

## Induction of Endogenous and of Spleen Focus-Forming Viruses During Dimethylsulfoxide-Induced Differentiation of Mouse Erythroleukemia Cells Transformed by Spleen Focus-Forming Virus

(Friend virus/Friend cells/globin mRNA/thymidine kinase/hemoglobin synthesis)

S. K. DUBE\*§, I. B. PRAGNELL†, N. KLUGE\*, G. GAEDICKE‡, G. STEINHEIDER\*, AND W. OSTERTAG\*¶

\* Max-Planck-Institut für Experimentelle Medizin, Göttingen, West Germany; † Royal Beatson Memorial Hospital, The Beatson Institute for Cancer Research, Glasgow, Scotland; and ‡ Molekularbiologisch-hämatologische Arbeitsgruppe der Universitätsklinik, Hamburg-Eppendorf, West Germany

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**ABSTRACT** Spleen focus-forming virus-transformed erythroleukemic cell clones, which have been established by infection of N type mice with NB tropic Friend virus, continue to release biologically active Friend virus of NB host range. Dimethylsulfoxide induces erythroid differentiation and a 10- to 100-fold increase in the release of biologically active Friend virus. The increase of Friend virus release is a function of the differentiating erythroleukemic cell. The induced Friend virus is not the NB tropic Friend virus complex, but shows N host range. The induction of the Friend virus complex is due to simultaneous induction of both spleen focus-forming and endogenous viruses.

Mouse erythroleukemia cells in culture offer an opportunity to study the role of the erythroid cell-transforming spleen focus-forming virus (SFFV) during dimethylsulfoxide (Me<sub>2</sub>SO)-induced erythroid differentiation of these cells. Differentiation is blocked by viral transformation; the block can be released specifically by aprotic solvents (1-3) or by steroids (4). There are two viral components in the Friend virus (FV) complex, the SFFV and a helper virus (LLV-F) (5, 6). The LLV-F can be replaced as helper virus by other C type viruses (6, 7) or by an endogenous virus which is inducible by BrdU (8).

In this paper we show that Me<sub>2</sub>SO, which induces erythroid differentiation in culture, also induces parallel release of an endogenous C type virus and SFFV. This induction is correlated in time with the induction of globin mRNA synthesis in the same cell (9). The release of the block of differentiation which is normally exerted by the SFFV, and the induction of endogenous N tropic and transforming virus but not of the original NB tropic helper virus during differentiation, can be used to study the interaction and normal function of both the endogenous virus and SFFV during differentiation and transformation.

### MATERIALS AND METHODS

**Tissue Culture.** The origin (DBA/2 mice, NB tropic Balb/c adapted FV complex) and maintenance of the erythroleukemic cell clone FSD1/F4 (=F4), as well as of the BrdU-

resistant, TK<sup>-</sup> subclone F4/B8 (=B8) have been described (2, 10). F4 has been kept permanently in culture for 4 years now. This line has been called F4A. After 4 years of continuous growth, F4A, after being passaged at a high cell density, largely lost the capacity to be induced by Me<sub>2</sub>SO. This subline is F4AO. Because of noninducibility of F4AO, we are now using the original cell clone F4, which has been kept frozen for almost 4 years, F4N (9). Cell Clone M2 is a subclone of Friend cell clone 707 (1, 11). The origin and properties of the FV (SFFV)-transformed cell lines are shown in Table 1. The cell lines are free of mycoplasma contamination.

**Induction of Differentiation.** Me<sub>2</sub>SO was added at 1-1.5% at day 0 to logarithmically growing cells at a density of  $5 \times 10^5$ - $1 \times 10^6$  cells per ml. Part of the medium and cells were discarded at day 2 of exposure to Me<sub>2</sub>SO and new medium containing Me<sub>2</sub>SO was added to maintain a constant cell density of about  $1 \times 10^6$  cells per ml. The degree of differentiation was measured by the synthesis of hemoglobin 4-5 days after the inducer was added (2).

**Virus Assay.** C type virus release was measured by the XC assay (12) with fetal Balb/c (A31) indicator cells (8). For each XC assay, serial 1:5 dilutions were made. One T75 flask was used for each step. Experiments were repeated at least twice. The release of transforming SFFV and of helper virus was determined by spleen focus formation in 2- to 6-month-old DBA/2 or Balb/c mice (13). The filtered tissue culture supernatants were serially diluted  $1/3$  in a 2% serum containing buffer solution (8). Three mice were used for each dilution. Each experiment was repeated at least once.

**Titration of SFFV by LLV-F.** NB tropic, Balb/c adapted LLV-F (NB) was obtained through the courtesy of Dr. Steeves (Albert Einstein College of Medicine, New York). LLV-F was propagated in newborn Balb/c mice. The titer of LLV-F was checked by the XC assay.  $1 \times 10^7$  plaque-forming units of NB tropic LLV-F were added to virus obtained from tissue culture supernatant and injected into the lateral tail vein of DBA/2 or Balb/c mice.

**Large-Scale Isolation of Virus.** F4 cells were grown to a density of  $2 \times 10^6$  cells per ml in roller cultures. Cells were collected by centrifugation and new medium was added. The supernatant was removed serially to collect virus. Virus was precipitated by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and separated from contaminants by banding the virus in a discontinuous sucrose gradient and

Abbreviations: FV, Friend virus complex; SFFV, spleen focus-forming virus; LLV, lymphatic leukemia virus; LLV-F, lymphatic leukemia virus of the Friend virus complex; Me<sub>2</sub>SO, dimethylsulfoxide.

§ Present address: California Institute of Technology, Division of Biology, Pasadena, Calif.

¶ To whom correspondence should be addressed.

TABLE 1. Friend virus-transformed cell lines, hemoglobin synthesis, spleen focus-forming virus release, and thymidine kinase activity

Clone or subclone	Treatment	% Globin synthesis <sup>a</sup>	SFFV/10 <sup>6</sup> cells <sup>b</sup>		Origin of cell line	Thymidine kinase <sup>c</sup>
			in DBA/2 mice	in Balb/c mice		
F4A	Uninduced	<1.1	1.2 × 10 <sup>4</sup>	1.0 × 10 <sup>4</sup>	4 years tissue culture F4	+++
	1.5% Me <sub>2</sub> SO	25	2 × 10 <sup>5</sup> -1.2 × 10 <sup>6</sup>	Not done		
F4N	Uninduced	<0.8	800-1500	1000-1600	1 year tissue culture F4	+++
	1% Me <sub>2</sub> SO	25.3	2 × 10 <sup>4</sup> -1 × 10 <sup>5</sup>	600-1200		
	1.5% Me <sub>2</sub> SO	21.5	5 × 10 <sup>3</sup> -2 × 10 <sup>4</sup>	Not done		
F4AO	Uninduced	<1.0	8 × 10 <sup>3</sup> -1 × 10 <sup>4</sup>	Not done	Derived from F4A, 5 years tissue culture	+++
	1% Me <sub>2</sub> SO	<1.2	8 × 10 <sup>3</sup>	Not done		
	1.5% Me <sub>2</sub> SO	1.8	1 × 10 <sup>4</sup>	Not done		
M2	Uninduced	2.5	0	Not done	Subclone of clone 707 (C. Friend)	+++
	1.5% Me <sub>2</sub> SO	26.0	0	Not done		
B8	Uninduced	2.2	1-5	0-3	Subclone of F4A	±
	1.5% Me <sub>2</sub> SO	23.5	0-3	Not done		
	0.6 mM BrdU	6	1-8 × 10 <sup>3</sup>	10-100		

The % globin synthesis is deduced by the amount of [<sup>14</sup>C]- or [<sup>3</sup>H]leucine label under the  $\alpha$  and  $\beta$  globin peaks separated by carboxymethylcellulose/urea column chromatography. This value is expressed as % of the total hot trichloroacetic acid-precipitable, 0.5% Nonidet-P40-soluble cytoplasmic leucine label in the cells. All experiments have been repeated at least once, some up to 10 times. The range of values that was obtained in different experiments with virus release is indicated. The % globin synthesis in different experiments has been averaged.

<sup>a</sup> Globin synthesis is determined at day 4-5 of Me<sub>2</sub>SO or BrdU treatment with treated cells.

<sup>b</sup> Spleen focus-forming units of treated cell during optimal induction by Me<sub>2</sub>SO or BrdU.

<sup>c</sup> Thymidine kinase activity was determined as described, and the thymidine kinase activity of different cell lines is published (8, 10).

by two sucrose density gradient centrifugations (14, 15). About 25  $\mu$ g of 60-70S RNA was obtained from the supernatant of 2 × 10<sup>10</sup> cells.

**Labeling of Viral RNA.** Cells were labeled with carrier-free <sup>32</sup>P (0.5 mCi/ml) in phosphate-free medium with 1.5% Me<sub>2</sub>SO 20-24 and 24-36 hr after Me<sub>2</sub>SO was added. Virus was separated as described above, and viral RNA was extracted (15). Thirty to 35S RNA was obtained by heating the total viral RNA for 45 sec at 100° (15). This RNA, together with unlabeled viral RNA, was applied to 2.2% acrylamide slab gels (2). After electrophoresis, the slab gels were exposed to x-ray films, and the 30-35S region and the regions corresponding to 8-16 S, where globin mRNA should separate, were eluted. This RNA, as well as the RNA obtained by sodium dodecyl sulfate/sucrose density gradients, was used for globin cDNA hybridization experiments.

**Globin cDNA and Globin cDNA Hybridization.** Globin mRNA isolated from reticulocytes of white Swiss (ICI) mice was used for preparing cDNA. Globin cDNA was prepared and purified (16). We used a titration technique in which a fixed amount of globin cDNA (1 ng) is annealed at 43° with increasing amounts of RNA in hybridization buffer

TABLE 2. Increase of FV release by Me<sub>2</sub>SO-stimulated F4N cells. <sup>32</sup>P label in virions

	<sup>32</sup> P cpm released per hr	Relative values
Control (4 hr)	70,000	1
1.5% Me <sub>2</sub> SO 20-24 hr	75,000	1.07
1.5% Me <sub>2</sub> SO 24-36 hr	600,000	8.6

F4N cells were labeled as described in *Materials and Methods*.

(0.5 M NaCl, 25 mM Hepes, 1 mM EDTA, 50% formamide, pH 6.7) (17). The concentration of globin mRNA in the RNA is deduced by comparing the initial slope of its titration plot with that of globin mRNA.

After hybridization for 72 hr, hybrid was detected by resistance to single-stranded nuclease (S1) (18). The number of globin mRNA sequences per viral genome is calculated from the percent of viral RNA comprising globin mRNA, allowing for the 50-fold increase in molecular weights of viral RNA relative to globin mRNA.

## RESULTS

**Induction of Globin Synthesis.** When cells of F4A, F4N, or M2 are induced by 1-1.5% Me<sub>2</sub>SO, 20-25% of the total hot trichloroacetic acid-precipitable, 0.5% Nonidet-P40-soluble material labeled with leucine is globin at day 4-5, while subline F4AO does not show induction of globin synthesis (Table 1).

**Induction of Virus Release in Erythroid Differentiating Cells.** One or 1.5% Me<sub>2</sub>SO, if added to F4N cells, induces differentiation and a 10- to 100-fold increase in spleen focus formation of the FV complex (Fig. 1a and Table 1). The same increase is obtained if F4A cells are used (19, 20). F4A has a 10-fold higher spontaneous virus titer than F4N. The relative virus and globin induction rate, however, is the same (refs. 19 and 20 and this paper). This increase parallels in time the appearance of newly labeled globin mRNA (9, 19). The increase in virus production during the first 2 days of Me<sub>2</sub>SO induction is confirmed by an 8.6-fold increase in the <sup>32</sup>P label of virions (Table 2) and by a 6.5-fold increase in viral RNA by absorbance measurements (Table 3). The discrepancy between the increase in biological titer and the physical measurements can be explained by our observations that the yield of intact 30-35S RNA relative to 70S RNA is increased after

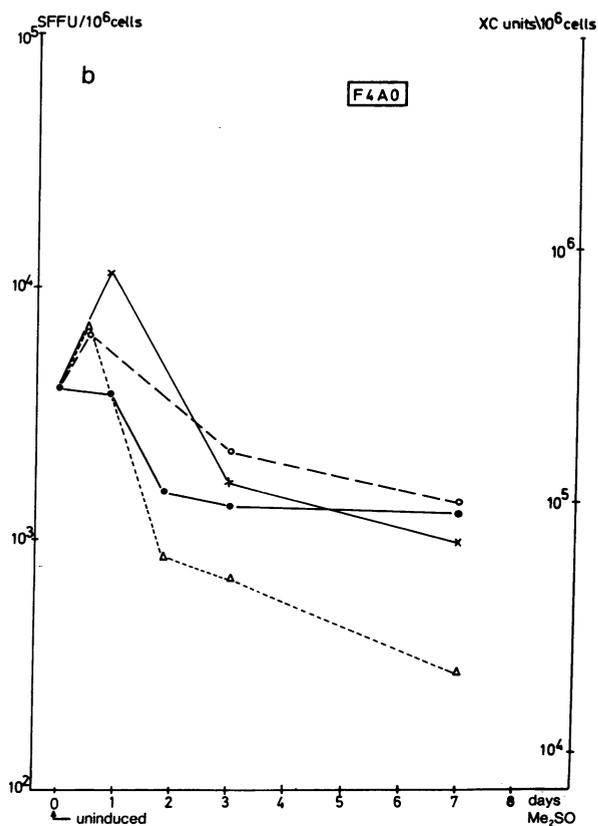
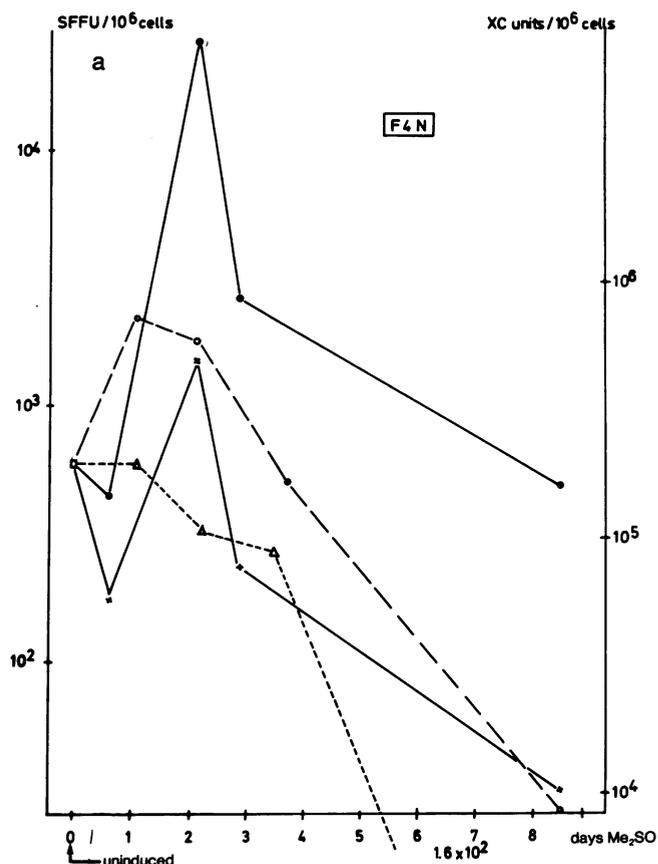


FIG. 1. Induction of virus release in erythroid differentiating cells as determined by spleen focus-forming units in N type DBA/2 mice. The increase in virus release is the same if medium is exchanged daily (20). (a) F4/N cells differentiate on exposure to

TABLE 3. Increase of FV release by Me<sub>2</sub>SO-stimulated F4N cells

	A <sub>260</sub> viral RNA released per hr	Relative values
Control (7 hr)	2	1
1% Me <sub>2</sub> SO (0-16 hr)	3.5	1.7
1% Me <sub>2</sub> SO (16-25 hr)	9	4.5
1% Me <sub>2</sub> SO (25-36 hr)	13	6.5

F4N cells (10L) were grown to a density of 2 × 10<sup>6</sup> cells per ml in Roller cultures. Cells were centrifuged. New medium (5L) was added and incubation continued for 7 hr. After 7 hr, cells were pelleted. The supernatant was removed to collect virus. Incubation of the cells was continued with fresh medium containing 1% Me<sub>2</sub>SO for successive time periods with medium exchange as indicated in column 1. Virus was separated by the usual methods and viral RNA prepared. The relative values are corrected for cell numbers.

Me<sub>2</sub>SO stimulation. Degradation of the 70S RNA is decreased after stimulation and biological activity increased as a result (19). The induction and release of the FV complex in differentiating cells is temporary and precedes the time of maximum globin synthesis. After 2-3 days of exposure to Me<sub>2</sub>SO a steep drop in virus release is detected, and after 5-6 days cells release less virus than untreated cells.

*Induction of Virus Release Is Dependent on Erythroid Differentiation.* In order to show that Me<sub>2</sub>SO alone is not responsible for the increase in virus titers, we used the subline F4AO of cell clone F4. F4AO does not respond and differentiate as the parent cell line does in the presence of Me<sub>2</sub>SO. These cells were exposed to 1 or 1.5% Me<sub>2</sub>SO. The data are shown in Fig. 1b and Table 1. No increase in the number of spleen focus-forming units is observed.

*The Virus Induced by Me<sub>2</sub>SO in Differentiating Cells Is Not NB Tropic FV but an N-Tropic Endogenous FV Complex.* To determine the host range of the Me<sub>2</sub>SO-induced virus complex, we injected the supernatant from noninduced (0 point of Fig. 2) and of Me<sub>2</sub>SO-induced cells into the tail vein of N type DBA/2 and B type Balb/c mice (Fig. 2). The data show that the FV complex released by uninduced F4 cells is indeed NB tropic since no marked difference in spleen focus formation in Balb/c or DBA/2 mice could be detected (Fig. 2 and Table 1). FV complex released by induced cells is, however, almost exclusively N tropic (Fig. 2) since a 20-fold increase in spleen focus formation was obtained in N type DBA/2 mice, but no detectable increase over the titer observed in uninduced cells was found in B type Balb/c mice. This conclusion was confirmed in a second series of experiments when only a marginal increase in plaque formation in the XC B type cell assay was found, whereas the same sample elicited a 60- to 80-fold increase in spleen foci in N type mice (Fig. 1a). The slight 2-fold increase in the XC assay at day 2 of Me<sub>2</sub>SO exposure is

Me<sub>2</sub>SO (Table 1). They show a 60- to 80-fold increase in virus release with spleen focus formation. ●, 1% Me<sub>2</sub>SO, SFFU; ○, 1.5% Me<sub>2</sub>SO, SFFU; ×, 1% Me<sub>2</sub>SO, XC; △, 1.5% Me<sub>2</sub>SO, XC. (b) F4AO cells do not differentiate on exposure to Me<sub>2</sub>SO (Table 1). They do not show an increase in virus release with spleen focus formation. ○, 1% Me<sub>2</sub>SO, SFFU; ●, 1.5% Me<sub>2</sub>SO, SFFU; ×, 1% Me<sub>2</sub>SO, XC; △, 1.5% Me<sub>2</sub>SO, XC.

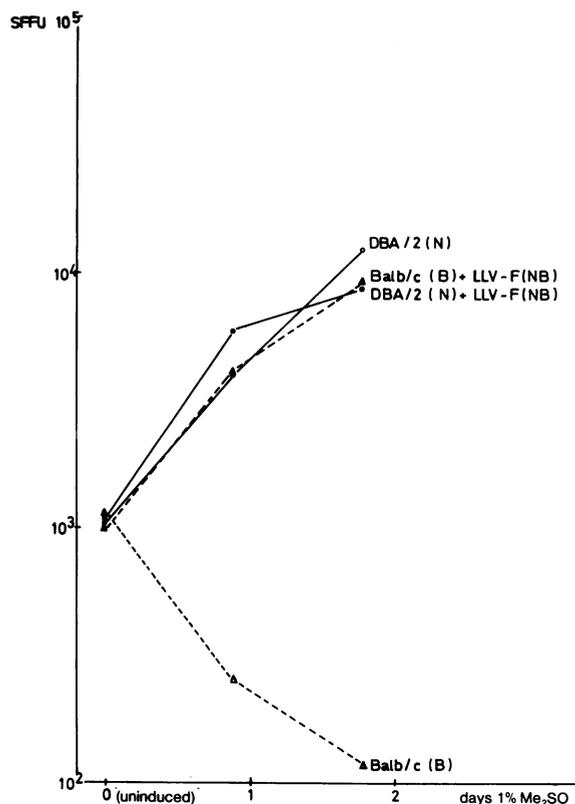


FIG. 2. The host range of the virus of noninduced F4N cells is NB as shown by equal numbers of spleen foci induced in N type DBA/2 and B type Balb/c mice (0 point of curve). Other values are with 1% Me<sub>2</sub>SO. This shows the N-tropism of the induced endogenous helper virus. Titration of SFFV with NB tropic LLV: ○, spleen foci in N type DBA/2 mice; ●, spleen foci in N type DBA/2 mice, 10<sup>7</sup> XC units NB tropic LLV-F added; △, spleen foci in B type Balb/c mice; ▲, spleen foci in B type Balb/c mice, 10<sup>7</sup> XC units NB tropic LLV are added.

probably due either to the protective properties of Me<sub>2</sub>SO of virions in Me<sub>2</sub>SO-containing medium or to the incomplete resistance of B type mice to N tropic virus. We conclude that the induced virus is not the NB-tropic helper virus of uninduced cells, but an N-tropic endogenous virus. The yield of Me<sub>2</sub>SO-induced endogenous virus in F4 cells is very high, much higher than that obtained by BrdU treatment of normal mouse fibroblasts (21) or TK<sup>-</sup> erythroleukemia cells (8).

*SFFV Is Increased in Differentiating F4 Cells.* To determine whether the induced virus is exclusively N tropic endogenous virus or also SFFV, we need to know whether the helper virus or the SFFV is limiting in the spleen focus formation. We therefore added to the cell supernatant excess NB tropic LLV-F. The data are shown in Fig. 2. LLV-F does not increase the number of spleen foci in N type DBA/2 mice. Added NB tropic LLV-F does not increase the number of spleen focus-forming units released by either uninduced (0 point of curve of Fig. 2) or induced F4 cells (Fig. 2). The induction of virus in Me<sub>2</sub>SO-exposed cells is therefore not only an induction of N tropic endogenous virus, but also of SFFV. The titer of SFFV is raised in B type Balb/c mice if NB tropic LLV-F is added to the viral supernatant. The increase of spleen focus-forming units in B and N type mice is then identical.

TABLE 4. Globin mRNA sequences in Friend virus RNA of virus released during Me<sub>2</sub>SO induction

Viral RNA	% Globin mRNA
Total	0.004
60-70S	0.009
9-14S	0.0023
30-35S	<0.0001

RNA was prepared and hybridizations were performed as described in *Materials and Methods*.

*Globin mRNA Sequences in the FV Complex.* Conflicting results concerning the presence of globin mRNA in FV released by SFFV-transformed cells have been reported for two different cell lines (11, 22). We have re-examined this question both with the Friend M2 cell line (11) and with F4. We have used virions released during a 7-hr period of 1% Me<sub>2</sub>SO stimulation in order to avoid the use of old virions that contain 70S RNA with internal single-strand breaks (23). No globin mRNA sequences were obtained in M2 virus, whereas some globin mRNA sequences were detected in viral RNA from Me<sub>2</sub>SO-stimulated F4 cells (Table 4). However, these sequences are largely (72%) in the 9-14S region, where globin mRNA should appear, with only a small amount (28%) in the 60-70S RNA (Table 4). The data for 60-70S RNA correspond to approximately one globin mRNA sequence per 10,000 viral genomes. No sequences complementary to globin cDNA were found in 30-35S viral RNA after melting and fractionation of the 70S RNA on acrylamide gels (Table 4).

<sup>125</sup>I-Labeled globin mRNA was hybridized to viral RNA to detect sequences complementary to globin mRNA. At ratios of viral RNA to globin mRNA of up to 5000:1, only 2.6% hybrid was observed (the background level for this assay), although in a control experiment the <sup>125</sup>I-labeled mRNA was rendered 60% resistant to RNase treatment in the presence of excess globin cDNA. We conclude that no "anti-strands" to globin mRNA exist in the viral genome. We, therefore, believe that the association between mRNA and viral RNA observed by other investigators (22) and observed by us to a much lesser extent, is noncovalent and artifactual.

A considerable proportion (10-20%) of the RNA isolated from preparations of Friend virus is ribosomal RNA (ref. 19 and Pragnell and Crossley, unpublished). Ribosomal RNA in C type virus has been ascribed to the presence of host ribosomes fortuitously trapped in budding virions (24). These ribosomes in Me<sub>2</sub>SO-induced cells may contain globin mRNA which might artifactually bind to the 60-70S RNA, especially if the virus particles contain nicked exposed 60-70S RNA, as would be the case in 4- to 5-day-old virions.

## DISCUSSION

The fact that there is no virus induction in a clone of SFFV-transformed cells that do not undergo erythroid differentiation when exposed to Me<sub>2</sub>SO indicates that the increased release of virus observed in the F4 parent cell lines is dependent upon erythroid differentiation taking place. However, induced release of a C type virus is not a prerequisite for differentiation of SFFV-transformed cells. We have described several BrdU-resistant, TK<sup>-</sup>, SFFV-transformed cell lines that do not re-

lease C type particles spontaneously or on exposure to Me<sub>2</sub>SO, but nevertheless synthesize hemoglobin (8) (Table 1). Similarly, F4 cells treated with interferon or with a thymidine analogue, azidothymidine, together with Me<sub>2</sub>SO do differentiate but release 50- to 100-fold less virus than cells that were treated with Me<sub>2</sub>SO alone (9, 19, 20). However, a 10- to 20-fold increase in intracisternal virus-like A particles is found during differentiation of "virus-negative" B8 cells and during inhibition of virus release during differentiation of virus-positive F4 cells (19).

We have shown that the FV complex that is induced by Me<sub>2</sub>SO is N-tropic, whereas the virus released by uninduced cells is NB tropic (Fig. 2 and Table 1). Therefore, the virus induced as a result of Me<sub>2</sub>SO-stimulated differentiation of F4 cells is not the NB tropic helper virus of uninduced cells but an endogenous N-tropic helper virus. In some experiments, a 2- to 5-fold increase in focus-forming units even in B type mice can initially be observed. This is probably a reflection of the excess of NB helper virus. At higher Me<sub>2</sub>SO concentrations or with lower cell densities, we usually obtain a steep decline of the NB tropic helper as compared to that released by uninduced cells.

We here report induction of an endogenous virus during differentiation, in contrast to induction by IdU or BrdU (21, 8), radiation (25), or activation by relaxation of immune mechanisms (26). The induction of virus in the FV complex is not restricted to the endogenous virus. An increased number of transforming SFFV are released as well, as shown by the same increase in spleen focus formation in N type mice regardless of the number of externally added NB tropic LLV-F. Accordingly, the increase in spleen focus formation was also obtained with B type Balb/c mice if excess NB tropic LLV-F as a helper was added (Fig. 2).

The joint induction of both endogenous and SFFV virus could also mean that the transformed cells release a non-defective endogenous virus with the ability to transform erythroid cells. These data indicate that the endogenous virus and the erythroid focus-forming virus interact during Me<sub>2</sub>SO-induced erythroid differentiation.

Let us assume that the endogenous virus that can be induced by BrdU in TK<sup>-</sup>, B8 cells (8) (Table 1) is the same in differentiating TK<sup>+</sup>, F4 cells. Then the most likely explanation of the lack of inducibility of endogenous virus in TK<sup>-</sup>, "virus-negative," SFFV-transformed cells during Me<sub>2</sub>SO-induced differentiation seems to us to be that the presence of thymidine kinase activity is needed during induced differentiation to rescue endogenous virus. Thymidine kinase activity is present in TK<sup>+</sup>, F4 cells and can be induced by BrdU but not by Me<sub>2</sub>SO in the inducible TK<sup>-</sup>, B8 cells (8) (Table 1).

**Note Added in Proof:** We have now isolated a Balb/c SFFV-transformed virus negative cell line, FLD-3, which, on exposure to Me<sub>2</sub>SO releases a large titer of endogenous SFFV.

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