-inducible sibling, we also observed a single subclone of a DMSO-resistant cell line that did not have elevated levels of extrachromosomal SFFV DNA (unpublished data). Also, the removal of DMSO from the growth medium in which a DMSO-resistant cell line was maintained resulted in a decrease in the amount of extrachromosomal SFFV DNA without any large change in the DMSO inducibility phenotype. These results indicate that no simple relationship exists between the number of copies of extrachromosomal SFFV DNA and the ability of cells to respond to DMSO by ceasing proliferation and expressing erythroid phenotypes.

An immediate reduction in the amount of extrachromosomal SFFV DNA was observed after the addition of DMSO to the medium in which DMSO-sensitive cells were growing. This makes it unlikely that the increased amounts of extrachromosomal SFFV DNA observed in two DMSO-resistant variants were due to an immediate effect of DMSO in increasing the synthesis of this DNA or in prolonging the maintenance of this DNA. A cell line resistant to the induction of terminal differentiation by DMSO (4% benzidine positive after 4 days in 1.8% DMSO) showed a similar decrease in the amount of extrachromosomal DNA 20 h after DMSO was added to the growth medium (unpublished data). Therefore, this decrease did not appear to be related to the induction of differentiation by this

agent unless one also postulates that there were differences in the threshold amounts of extrachromosomal SFFV DNA that were required to maintain differentiation arrest in sensitive and resistant lines.

More likely, this immediate decrease in the amount of extrachromosomal SFFV DNA was related to the transient delay in the G1 phase of the cell cycle that is observed in MELC after the addition of DMSO to the growth medium (11, 40). The data of Varmus et al. indicate that cellular factors that may be present only during a particular phase of the cell cycle are required for the synthesis of complete DNA copies of the retrovirus genome during acute infection (43). The reduced amount of extrachromosomal SFFV DNA observed in both DMSO-sensitive and DMSO-resistant cell lines at 18 to 24 h after DMSO treatment and at 48 to 96 h after the addition of DMSO to cells that were sensitive to the differentiation-inducing effects of this agent may therefore reflect the fact that fewer cells are traversing the cell cycle during these intervals due to the transient delay in G1 observed after DMSO addition (11, 40) and to the growth arrest of cells that have terminally differentiated (18).

The source of the extrachromosomal viral DNA and the reason for the elevated levels of this DNA usually observed in DMSO-resistant variants remain to be determined. It is possible that the elevated levels of extrachromosomal SFFV DNA reflect increased viral transcription and secondary reverse transcription similar to the situation observed in dexamethasone-treated rat hepatoma cells infected with murine mammary tumor virus (36). It is also possible that the effects of prolonged DMSO treatment on the cell membrane resulted in an increased rate of superinfection which, in turn, could account for the increase in extrachromosomal SFFV DNA observed in DMSO-resistant variants. If this was the case, however, it did not result in an increase in the number of integrated copies of SFFV since we could not detect any additional cell-virus junction fragments in DMSO-resistant cells that were not also present in cells sensitive to DMSO. Furthermore, we could not detect any increase in the intensities of internal virus restriction fragments with DNA taken from DMSO-resistant cells.

The stable integration of endogenous proviral sequences in differentiated tissues has previously been reported (2), and revertants of avian sarcoma virus-transformed rat cells that retain the integrated proviral information have been described elsewhere (5, 8). Similarly, no change was observed in the number or intensity of cell-virus junction fragments in DNA taken from a terminally differentiated population of MELC when compared with the uninduced population.

Although we did not use stabilized cells (44) in this study, we consider it unlikely that the continued presence of cell-virus junction fragments is due to an outgrowth of cells that fail to respond to the inducer since the visual inspection of the cell pellet used to extract the DNA indicated a majority of differentiated hemoglobin-containing cells. These findings would appear to rule out a simple excision of complete SFFV proviral information during the induction of differentiation. The confirmation of continued viral integration in differentiated MELC must await the development of SFFV-specific hybridization probes that can firmly establish that the extra hybridizing bands observed in MELC DNA, and not observed in the endogenous background, do indeed contain SFFV proviral sequences and are not due to chromosomal rearrangements of related endogenous sequences.

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