Flow Cytometric Analysis of Megakaryocyte Ploidy. Comparison With Feulgen Microdensitometry and Discovery That 8N is the Predominant Ploidy Class in Guinea Pig and Monkey Marrow

By R. F. Levine, P. A. Bunn, Jr., K. C. Hazzard, and M. L. Schlam

Flow cytometry (FCM) was adapted to measure ploidy values in megakaryocytes isolated from normal guinea pigs and African green monkeys. The FCM approach was validated by microscopic identification of electronically sorted 8N and higher ploidy cells and by comparisons with Feulgen microdensitometry on the same samples. Sorted 8N populations contained only megakaryocytes and were completely free of cell clumps; clumping was also absent in cell suspensions of African green monkeys. The FCM approach was validated by microscopic identification of electronically sorted 8N and examined prior to FCM. Discrete megakaryocyte peaks were found at 8N, 16N, and 32N but not at intermediate values. The ploidy patterns of isolated megakaryocytes were similar by both methods (approximately 15% 8N, 58% 16N, and 27% 32N in guinea pigs). Unexpectedly, FCM analysis of unseparated marrow cell suspensions found that the most frequent ploidy class of megakaryocytes was 8N (50%). In contrast, by microdensitometry, the 8N class accounted for only 21% of the megakaryocytes in unseparated marrow cell suspensions. Since the traditional method depends on measurement only of easily recognizable megakaryocyte nuclei, a microdensitometric analysis of unselected marrow cells was carried out. Many more smaller 8N megakaryocytes (48%) were found by this approach, confirming the FCM data and the bias of the traditional sampling method. The decrease in 8N megakaryocytes (50%–15%) with enrichment was accounted for by the consistent finding that cells lost during isolation were predominantly 8N, with relative sparing of the 32N class. FCM provides more rapid and objective analysis of ploidy patterns in much larger samples of megakaryocytes than previous methods.

Megakaryocytes undergo synchronous nuclear doublings without cell division (endomitoses), resulting in increased DNA content or polyploidy. Microdensitometric quantitation of DNA content in Feulgen-stained megakaryocytes in marrow smears or sections has shown ploidy levels of 4N, 8N, 16N, 32N, 64N, and (rarely) 128N, where 2N is the normal diploid value. The relative distribution of megakaryocytes among these ploidy classes in normal animals has been similar in previous reports: 4N, 1%–2%; 8N, 10%–25%; 16N, 46%–76%; 32N, 15%–32%; and 64N, 1%–2%. No substantial species differences have been reported.

The distribution of megakaryocyte ploidy values is believed to be shifted by platelet demand. Thrombocytopenia is associated with increased megakaryocyte ploidy levels, which are thought to lead to larger cell size and therefore to more platelets per megakaryocyte. Thrombocytosis, with a decreased demand for platelets, is associated with smaller size and decreased ploidy values. Thus, changes in platelet production appear to reflect changes in DNA replication in megakaryocytes. Data on factors regulating this ploidy modulation are meager because of the difficulty in measuring DNA content of megakaryocytes. Because megakaryocytes comprise a fraction of 1% of all marrow cells, visual identification of Feulgen-stained megakaryocyte nuclei is often difficult and some of the younger megakaryocytes may not be detected. Because the procedure is tedious, few densitometric studies have analyzed more than 150 megakaryocytes per sample.

Flow cytometry (FCM) is capable of rapid, objective analysis of DNA content in large numbers of marrow cells. We have adapted this new technology to measure megakaryocyte ploidy values and have compared the results with standard Feulgen microdensitometric analysis.

MATERIALS AND METHODS

Marrow cell suspensions were prepared from the humeri and femora of two guinea pigs or one African green monkey (Cercopithecus aethiops)* per experiment, as previously described. Marrow was scooped out of the bones and into calcium- and magnesium-free Hanks' solution containing citrate, theophylline, and adenosine; the gelatinous material was finely minced and then disaggregated by pipetting. Prior to further studies the marrow cell suspensions were passed through a stainless steel sieve with openings of 100 μm. Megakaryocytes were successively purified from the marrow suspensions by a density gradient centrifugation and one or two velocity sedimentations. At each step megakaryocytes and total cells were counted in hemocytometer chambers by phase contrast at ×250. In cell suspensions, megakaryocytes were identified by their size, nuclear configuration, and refractility. The calcium-free medium, sieving, and repeated pipetting at each step virtually eliminated cell clumping.

*Animals were maintained in animal research facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.
Feulgen staining was carried out on cell suspensions centrifuged onto cover slips and air-dried. The cells were subjected to hydrolysis at room temperature with 5N HCl for 40 min. Quantitation of the Feulgen stain reaction in single cells was carried out with a Vickers M-85 Scanning Microdensitometer (Vickers Instruments, Inc., Woburn, Mass.). Integrated nuclear density measurements were obtained at a wavelength of 550 nm, with the background setting adjusted to zero. In routine ploidy analysis, the slide was scanned for megakaryocyte nuclei, identified by their size, configuration, and staining density at the rate of one ×400 field (20–40 cells) per second. From 120 to 250 megakaryocytes and about 20 polymorphonuclear or band form neutrophils were examined in each sample. The latter cells were taken as the 2N standards. When the data were plotted as histograms, each megakaryocyte was assigned to the ploidy class of the nearest modal value. Clumps of two or more cells were quite rare and were not analyzed.

In one experiment a different sampling approach was taken; an effort was made to measure a more complete sample of all cells present, to avoid the bias of measuring only obvious megakaryocytes. Cover slips were prepared from a suspension of unseparated marrow cells from a single guinea pig, such that there were only 10–15 cells per field (×400). Moving the stage laterally, neighboring cells were successively measured, with attention to measure all cells not obviously small lymphocytes, maturing erythroblasts, or polymorphonuclear or band-form neutrophils. This approach will be referred to as a "comprehensive sample," in contrast to the traditional "sampling by scanning."

FCM analysis of DNA content was performed on each specimen analyzed by microdensitometry as well as on additional samples. The cell suspensions were stained directly with propidium iodide by the method of Krishan.14 Specimens for the sorting studies were fixed in ethanol, treated with RNase, and stained with propidium iodide according to the method of Crissman and Steinkamp.15 Cytoplasmic fluorescence and cell clumping in stained cell suspensions were absent routinely by fluorescence and phase-contrast microscopy. From 2 × 10⁵ to 10⁶ cells in each sample were analyzed in a Coulter TPS-I Cell Sorter (Coulter Electronics, Hialeah, Fla.).

The principles of FCM and the validity of the methodology for determination of relative DNA content in cells have been previously described.16 In this instrument, an argon ion laser with an output of 30 mW at 488 nm is used to excite fluorescence. The fluorescent response is collected and translated into electrical signals through a combination of filters and photomultipliers. The electrical signals are stored in a pulse-height distribution analyzer divided into 128 channels. The sample flow rate was 0.02 ml/min, and the exit orifice was 70 μm in diameter. Guinea pig and monkey blood leukocytes were used initially as 2N standards; the instrument controls were routinely set so that 2N cells were recorded in channel 6 and 32N cells were seen around channel 96. If the instrument settings were adjusted differently, a small number of 64N and 128N cells could be seen, but this adjustment was not used routinely. The number of cells in each of 128 relative fluorescence channels (linear scale) was entered into the NIH DEC 10 computer. The frequency distribution of fluorescence intensities was displayed in a histogram; a computer-derived Gaussian fit (M-Lab program)17 was applied to each data curve to determine accurately the number of cells in each peak or ploidy class.

Five electronic sorting experiments were performed. DNA histograms of these ethanol-fixed cells were not different from histograms of hypotonically lysed cells. There were no 6N or 10N peaks or other evidence of significant clumping. In these experiments, 8N cells and 16N plus 32N cells (all cells with ploidy ≥ 14N) were sorted simultaneously into plastic beakers. The cells were centrifuged onto separate glass slides and stained with Giemsa.

RESULTS

Isolated Megakaryocytes

Direct comparisons between Feulgen microdensitometry and FCM were made in enriched populations in which 28%–48% of the cells were megakaryocytes. Figures 1 and 2 are histograms of data obtained by microdensitometry and FCM on portions of the same sample of isolated guinea pig megakaryocytes. In Fig. 1, a total of 216 definite megakaryocytes was analyzed by Feulgen-microdensitometry, along with 27 polymorphonuclear neutrophils (left-most peak) taken as the 2N standards. The megakaryocytes fell into fairly discrete groups, each with a modal ploidy value twofold greater than the preceding peak. In this

![Fig. 1. Histogram of relative DNA content of isolated guinea pig megakaryocytes, determined by Feulgen microdensitometry. The first peak is due to polymorphonuclear neutrophils included as 2N standards. The small arrows beneath the scale indicate ploidy values that are exact multiples of that of the neutrophils. Most of the megakaryocytes fell into groups centered about the values 4N, 8N, 16N, and 32N. The vertical lines above the scale indicate the arbitrary cut-offs between the different ploidy peaks.](image-url)

![Fig. 2. Histogram of relative DNA content in isolated guinea pig megakaryocytes (same specimen as in Fig. 1), determined by FCM. The solid line is overall data obtained from analysis of 206,000 cells; top of scale is 50,000 cells/channel (entire abscissa comprises 128 channels). Five distinct peaks or ploidy classes of cells were identified. The tall peak of diploid or 2N marrow cells was centered at channel 6. The 4N class, mostly nonmegakaryocytic marrow cells in G₀ and M₁, was a prominent shoulder over channel 12. The 8N, 16N, and 32N populations of megakaryocytes were centered over channels 24, 48, and 100. When the same data were magnified (expanded scale, dotted line) the top of the scale was 3000 cells/channel. Discrete 8N, 16N, and 32N ploidy classes were well separated from the 2N and 4N peaks (off-scale). The computer-derived Gaussian fits are shown by the dot-dashed line. Arrows indicate ploidy values which are exact doublings of the modal 2N value.](image-url)
sample, 4N megakaryocytes comprised 2%, 8N were 13%, 16N were 56%, 32N were 28%, and 64N were 1% of the recognized megakaryocytes. A small number of megakaryocytes were probably in S phase, in transition between one ploidy level and the next higher one. In accordance with established practice, however, these cells were assigned to the nearest peak and every megakaryocyte was thus counted in one of the doubling ploidy classes. Approximately 2 hr was required to determine the relative DNA content of 200 megakaryocytes.

Figure 2 shows the results of an FCM analysis of 55,000 megakaryocytes from the same specimen. The three major ploidy classes, 8N, 16N, and 32N, were readily apparent. Quantitation of the number and percent of cells under each peak was obtained by summing all the cells beneath the computer-fitted Gaussian curves. In this sample, 15% of the megakaryocytes were in the 8N peak, 46% were 16N, and 39% were 32N. Lower ploidy megakaryocytes (4N and perhaps even 2N) were not distinguished from the other cell types in the 2N and 4N classes; consequently the FCM data included only the 8N, 16N, and 32N classes. About 5–10 min was required to analyze 50,000 megakaryocytes (1–2 × 10⁷ total cells). The data in Figs. 1 and 2 are included in Table 1 (first specimen).

Data from direct comparisons between Feulgen microdensitometry and FCM on the same specimens of isolated megakaryocytes are given in Table 1. In the guinea pig specimens, the proportion of 8N megakaryocytes was 13% and 12% by microdensitometry and 15% and 18% by FCM; the 32N were 29% and 16% by microdensitometry and 39% and 26% by FCM. In the monkeys also, FCM detected only a few percent more 8N and about 10% more 32N cells than were observed by microdensitometry. Thus, with isolated megakaryocytes, these two methods agreed fairly closely, despite the small number of 8N cells analyzed by microdensitometry.

To confirm the identity of the cells considered to be megakaryocytes on the basis of their ploidy values, electronic sorting of cells in the 8N class and from 16N to 32N was carried out. Examples of sorted cells are shown in Fig. 3. Morphologically, all the sorted 8N cells were megakaryocytes. No clumps of nonmegakaryocytes were seen in 5 separate sorting experiments. The higher ploidy megakaryocytes (16N–32N) were consistently larger than the 8N megakaryocytes.

**Unseparated Marrow**

In contrast to the similarity of results of Feulgen microdensitometry and FCM on isolated megakaryocyte suspensions, the two methods gave rather different distributions of megakaryocyte ploidy classes when unseparated marrow cells were studied (Table 2). Microdensitometry found 21% of the megakaryocytes were 8N, 56% were 16N, and 23% were 32N in the guinea pig marrow samples, but FCM found 66% to be 8N, 30% 16N, and 4% 32N in the same specimens. The discrepancies in the monkey specimens were as great.

These unexpected results led us to examine the adequacy of the routine densitometric sampling technique in detecting megakaryocytes in marrow, where they were 0.06%–0.26% of all cells. We developed a comprehensive sampling approach for analysis of an unbiased sample of the marrow cells. The results are shown in Fig. 4. Almost all of the more than 6000 marrow cells were 2N–4N. One-hundred twenty-four cells had ploidy values of 5.2N or more and were considered to be megakaryocytes; this threshold was 29% greater than the 4N modal value. By assigning all cells to the nearest ploidy peak, 48% of the higher ploidy cells were included in the 8N class of megakaryocytes, 42% were 16N, 8% were 32N, and 2% were 64N. When a portion of the same unseparated marrow specimen was examined by FCM (Fig. 5), the proportion of 8N megakaryocytes was again higher than the 16N or 32N fractions. From the computer-fitted curves, 65% of the megakaryocytes were 8N, 31% were 16N, and 3.4% were 32N. Thus, the distribution of megakaryocytes among these three ploidy classes was similar (8N > 16N > 32N) by comprehensive microdensitometry and FCM. In 12 guinea pig specimens analyzed by FCM, the mean proportion of 8N megakaryocytes in unseparated marrow was 50%, and 52% in the 2 monkeys.

In these experiments FCM analysis of 10³–10⁴ megakaryocytes was performed by electronic sorting of each specimen. Half of each specimen was examined by Feulgen microdensitometry and the remainder was analyzed by FCM. The purity of the 4 specimens ranged from 28%–48% megakaryocytes. Only 8N, 16N, and 32N megakaryocytes were included in these data, as explained in Materials and Methods. Data on the megakaryocytes in the unseparated marrow cell suspensions from which the above specimens were isolated are given in Table 2.

### Table 1. Comparison of Methods: Ploidy of Isolated Megakaryocytes

<table>
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<th>Sample</th>
<th>Microdensitometry</th>
<th>Flow Cytometry</th>
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<tbody>
<tr>
<td></td>
<td>8N</td>
<td>16N</td>
</tr>
<tr>
<td>Guinea pig</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>13</td>
<td>57</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
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<tr>
<td>Monkey</td>
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<td></td>
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Fig. 3. Photomicrographs of results of electronic sorting of 8N cells (A) and 16N plus 32N cells (B), both 1000x. Sorted cells were deflected to the left and right, respectively, of the main stream of discarded cells (2N and 4N), put on glass slides, air-dried, and stained with Giemsa. In the 8N group, no nonmegakaryocytes, clumped or free, were seen, only small megakaryocytes (approximately 10-20 μm in diameter). A rare single myeloid cell was found with the 16N plus 32N megakaryocytes; these 2N cells may have been splashed from the central discard stream of lower ploidy cells.
unseparated megakaryocytes took 1–2 hr. The comprehensive sample data required 50 person-hours.

The Effect of Isolation on the Megakaryocyte Ploidy Pattern

Shifts in the pattern of megakaryocyte ploidy values were observed during isolation of megakaryocytes (compare Tables 1 and 2). To determine the consistency of these results and to explain them, six additional FCM experiments were performed in which the ploidy pattern of the megakaryocytes was examined after each enrichment step, along with the ploidy distribution of those megakaryocytes discarded after each step. The results are shown in Fig. 6. The mean percentage of 8N megakaryocytes in unseparated guinea pig marrow was 53%, after the first-purification step it was 31%, after the second step it was 28%, and after the third it was 23%. These results were quite consistent, as is evident by the small standard errors. While the 8N proportion was thus halved, the 32N fraction tripled. This ploidy shift accompanied a 400-fold increase in megakaryocyte frequency, from 0.18% to 79% purity. After the first and second isolation steps, 70% and 32%, respectively, of the 8N megakaryocytes were discarded with the bulk of the nonmegakaryocytes, with much lower numbers of 16N and 32N cells lost at each step. Since a majority of the starting number of megakaryocytes were lost during isolation, the predominant loss of 8N cells with relative sparing of 32N cells accounted for the shift in the population ploidy pattern from lower to higher ploidy values.

Table 2. Comparison of Methods: Ploidy of Megakaryocytes in Unseparated Marrow Cell Suspensions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Microdensitometry</th>
<th>Flow Cytometry</th>
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<tbody>
<tr>
<td></td>
<td>8N</td>
<td>16N</td>
</tr>
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<td>Guinea pig</td>
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<td>Monkey</td>
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<tr>
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<td>34</td>
</tr>
<tr>
<td>Sample sizes</td>
<td>79–131</td>
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</table>

The concentrations of megakaryocytes ranged from 0.06% to 0.26%.

Data on megakaryocytes isolated from these unseparated marrow cell suspensions are given in Table 1.

DISCUSSION

We have applied a new technique, FCM, to the rapid analysis of megakaryocyte ploidy levels. Our results showed (1) that FCM allowed rapid and reproducible ploidy analysis of all cells in marrow samples, (2) that 8N was the predominant ploidy class of megakaryocytes in unseparated marrow, in contrast to earlier reports, and (3) that megakaryocyte isolation
shifted the ploidy pattern toward higher values. The FCM technique with a combination of laser-induced fluorescence and a flow sampling system is ideal for rapid measurement of relative DNA content of every cell in the marrow population. Identification of megakaryocytes was based on a specific objective criterion, DNA content, rather than on visual recognition. The validity of this approach was established by direct comparison of FCM with the standard Feulgen method on populations of isolated megakaryocytes, by comparison with a comprehensive sampling approach to microdensitometry on unseparated marrow, by extensive morphologic-densitometric correlations, and by examination of sorted populations.

The traditional method of single cell microdensitometry has had numerous disadvantages. Although various types of equipment have been used, all these instruments are expensive and have not been widely available. The Vickers M85 Scanning Microdensitometer used in our study is one of the newer and more convenient designs. The Feulgen staining reaction comprises several steps and takes 3 hr to perform. A sample size of 100–200 megakaryocytes could be measured in 1–3 hr. The number of cells was limited primarily by the tedious microdensitometry. The most serious drawback of routine microdensitometry, however, was the sampling bias. When we measured only the obvious megakaryocytes, as in traditional practice, we found in unseparated marrow a similar proportion of 8N cells to that previously reported. When we methodically attempted to perform microdensitometric quantitation on virtually every marrow cell, many megakaryocytes of subtle or uncertain morphology were detected by their polyploidy. The frequent exclusion of these cells from analysis was the source of the failure of routine microdensitometry to detect as many 8N megakaryocytes as FCM. With FCM, no cell is excluded; every cell is measured.

Flow cytometers, although also expensive, are found in many research centers. The staining procedure for FCM was short (5 min) and simple. Very large samples of megakaryocytes were analyzed (10^7–10^8 megakaryocytes in approximately 10^5–10^6 total cells) in relatively short times (5–60 min). Although the entire cell populations were examined, the 4N cells recognizable as megakaryocytes with the Feulgen stain were not distinguished by FCM. However, 4N megakaryocytes have never accounted for more than 1%–2% of the identifiable megakaryocyte population and are probably even more easily missed than the 8N megakaryocytes by routine microdensitometry.

An important issue in FCM methodology has been the certainty that each measurement was from a single cell, not a clump of 2N (and/or 4N) cells. Clumps of nonmegakaryocytes would have been counted as 6N, 8N, or 10N, etc. No definite 6N or 10N classes were observed in the FCM histograms; quantitative analysis of the occasional slight peaks at these values found that clumped nonmegakaryocytes could never have been more than 5% of the 8N class of cells. No such clumps were seen by fluorescence or phase microscopy prior to analysis, and none of the sorted cells were found to be clumps.

Clumping appeared to have been a significant artifact in the recent publication by Nakeff et al., where major 6N and 10N peaks were present and where large numbers of nonmegakaryocytes were found in sorted samples. Differences in the frequency of clumping may have been due to differences in sample preparation and/or differences in staining techniques. We
have found calcium-free media, frequent pipetting of cell suspensions, low flow rates, and hypotonic lysis to be important in avoiding clumping.

The possibility of identification of megakaryocytes in single cell suspensions by a threshold ploidy value was apparent from the distribution of 4N cells in Fig. 4 and 5 (unexpanded scales). A threshold of 5.65N was predicted by Paulus et al. With the FCM data, the distinction of 6N–8N megakaryocytes from the statistical tail of the very large 4N peak required the application of Gaussian fits to the 8N peaks, especially with unseparated marrow. The computer-derived curves were quite reproducible and much less arbitrary than assignment of megakaryocytes to ploidy classes from a visual assessment of the histogram. The excellence of fit for the 8N, 16N, and 32N peaks indicated a true normal or Gaussian distribution for the non-S-phase megakaryocytes, in contrast to Paulus’ expectation of a log normal distribution, but similar to the findings of Odell et al. The number of megakaryocytes in S phase, with intermediate ploidy, not included in these peaks was a very small percent of the total (Fig. 4 and 5, expanded scales). Exclusion of these cells from the FCM ploidy class data might account, however, for some of the minor differences from the microdensitometric results.

The assumption that marrow cells significantly >4N must be megakaryocytes was supported by three different observations. Extensive morphological studies of normal marrow populations have found no other known polyploid cells (>4N) other than osteoclasts, which were extremely rare in these preparations. In the study in which 6276 marrow cells were examined morphologically and simultaneously examined for ploidy values, about 85% of the cells 5.2N or greater were clearly megakaryocytes; the remaining higher ploidy cells had no features indicating a distinct lineage but were consistent in size and nuclear configuration with precursors of the youngest definite megakaryocytes. Finally, electronic sorting found no other cell types in the 8N ploidy class.

The major finding of the present study was that 8N was the predominant ploidy class of megakaryocytes in unseparated marrow from two different species. We found a 2.5 times higher proportion of 8N megakaryocytes with both FCM and a new approach to microdensitometry as with the standard Feulgen method. This information has significance for the understanding of megakaryopoiesis. Because an 8N megakaryocyte can either produce platelets in that ploidy state or become 16N or 32N before maturation is completed the 8N class might have been expected to be the most frequent. Previous reports that 16N was the predominant class have been interpreted as a reflection of a faster turnover (maturation time or loss to higher ploidy classes) in 8N megakaryocytes compared to the 16N class. Our data would require a new kinetic model.

This is the first time that megakaryocyte ploidy shifts due to isolation methods have been documented. The loss of 8N megakaryocytes, and relatively greater enrichment of 32N cells, was a consistent finding. Any experiments using isolated megakaryocyte populations must take into account this shift before extrapolating to the in vivo population, for a megakaryocyte property or function under study might vary with the ploidy level, as Pennington has postulated.

Although only two monkey specimens were studied, it appeared that their megakaryocytes had higher overall ploidy values, i.e., more 32N and fewer 8N, than that seen in guinea pigs. These differences were noted in unseparated and in purified populations and by both Feulgen microdensitometry and FCM. Further study will be needed to confirm species differences in ploidy distribution patterns.

With the capacity to rapidly measure ploidy values of large numbers of cells, FCM has made possible routine study of megakaryocyte ploidy patterns in various human disease states in which platelet production is altered and in tissue culture experiments with different agents affecting megakaryopoiesis.

ACKNOWLEDGMENT

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