Concentrations of High-mobility-group Proteins in the Nucleus and Cytoplasm of Several Rat Tissues

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ABSTRACT Nuclear and cytoplasmic fractions were isolated from various tissues of the rat by a nonaqueous technique. The high-mobility-group (HMG) proteins were extracted from these fractions with acid and separated by one- and two-dimensional PAGE. The concentrations of high-mobility-group proteins HMG1, HMG2, and HMG17 in the nucleus and cytoplasm were then estimated from the staining intensities of the electrophoretic bands. The cytoplasmic concentrations of these proteins were very low—usually less than \( \frac{1}{30} \) of those present in the corresponding nuclear fractions. For the tissues studied (liver, kidney, heart, and lung), the concentrations of HMG proteins in the nucleus did not differ significantly from one tissue to another. Averaged over the four tissues investigated, there were 0.28 molecule of HMG1, 0.18 molecule of HMG2, and 0.46 molecule of HMG17 per nucleosome. These values are considerably higher than those that have been reported previously.

The high-mobility-group (HMG) proteins are a family of well-characterized proteins which are present in relatively large amounts in the chromatin of all higher organisms (reviewed in reference 1). The cells of higher eukaryotes contain four major HMG proteins: HMG1, HMG2, HMG14, and HMG17. These four proteins constitute two groups: one containing HMG1 and HMG2, and the other, HMG14 and HMG17. The two proteins belonging to each group are of similar size, show considerable sequence homology, and may bind to similar or identical sites on the chromatin. Two major HMG proteins in trout testis, HMG-T and H6, have been extensively investigated. HMG-T is homologous to HMG1 and HMG2, and H6, to HMG14 and HMG17.

Considerable interest in the HMG proteins has been generated by reports that they are preferentially associated with transcriptionally competent portions of the genome (2-5), and a great deal of effort is currently being expended to define the role which these proteins play in transcription. Not all investigators have found such a relationship. Recently, Seale et al. (6) reported that there is little correlation between HMG protein content and transcriptional activity in HeLa cell chromatin, and Gabrielli et al. (7) have reported that, in mouse P815 cell chromatin, transcriptional activity is correlated with the presence of HMG14, but not of HMG1 and HMG2 or of HMG17.

Hypotheses concerning the function and metabolism of the HMG proteins frequently involve assumptions concerning their intracellular concentrations and subcellular distributions. Definitive information on these points, however, is unavailable. It has been suggested that there are \( \sim 10^6 \) molecules of each of the major HMG proteins in a typical mammalian cell (8, 9), corresponding to about one molecule each of HMG1, HMG2, and HMG17 for every 30 nucleosomes. This value is apparently based on the yields of the HMG proteins which have been obtained from calf thymus using standard isolation techniques. Because yields were not corrected for losses sustained during isolation, the estimate of \( 10^6 \) molecules per cell represents the minimum intracellular concentration of these proteins. A more recent determination of the concentrations of HMG1 and HMG2 in calf thymus yielded values nearly three times as high as the previous ones, but even in this study losses during isolation could only be partially corrected for (10). In the same study the concentrations of HMG1 and HMG2 in HeLa cells were reported to be \( 2.3 \times 10^6 \) and \( 1.2 \times 10^6 \) molecules per nucleus, respectively, values in the same general range as those found for calf thymus.

Gabrielli et al. (7) have reported that in a nontranscribing chromatin fraction from mouse P815 cells, there are 0.09 molecule of HMG1 plus HMG2 and 0.027 molecule of HMG17 per molecule of histone H4. This corresponds to about one molecule of HMG1 plus HMG2 per six nucleosomes and one molecule of HMG17 per 19 nucleosomes. The
concentrations of these proteins were significantly lower in a transcribing chromatin fraction. Finally, Seale et al. (6) have reported that the concentration of HMG17 in HeLa cell chromatin is 2.5-5% of the total core histone mass, corresponding to about one molecule of HMG17 for every 1.5-3 nucleosomes. The concentration of HMG14 was lower by about a factor of 10. No other estimates of the absolute concentrations of HMG proteins in cells appear to have been made, although many investigators have determined the ratios of the various HMG proteins in different cell types under a variety of physiological conditions (11–14).

The HMG proteins were originally prepared from chromatin and, therefore, presumed to be chromosomal proteins. Most investigators now agree that HMG14 and HMG17 are localized in the nucleus and not found in significant concentrations in the cytoplasm. There is still considerable disagreement, however, concerning the subcellular distribution of HMG1 and HMG2. Although they are present in isolated nuclei and chromatin (11, 12, 15–17), they have also been identified in cytoplasmic fractions (10, 11, 18–20), and it has been suggested that a large proportion of the cellular HMG1 and HMG2 may be cytoplasmic (18, 20).

The identification of HMG1 and HMG2 in cytoplasmic fractions prepared in aqueous media does not, however, justify the conclusion that they are present in the cytoplasm in vivo inasmuch as these proteins are only loosely bound to the chromatin and may be lost from the nuclei when cells are disrupted in aqueous media. When 125I-labeled HMG1 or HMG2 is introduced into the cytoplasm of a cultured cell, the radioactivity concentrates in the nucleus; upon disruption of the cell, however, most of the label is released into the cytoplasmic fraction (10, 21). In another interesting study relating to this point, Gordon et al. (22) enucleated chick embryo fibroblasts with cytochalasin B and observed that nearly all of the HMG1 and HMG2 remained with the nuclei; when nuclei were isolated by conventional techniques, in contrast, there was extensive loss of these proteins into the cytoplasm.

Several groups of investigators have used antibody-staining techniques to establish the intracellular location of the HMG proteins. Smith et al. (23), using an indirect immunoperoxidase method with antibodies to HMG1 and HMG2, detected these proteins in nuclei and mitotic chromosomes, but did not find them in the cytoplasm of cultured cells. In a similar study, Bhullar et al. (24), using antibodies against HMG-T, showed that this protein is localized primarily in the nuclei of cultured cells of rainbow trout. Bustin and Neihart (18), on the other hand, using fluorescent antibodies to HMG1, detected considerable amounts of this protein in the cytoplasm of several lines of cultured cells. In some instances the cytoplasmic fluorescence was more intense than that in the nucleus. As in the case of the fractionation studies, a potential problem with the immunochemical techniques is the redistribution of proteins during the experimental procedure (10).

When 125I-labeled HMG1 and HMG2 are microinjected into cultured cells, they concentrate in the nucleus (10, 21). Between 10 and 30% of the injected label, however, is associated with the cytoplasm at equilibrium, suggesting that there is a significant cytoplasmic pool of these proteins.

In the present investigation, we have determined the concentrations of HMG1, HMG2, and HMG17 in nuclear and cytoplasmic fractions isolated from several tissues of the rat. The subcellular fractions were prepared by a nonaqueous technique, which eliminates the redistribution of proteins during the isolation procedure (25). Appropriate controls were run so that corrections could be made for losses incurred during the extraction and fractionation procedures. Two main conclusions can be drawn from our results: first, the cytoplasmic concentrations of HMG proteins are very low, and; second, the amounts of these proteins in the nucleus are considerably larger than previously believed.

MATERIALS AND METHODS

Isolation of HMG Proteins: HMG1 and HMG2 were prepared from calf thymus by the method of Goodwin et al. (15); HMG17 was isolated from thymus as described by Walker et al. (26).

Preparation of Subcellular Fractions: Liver, kidneys, heart, and lungs were obtained from male Holtzman albino rats weighing 250–300 g. After fasting overnight, the animals were decapitated; tissues were immediately excised and transferred to liquid nitrogen. The frozen tissues were lyophilized then separated into cytoplasmic and nuclear fractions by a nonaqueous technique as described previously (27).

Extraction of HMG Proteins from Nuclear and Cytoplasmic Fractions: Each subcellular fraction was extracted at 0°C with 0.2 M H2SO4–0.2 M NaCl (6 ml/500 mg cytoplasman; 2 ml/10 mg nuclei). The residue was then reextracted with half the initial volume of H2SO4–NaCl solution and the two extracts were combined. To the resulting solution was added 100 wt/vol trichloroacetic acid solution to a final concentration of 2%. Insoluble material was removed by centrifugation and the supernatant, which contained the HMG proteins, was dialyzed against water in dialysis tubing of 3,500-mol weight cutoff and lyophilized.

PAGE: Lyophilized preparations of crude HMG proteins were fractionated by one- or two-dimensional polyacrylamide gel electrophoresis on 0.15–cm-thick gel slabs. One-dimensional gels were run using the acid-urea system of Panyim and Chalkley (28). The urea concentration was 6.25 M. Electrophoresis was for 4.5 h at 250 V.

Proteins were reduced before electrophoresis. This was accomplished by dissolving the samples in freshly prepared 6 M urea–0.01 M Tris, pH 7.6, adding 2 μl of 2-mercaptoethanol to a 30-μl portion of the resulting solution, and incubating for 30 min at 24°C. After reduction, 1.6 μl of acetic acid was added and an appropriate aliquot of the acidified solution was applied to the gel. After electrophoresis the gels were stained for 30–45 min in 0.25% Coomassie Brilliant Blue R250–9.2% acetic acid–45% methanol and then destained in 7.5% acetic acid–22.5% methanol.

For two-dimensional electrophoresis, the first dimension was run as described above except that the urea concentration was reduced to 2.5 M. A lane cut from the acid-urea gel was stained for 15 min in the staining solution described above and then transferred to a 30-ml portion of 0.067 M Tris, pH 6.8, 0.1% SDS and allowed to equilibrate for 60–80 min with gentle shaking. During the equilibration, the gel was stained in 10% acetic acid. After equilibration, the gels were cut and placed into 10% acetic acid for 15 min and then destained in 7.5% acetic acid–22.5% methanol.

For the second dimension, the SDS gel system of Laemmli was employed (29). The stacking gel contained 3.75%, and the separating gel 15%, acrylamide. Electrophoresis was at 25 mA for ~12 h. After electrophoresis, the gels were stained by the same method used for the one-dimensional gels and dried on a sheet of filter paper.

Quantitation of Proteins on Stained Gels: The amount of protein in a stained band on a polyacrylamide gel was determined as follows: the band of interest was cut from the gel and shaken overnight with a measured volume of 25% pyridine in water (30). The absorbance of the resulting solution at 595 nm was determined and, from this value thus obtained, the amount of protein in the original band was calculated by reference to a standard curve prepared by electrophoresing known amounts of the appropriate homogenous protein (12).

Analytical Methods: Cross-contamination of the nuclear and cytoplasmic fractions was estimated by using catalase as a marker enzyme for cytoplasm and DNA as a marker for nucleus. Measurement of catalase activity was done essentially as described by Beers and Sizer (31). DNA was assayed as described previously (32).

Labeling and Radioactivity Measurements: HMG1, HMG2, and HMG17 were labeled with 125I using the lactoperoxidase method as previously described (21). Since HMG17 contains no tyrosine, it cannot be...
iodinated directly. To label this protein, a limited number of its lysine residues were reacted with N-succinimidyl-3-(4-hydroxyphenyl)propionateto provide a substrate for the lactoperoxidase (33, 34). Samples labeled with $^{125}$I were counted in a Packard model 5210 Auto-Gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). Autoradiography of polyacrylamide gels was accomplished by drying the gel onto a sheet of filter paper and exposing a sheet of Kodak X-Omat XR-1 x-ray film (Eastman Kodak Co., Rochester, NY) to the dried gel at -70°C.

RESULTS

Cross-contamination of Subcellular Fractions

To estimate the extent of the cross-contamination of nuclear and cytoplasmic fractions prepared in nonaqueous media, catalase was used as a marker enzyme for cytoplasm, and DNA served as a marker substance for nuclei (35). On the assumption that catalase is restricted to the cytoplasm (35), and that 0.5% of the total cellular DNA originates from the mitochondria (36), we were able to calculate the cross-contamination of each nuclear and cytoplasmic fraction from their contents of DNA and catalase. Results obtained in this manner are presented in Table I. Values for the concentrations of HMG proteins in the various fractions have been corrected for cross-contamination.

Recovery of HMG Proteins from Nuclear and Cytoplasmic Preparations

To determine the efficiency of our extraction procedure, $^{125}$I-labeled HMG proteins were added to nonaqueous preparations of nuclei and cytoplasm and the fraction of the added radioactivity recovered upon extraction was determined. The results of these experiments, which are presented in Table II, demonstrate recoveries ranging from 74 to 95%. These results are similar to those which would be anticipated if losses were mainly due to mechanical trapping of the aqueous phases in the pellets: losses were smaller for the nuclear preparation, which yielded smaller pellets. The experiments reported in Table II were done with preparations of nuclei and cytoplasm from liver; we have assumed that similar results would be obtained for other tissues and have corrected all our results using the yields reported in Table II.

Concentrations of HMG Proteins in Nuclei and Cytoplasm as Estimated from One-dimensional Gels

When portions of the crude HMG proteins extracted from nonaqueous cytoplasm and nuclei were applied to acid-urea gels, patterns like those of Fig. 1 were obtained. Bands corresponding to HMG1, HMG2, and HMG17 are clearly identifiable in the lanes to which nuclear proteins have been applied (Fig. 1B), but not in the patterns produced by cytoplasmic proteins (Fig. 1A); apparently the concentrations of HMG proteins in cytoplasm are, in most cases, too low to permit the proteins to be visualized on the gels. In the nuclear patterns, the identity of the protein in each of the putative HMG-containing bands was confirmed by cutting the band from the gel, hydrolyzing the material in the band, and performing an amino acid analysis on the resulting hydrolysate (data not shown).

The amount of protein in each band of interest was determined from its staining intensity as described under Materials and Methods, and the resulting data were used to calculate the concentrations of HMG1, HMG2, and HMG17 in the cytoplasm and nucleus of each of the tissues investigated. These results are presented in the upper portion of Table III. HMG14, which is present in considerably lower concentrations than HMG1, HMG2, and HMG17, was not determined.

For every tissue examined, the cytoplasmic concentrations of both HMG1 and HMG17 are very low relative to the concentrations of the same proteins in the nucleus. Considerable amounts of HMG2, on the other hand, were found in the cytoplasm as well as in the nucleus of all tissues except lung. Because the acid-soluble proteins from cytoplasmic fractions yield complex electrophoretic patterns, it seemed possible that the putative cytoplasmic HMG2 might be an artifact caused by the presence, in the acid extracts, of a contaminating protein with an electrophoretic mobility similar to that of HMG2. Examination of Fig. 1A reveals that there are, in fact, bands with mobilities slightly different from that of HMG2 in every lane to which cytoplasmic proteins have been applied, and that these bands overlap the HMG2 position. To obtain a better estimate of the concentrations of the HMG proteins in cytoplasm, the cytoplasmic extracts were examined by two-dimensional polyacrylamide gel electrophoresis in which the possibility of interference by contaminating proteins is much less than for one-dimensional gels (see below).

As is evident from Table III, there is considerable scatter in

| Tissue | Cytoplasm | Nuclei |
|--------|-----------|--------|
| Liver  | 98.6      | 95.9   |
| Kidney | 94.5      | 92.5   |
| Heart  | 90.1      | 98.6   |
| Lung   | 92.7      | 99.0   |

* The degree of cross-contamination of the nuclear and cytoplasmic fractions was estimated from their contents of catalase, a marker enzyme for cytoplasm, and DNA, a marker for nuclei (see text).

Table I

Purity of Nuclear and Cytoplasmic Preparations*
Figure 1 Resolution of HMG proteins on acid-urea gels. Acid-soluble proteins were prepared from cytoplasmic and nuclear fractions as described in Materials and Methods and run on acid-urea gels. (A) Cytoplasmic proteins; (B) nuclear proteins. Lane 7, liver; lane 3, kidney; lane 5, heart; lane 7, lung. Lanes 2, 4, 6, and 8, standard mixture containing equal concentrations of HMG1, HMG2, HMG17, and BSA.

Table III
Concentration of HMG Proteins in the Nucleus and Cytoplasm of Various Rat Tissues as Determined by One-dimensional Gel Electrophoresis

| Tissue | HMG1 | HMG2 | HMG17 | HMG1 | HMG2 | HMG17 |
|--------|------|------|-------|------|------|-------|
| Liver  | 6.8  | 6.9  | 4.6   | 1.3  | 2.8  | 0.0   |
|        | (2.8-9.1) | (4.7-8.9) | (2.1-7.1) | (0.1-2.1) | (1.6-3.8) | (0.0-0.0) |
| Kidney | 11.5 | 5.8  | 3.7   | 0.9  | 6.2  | 0.3   |
|        | (8.2-17.5) | (4.4-8.2) | (2.2-6.1) | (0.5-1.3) | (1.4-10.0) | (0.0-0.6) |
| Heart  | 11.8 | 5.5  | 5.4   | 0.3  | 3.9  | 0.1   |
|        | (7.8-15.9) | (5.3-5.7) | (2.5-9.6) | (0.0-0.8) | (2.3-7.0) | (0.0-0.3) |
| Lung   | 7.5  | 3.8  | 7.3   | 0.0  | 0.0  | 0.1   |
|        | (6.1-8.9) | (3.1-5.2) | (6.3-8.1) | (0.0-0.1) | (0.0-0.0) | (0.0-0.3) |

*Values in the upper portion of the table represent, in most cases, the mean of three separate determinations; in a few instances, only two determinations were made. The range of values obtained is reported in parentheses under the mean. Data from all tissues were averaged to yield the values in the lower portion of the table. The DNA content of a rat kidney, heart, or lung cell was assumed to be 6.5 pg, and that of a liver cell was taken as 10.1 pg (37). Each nucleosome was assumed to contain 200 base pairs of DNA (38).

Concentration of HMG Proteins in Cytoplasm as Estimated from Two-dimensional Gels

Fig. 2A is a photograph of the pattern obtained upon electrophoresis, on a two-dimensional gel, of a mixture containing HMG1, HMG2, and HMG17; HMG1 and HMG2 have each separated into two subfractions on the gel. Fig. 2B shows the pattern obtained upon electrophoresis of the acid-soluble proteins from rat liver cytoplasm; the positions to which HMG1, HMG2, and HMG17 migrate have been outlined on the photograph. Note that the outlined areas are nearly devoid of staining material. The pattern of Fig. 2B clearly demonstrates the value of two-dimensional electrophoresis for separating the HMG proteins from other acid-soluble cytoplasmic proteins. In contrast, two-dimensional gels offered no advantage over one-dimensional acid-urea gels in resolving acid-soluble nuclear proteins.

On the basis of the above observations, we separated the data; differences between tissues, although in some cases appreciable, are not statistically significant. We have, therefore, combined the data for all tissues to calculate the number of molecules of each HMG protein per nucleus and per nucleosome. These values are given in the bottom portion of Table III. The differences between these values are significant at the 0.001 level by Student's t test.
Figure 2 Separation of acid-soluble cytoplasmic proteins by two-dimensional gel electrophoresis. (A) Reference pattern—standard mixture containing HMG1, HMG2, and HMG17 applied to the gel. (B) Acid-soluble proteins from liver cytoplasm; the positions of HMG1, HMG2, and HMG17 are indicated. The first dimension (acid-urea) was run from left to right, and the second dimension (SDS), from top to bottom.

Acid-soluble proteins from all of the cytoplasmic fractions on two-dimensional gels, excised the areas corresponding to HMG1, HMG2, and HMG17, and, based on the amount of stain in each excised gel segment, calculated, for each tissue, the cytoplasmic concentration of HMG1, HMG2, and HMG17. Two-dimensional gels to which known amounts of HMG1, HMG2, and HMG17 had been applied served as standards. To aid in establishing the positions of the HMG proteins on the gels, a trace amount of 125I-labeled HMG1 was added to each sample to be electrophoresed. Subsequently, an autoradiograph of finished gel was prepared and compared with the staining pattern.

The cytoplasmic concentrations of HMG1, HMG2, and HMG17 as determined by two-dimensional gel electrophoresis are given in Table IV. In every case, the concentration of the HMG protein lay near or below the level which could be measured by the analytical method employed. A comparison of the values of Tables III and IV indicate that the concentration of a particular HMG protein in the cytoplasm is usually less than \( \frac{1}{10} \) and never significantly more than \( \frac{1}{10} \) that in the nucleus.

**DISCUSSION AND CONCLUSIONS**

In the present investigation we have determined the concentrations of the major HMG proteins in nuclear and cytoplasmic fractions from several tissues of the rat by using a procedure that avoids, or makes corrections for, important sources of error commonly associated with this type of analysis. Subcellular fractions were isolated by a nonaqueous technique, which prevents loss or redistribution of proteins during the isolation (25). The purity of the nuclear and cytoplasmic fractions was assessed, and appropriate corrections were made for cross-contamination. Finally, the yields of HMG proteins obtained upon acid extraction of the subcellular fractions were determined, and corrections were made for losses.

For all tissues studied, the cytoplasmic concentrations of HMG1, HMG2, and HMG17 have been found to be very low, often too low to be measured by the analytical method employed. In general, the concentration of a particular HMG protein is more than 30 times as great in the nucleus as in the cytoplasm, and in no case was the nuclear/cytoplasmic concentration ratio much lower than 10:1.

The above results are in substantial disagreement with reports from several laboratories that suggest that HMG1 is present in high concentrations in the cytoplasm of various cell types (18–20). The identification of HMG1 in cytoplasmic fractions prepared in aqueous media has served, in part, as the basis for these reports. This, however, is not justified, since, as discussed at the beginning of this article, HMG1 and HMG2 are only loosely bound to chromatin and may be lost from the nuclei when cells are disrupted in aqueous media (10, 22). Bustin and Neihart (18) have also concluded, on the
basis of immunofluorescence studies, that the cytoplasmic
and nuclear concentrations of HMG1 are comparable in
several lines of cultured cells. It has subsequently been re-
ported that HMG1 may leak from the nucleus when cells are
fixed in methanol, the fixative employed by these investigators
(10). Furthermore, immunofluorescence studies done in other
laboratories suggest that HMG1 and HMG2, and the homol-
ogous protein HMG-T, are not present in significant con-
centrations in the cytoplasm of several lines of cultured cells (23, 24).

Teng and Teng (20) measured, by an immunological tech-
nique, the concentrations of HMG1 in nuclear and cyto-
plasmic fractions isolated from chicken oviduct in aqueous
media and found high concentrations of the protein in the
cytoplasm. They also measured the concentration of HMG1
in oviduct nuclei prepared in nonaqueous media and found
that it was only slightly lower than that in nuclei prepared in
aqueous solutions. From this they concluded that the cyto-
plasmic HMG1 was not the result of leakage from the nucleus
and that there are substantial concentrations of HMG1 in
oviduct cytoplasm. The intracellular distribution of HMG
proteins in oviduct might, of course, be different from that in
the tissues which we studied; further work will be required to
determine whether this, or some other explanation, accounts
for the differences between our two laboratories.

When $^{125}$I-labeled HMG1 or HMG2 is introduced into the
cytoplasm of a cultured cell, the radioactivity rapidly concen-
trates in the nucleus (10, 21). At equilibrium, between 10 and
30% of the label remains in the cytoplasm, a higher value
than might be expected on the basis of the results reported in
the present communication. The microinjection experiments,
however, overestimate the concentration of the HMG protein
in the cytoplasm, inasmuch as molecules damaged during the
isolation and labeling procedures may no longer be capable of
concentrating in the nucleus (L. Kuehl, L. Wu, and M.
Rechsteiner, unpublished observations).

The results of the present study suggest that the concentra-
tions of HMG1, HMG2, and HMG17 in a typical cell nucleus
are much higher than previously believed. A recent paper by
Seale et al. (6) also reports HMG17 concentrations in mouse
P815 cells similar to those found in the present study. Our
findings have important implications relating to the function
of the HMG proteins. These proteins are associated prefer-
entially with transcriptionally competent chromatin (2-5) and
are thought to modify the basic structure of chromatin in
such a way as to allow transcription to occur (9). Earlier
estimates, however, suggested that the quantities of HMG
proteins in the cell were not sufficient to saturate the high-
affinity binding sites on each transcriptionally competent
nucleosome (9). The data presented in the present report
suggest just the opposite, namely, that the intracellular
concentrations of HMG proteins are so high that not all of these
proteins can be associated with transcriptionally active
nucleosomes.

The DNA content of a typical somatic cell of a rat is ~6.5
pg (37). If it is assumed that all of this DNA is packaged in
nucleosomes and that each nucleosome contains 200 base
pairs of DNA (38), then there are ~30 $\times 10^9$ nucleosomes per
cell. Our results indicate that there is about one molecule of
one of the large HMG proteins (HMG1 or HMG2) and about
one molecule of HMG17 for every two nucleosomes (Table
III). Each nucleosome contains a single HMG1/HMG2-bind-
ing site (39) and two binding sites for HMG14/17 (39-41);
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