SMALL NUCLEAR RNA LOCALIZATION DURING MITOSIS

An Electron Microscope Study

LESTER GOLDSTEIN, GARY E. WISE, and CHRISTINE KO

From the Department of Molecular, Cellular, and Developmental Biology, University Colorado, Boulder, Colorado 80309 and the Department of Biological Structure, University of Miami School of Medicine, Miami, Florida 33152

ABSTRACT

The localization of small nuclear ribonucleic acids (snRNAs) during mitosis in *Amoeba proteus* was studied by high voltage (1,000 kV) electron microscope autoradiography. By suitable micromanipulations, the snRNA's, labeled with [^{3}H]uridine, were made to be the only radioactive molecules in the cell and thus easy to follow autoradiographically.

During interphase the snRNA label, which is almost exclusively nuclear, is distributed fairly uniformly through the nucleus with a slightly higher amount of label over chromatin than over nonchromatin areas. During prophase the sn-RNAs, which continue to be largely nuclear, become highly concentrated in the condensing chromosomes. At metaphase, almost all of the snRNAs are cytoplasmic and essentially none are associated with the maximally condensed chromatin. Beginning in early anaphase, the snRNAs resume their association with the chromosomes, with the degree of association increasing throughout anaphase. Most of the snRNAs are back in the nuclei by telophase, but the intranuclear localization is hard to determine. We conclude that snRNAs have a great affinity for the partially condensed chromosomes of prophase and anaphase, but none for the maximally condensed chromosomes of metaphase.

A minor amount of snRNA localizations in association with nucleoli and the nuclear envelope are also reported.

On the basis of these findings a role of snRNAs in genetic "reprogramming" or chromosome organization is proposed.

The function(s) of the small nuclear ribonucleic acids (snRNAs) found in all eukaryotic cells in which they have been looked for in the past decade are essentially unknown. Their almost exclusive localization in cell nuclei suggests that they function in replication and/or transcription, but this remains to be established. The work we report here supports the view that the snRNAs are involved in transcription of chromosome organization.

The recent light microscope investigation (5) that showed the unusual pattern of snRNA association with mitotic chromosomes may be our most important clue to date and prompted this more detailed and higher resolution study. On the basis of the light microscope observations, the snRNAs were proposed to function as relatively generalized regulators of transcription that affect certain chromosomal loci by hybridizing with DNA sequences at those loci. The study reported here

confirms and extends the earlier observations, thereby supporting the earlier speculation, and establishes that snRNAs have a maximal affinity for the partially condensed chromosomes of prophase and anaphase but have no affinity for the maximally condensed chromosomes of metaphase.

As in the previous study (5), the significant technical feature of this work is that the snRNAs of amebas (our experimental organism) can be made essentially the only radioactive molecules in the cell (and hence easy to follow) by sequentially transplanting [³H]uridine-labeled nuclei through a series of unlabeled, enucleate cytoplasms. The snRNAs become the only labeled molecules in the cell because they are extremely stable metabolically (our unpublished finding) and are the only RNA molecules that do not move unidirectionally from nucleus to cytoplasm.

MATERIALS AND METHODS

Organisms and Culture Methods

Amoeba proteus, the organism used in this study, was cultured according to the method of Prescott and Carrier (12), except that the culture medium contained 3.7×10^{-5} M $CaHPO_4$, 8×10^{-5} M KCl, and 1.6×10^{-5} M $MgSO_4$.

Labeling of Cells

Randomly selected amebas from standard, log-phase cultures were labeled by feeding them [³H]uridine-labeled tetrahymenas (Tetrahymena pyriformis) for 40 to 50 h, or about one ameba cell generation. The tetrahymenas were labeled by growth at 29°C for 1 day in synthetic medim (3) devoid of pyrimidines except for 250 μCi/ml of [5-³H]uridine (40–60 Ci/mM, Amersham-Searle Corp., Arlington Heights, Ill.). At the end of their [³H]uridine-labeling period, the amebas were fed unlabeled tetrahymenas for about 2 days before the ameba nuclei were transplanted into unlabeled cytoplasms. Numerous previous studies (e.g., 7) established that well over 95% of the incorporated label under these conditions is in RNA and that essentially none is in DNA.

Construction of Cells in which only the snRNAs are Radioactive

In earlier work all the labeled non-snRNA molecules were "chased" by sequential transplantation of [³H]uridine-labeled nuclei into unlabeled cytoplasms about every other day for 3 such transfers. Since the nucleus occupies only 2% of the cell's volume, each transplantation can be thought of as representing an approximately 50-fold "dilution" of all labeled material that moves unidirectionally to the cytoplasm. We found

that after three such transplantations, essentially all the remaining radioactivity is in snRNAs (7). Because this kind of sequential transfer is tedious and must be done under difficult time constraints, the method of chasing was somewhat modified for this study as follows.

At the end of the approximate 2-day feeding on unlabeled food, the nuclei from the [³H]uridine-labeled amebas were transplanted (8) into unlabeled and enucleated cytoplasms. These recipient cells, and all subsequent ones, continued to be fed unlabeled tetrahymenas for the rest of the experiment until they were fixed. Beginning the day after the first transplantation and every other day for about a week, about half the cytoplasm was cut (6) from each experimental cell. This procedure prevents the cells from entering division (and sets their cell cycle progression essentially back to the beginning of interphase), although they apparently are otherwise normal and continued to grow between amputations. This procedure also continues the chase by removing more and more of the ³H that is in, or enters, the cytoplasm. At the end of 4 or so amputations, the nuclei were again transplanted into fresh, unlabeled cytoplasms, and the following day the every-other-day amputations were resumed for another 4 or 5 days. The distribution of ³H in such a cell is shown in Fig. 2. This almost exclusive localization of label in the nucleus compares with an almost equal concentration of radioactivity in nucleus and cytoplasm in cells just before the first nuclear transplantation (Fig. 1). Following the last amputation, the cells were allowed to grow unimpeded and were fixed when they entered a desired stage of mitosis.

These nuclei, in which the snRNAs were the only radioactive molecules, we call "microsurgically chased" (MC) nuclei.

Identification of Mitotic Stages

The stages of mitosis were identified from the external appearance of living cells essentially according to the criteria described by Chalkley and Daniel (2).

High Voltage Electron Microscope Autoradiography (HVEMAR)

HVEMAR was used primarily to shorten the exposure time required to achieve acceptable autoradiographic grain density, since the thicker the section we could examine, the more radioactive the specimen. Amebas containing the MC nuclei at various stages of mitosis were fixed for 1 h in Karnovsky's mixture of glutaraldehyde and paraformaldehyde in 0.2 M cacodylate buffer (9), after which the cells were rinsed in buffer and embedded in agar to facilitate the transfer of a small number of cells. Amebas were embedded in agar by an adaptation of the method of Flickinger (4). That is, agar was layered on a microscope slide, and, after gelling, a well was made therein. A group of 30–100 amebas were carefully placed in the well, covered with molten agar, and, after hardening, a block of agar containing amebas was trimmed to the smallest possible size. The agar-
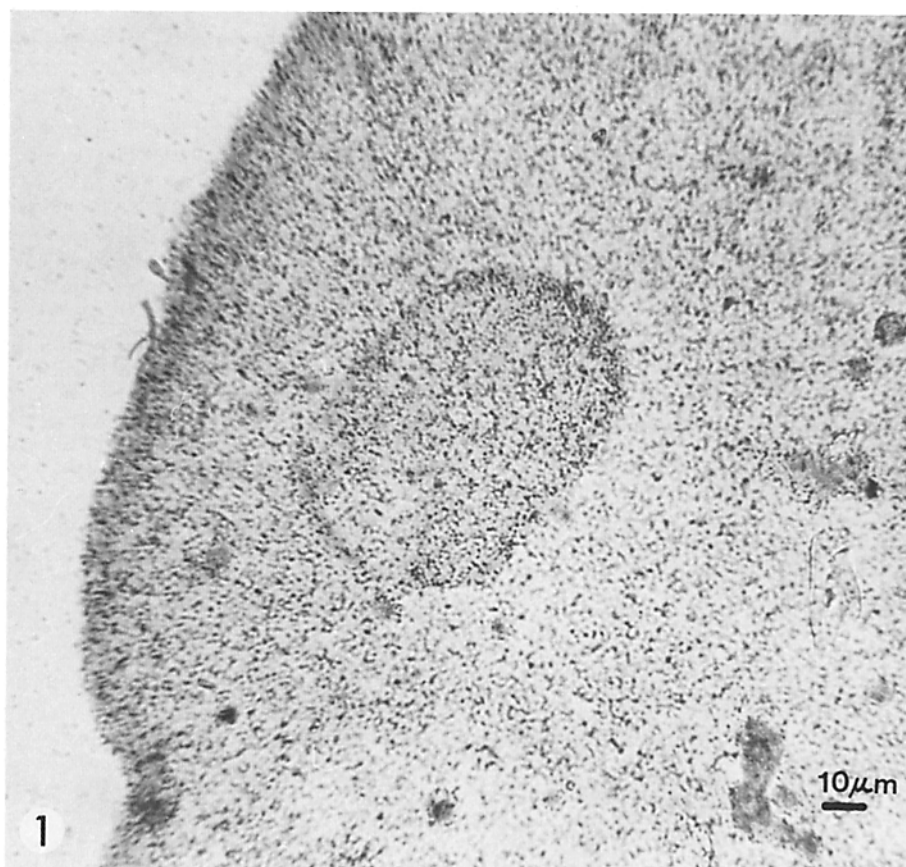
FIGURE 1   Light microscopic autoradiogram of part of a Giemsa's-stained and squashed ameba that was labeled with [³H]uridine for 2 days and then fed unlabeled food for about 2 more days. Note the essentially equal concentration of label over nucleus and cytoplasm. × 5,000.

embedded amebas were postfixed in 1% buffered OsO₄ for 1 h, dehydrated in a graded series of alcohols followed by propylene oxide, and finally embedded in Araldite.

For HVEMAR, about 0.3-μm sections were cut on a Porter-Blum MTII ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.) and placed on 150-mesh copper grids. The grids were mounted on glass rods and coated with a layer of Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, Eng.). In some experiments the emulsion was not diluted, and thus the layer of film over the sections was more than one layer of silver halide crystals thick. Because of the thickness of the sections (and the consequent relatively more intense source of radioactivity), many autoradiograms could be examined after only a 5-day exposure of the emulsion. The grids were developed according to the method of Stevens (17) and stained for 20 min in uranyl acetate followed by 10 min in lead citrate. The sections were then carbon-coated and examined on the JEOL JEM 1000 operating at 1,000 kV.

Conventional electron microscope autoradiography

was occasionally employed to corroborate the HVE-MAR findings. Sections were cut at about 800 Å and coated with a monolayer of Ilford L4 emulsion. After exposure for at least 28 days, coated sections were developed, stained, and examined with an AEI-801 electron microscope operating at 60 kV.

RESULTS

Cells containing MC nuclei in which snRNAs are the only labeled molecules (7) were allowed to enter mitosis and then fixed at selected stages. After suitable preparation for EMAR, the following electron microscope observations were made. The identification of mitotic stages was based on the descriptions given by Roth et al. (15) and more detailed findings from our studies (G. E. Wise, unpublished observations).

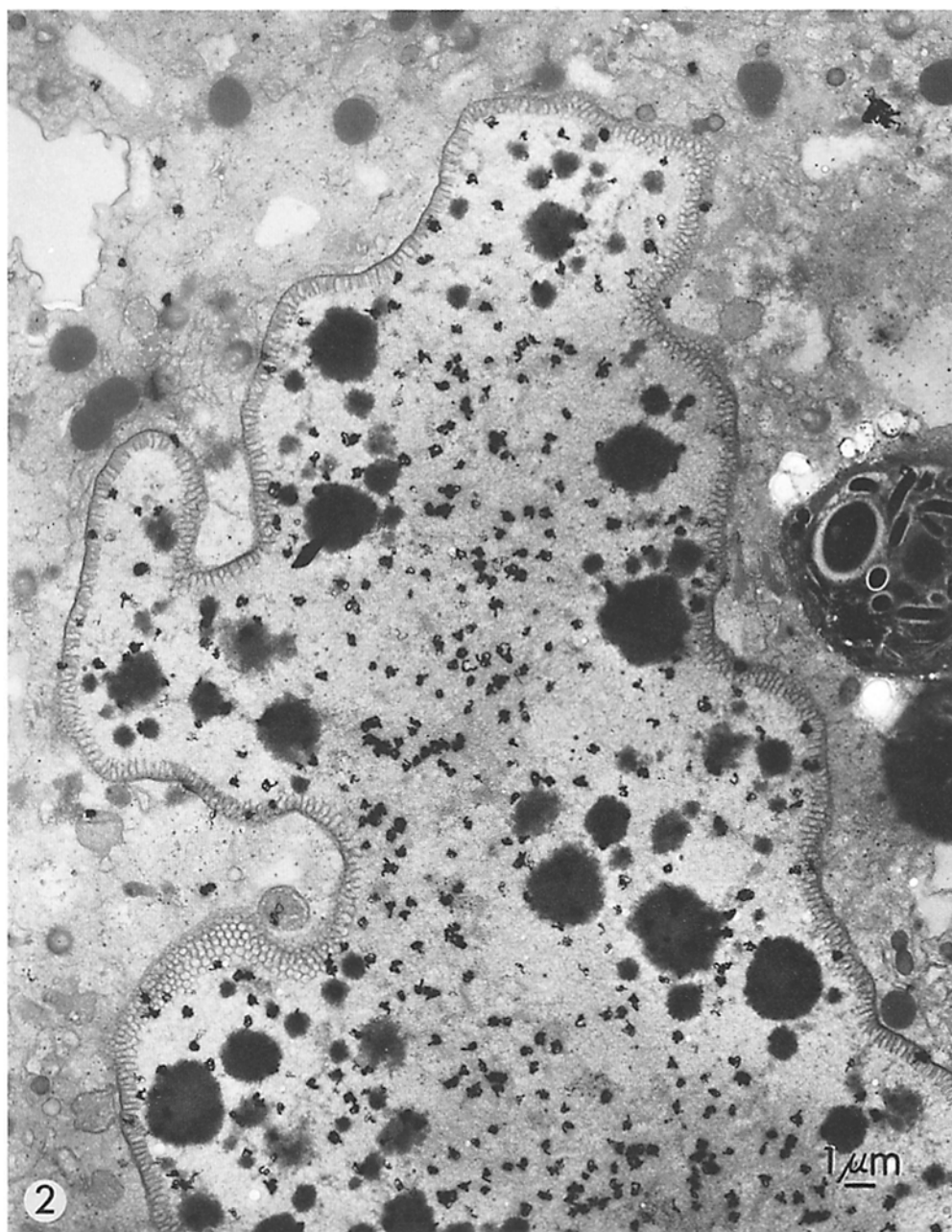### Interphase

As shown in Fig. 2, almost all the ³H in cells

FIGURE 2 EM autoradiogram of microsurgically chased [³H]RNA nucleus in unlabeled cytoplasm interphase, obtained as described in text. A somewhat higher than normal concentration of ³H in central region of nucleus where the chromatin is localized suggests that the cell may be in very early prophase. × 4,000.

containing MC nuclei is nuclear, with the bulk of that label being nucleoplasmic; only a few of the AR grains around the periphery of nucleoli can be attributable to those structures. We showed earlier

(18) that within the nonnucleolar parts of the nucleus the concentration of label of a particular class of snRNAs present over chromatin is slightly higher than that over the "nonstructural" nucleo-

plasm. In Fig. 2, a similar higher concentration of radioactivity over chromatin is evident, but conceivably that cell is in very early prophase.

Prophase

Prophase is characterized by: (a) the disappearance of the fibrous lamina (honeycomb layer of the nuclear envelope), (b) a swelling of the nucleus which makes it more spherical, (c), a marked reduction (from hundreds to perhaps dozens) in the number of nucleoli, which become somewhat enlarged, and (d) the condensation of chromatin into recognizable chromosomes. No spindle microtubules are visible during prophase. Autoradiograms (Fig. 3) show a dramatic concentration of label in chromosomes and nucleoli during prophase, the remainder of the nucleoplasm being almost devoid of radioactivity.

Metaphase

Metaphase is distinguished by: (a) the alignment of chromosomes in the middle of the spindle, the microtubules of which are now visible, (b) the dissolution of the nuclear envelope in many places,
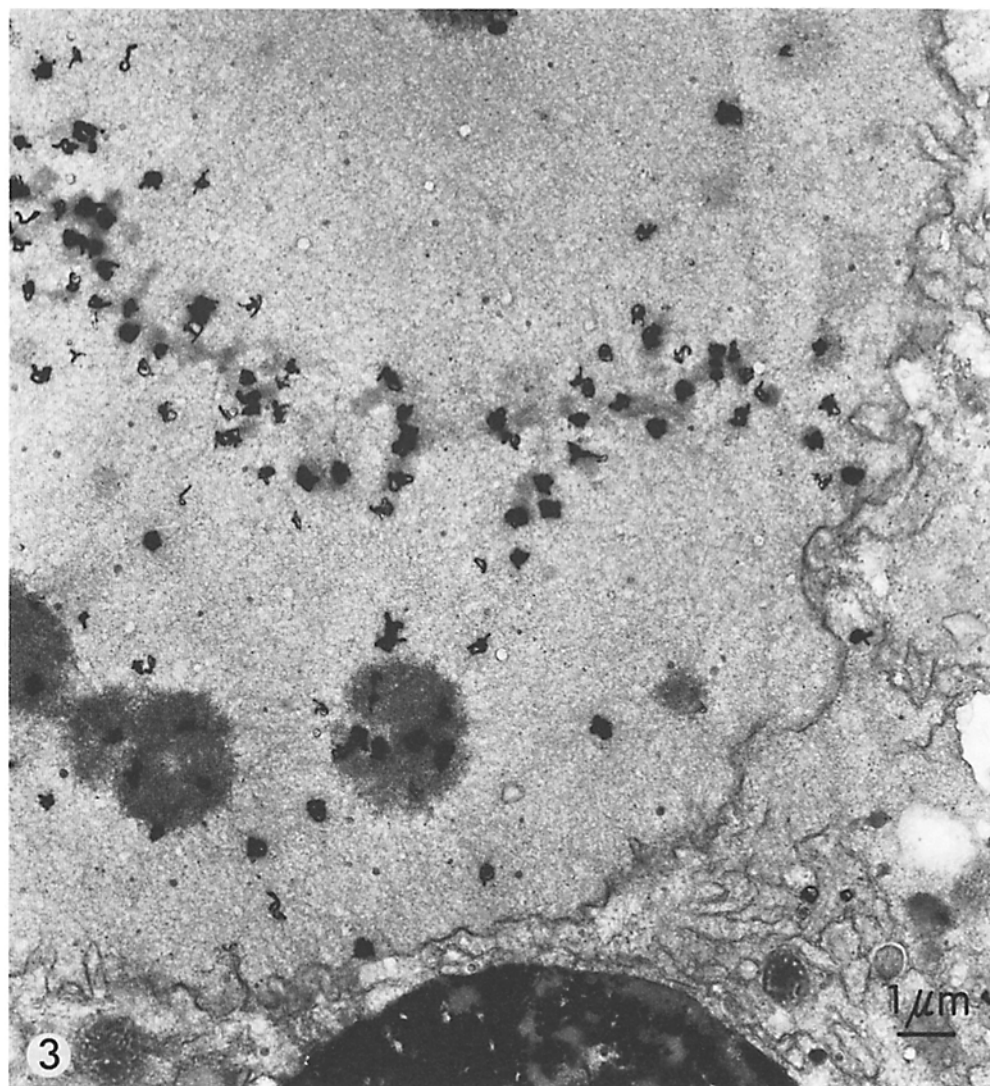

FIGURE 3   EM autoradiogram of [³H]snRNA localization in late prophase. The label is obviously highly localized in chromosomes and to some extent in the remaining nucleoli. × 8,000.

resulting in gaps in the boundary between nucleus and cytoplasm, and (c) an extensive convolution of the remaining nuclear envelope. A few nucleoli may persist through this stage, but often they are outside the ragged boundary demarcated by the residual nuclear envelope.

The distribution of radioactivity at metaphase (Fig. 4) is markedly different from that at prophase. Most striking is the almost total absence of any label associated with the chromosomes. At metaphase the snRNAs are largely cytoplasmic (seen best with light microscopic autoradiography [5]) but some are within the vicinity of the persisting fragments of the nuclear envelope which border and pervade the mitotic spindle. The few nucleoli that may remain continue to be labeled.

Anaphase

Ameba anaphase is like that of most animal cells, except that the remnants of the nuclear envelope persist and seem to be reforming at late anaphase. Moreover, remnants of the nuclear envelope are observed not only at the periphery of the spindle, but also among the chromosomes and between the separating sets of chromosomes.

During early anaphase [³H]snRNA is once again concentrating in chromosomes, although a substantial amount of radioactivity continues to be cytoplasmic. By late anaphase there is an even greater proportion of cellular radioactivity in chromosomes (Fig. 5). What we consider to be late anaphase is distinguished by more highly organized nuclear envelopes around the two sets of chromosomes, although the nuclear envelopes are not yet continuous. Note that a relatively high concentration of [³H]snRNA is found just inside the nuclear envelope, as is the case during metaphase.

Telophase

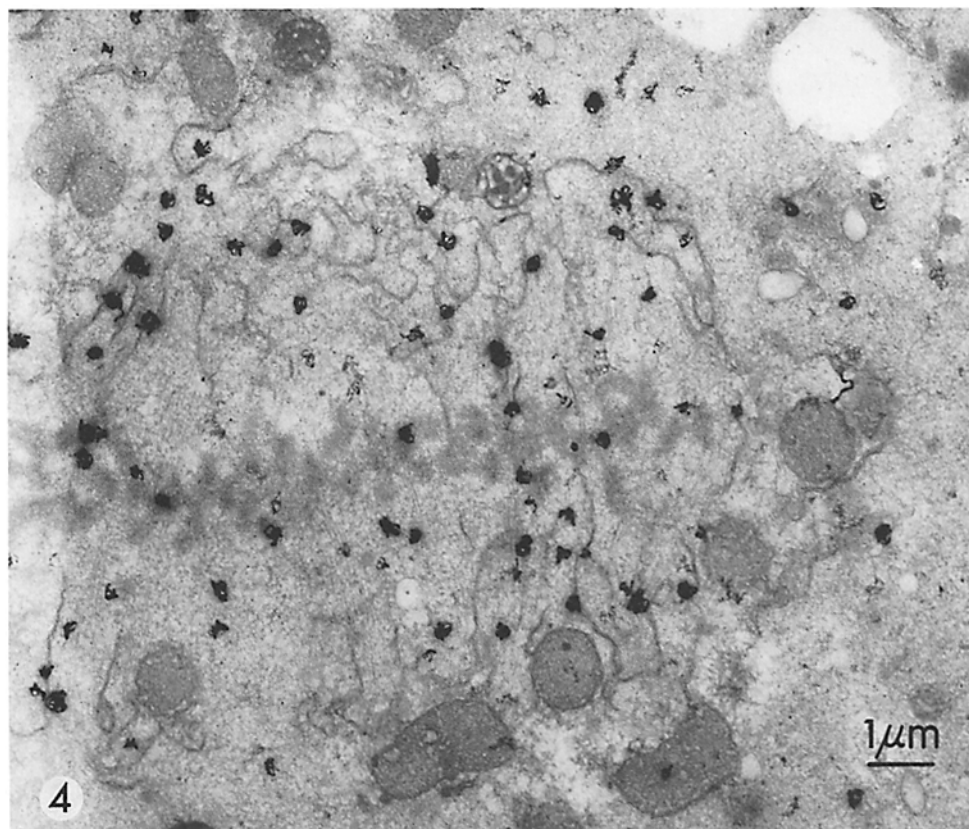Telophase is recognized by: (a) continuous nuclear envelopes, although sometimes with continu-

FIGURE 4   EM autoradiogram of [³H]snRNA localization at metaphase. The label is almost totally absent from the chromosomes. × 9,000.
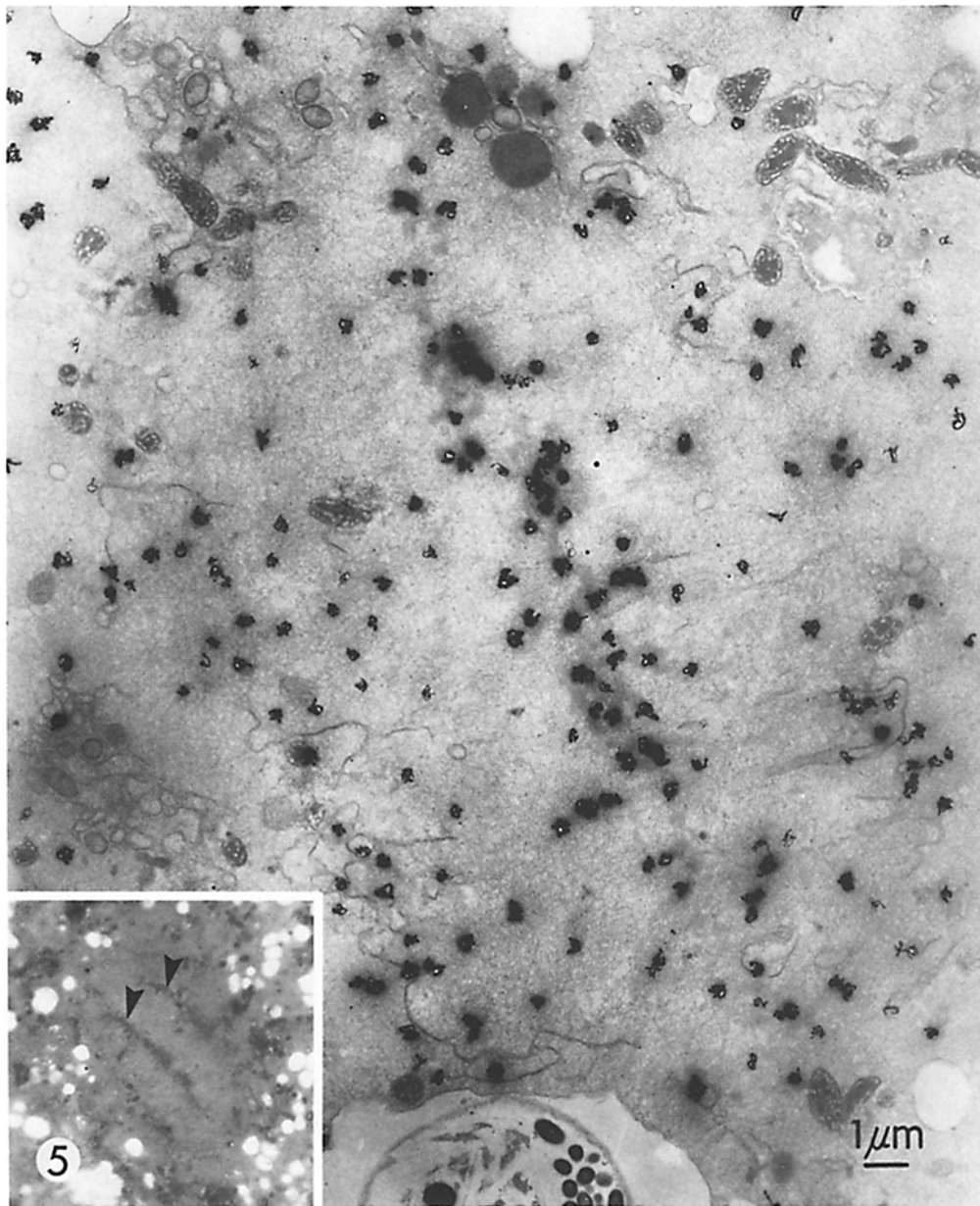
FIGURE 5 EM autoradiogram of [³H]snRNA localization at late anaphase. The radioactivity is once again highly concentrated in chromosomes. Because the EM section contains only one "complete" chromosome set, a light microscopic view of an adjacent toluidine blue-stained, thick section is given in the insert to show (at the arrows) that the cell is indeed in late anaphase. The EM section is × 6,000.

ity dependent upon gaps being filled by rough endoplasmic reticulum, (b) a rather flattened nuclear shape, (c) the presence of small nucleoli at the nuclear periphery, and (d) mostly decondensed chromosomes.

Most of the radioactivity is back in the nuclei by telophase (Fig. 6), but the intranuclear localization is difficult to distinguish. Most of the intranuclear radioactivity is seen to be nonnucleolar, however.
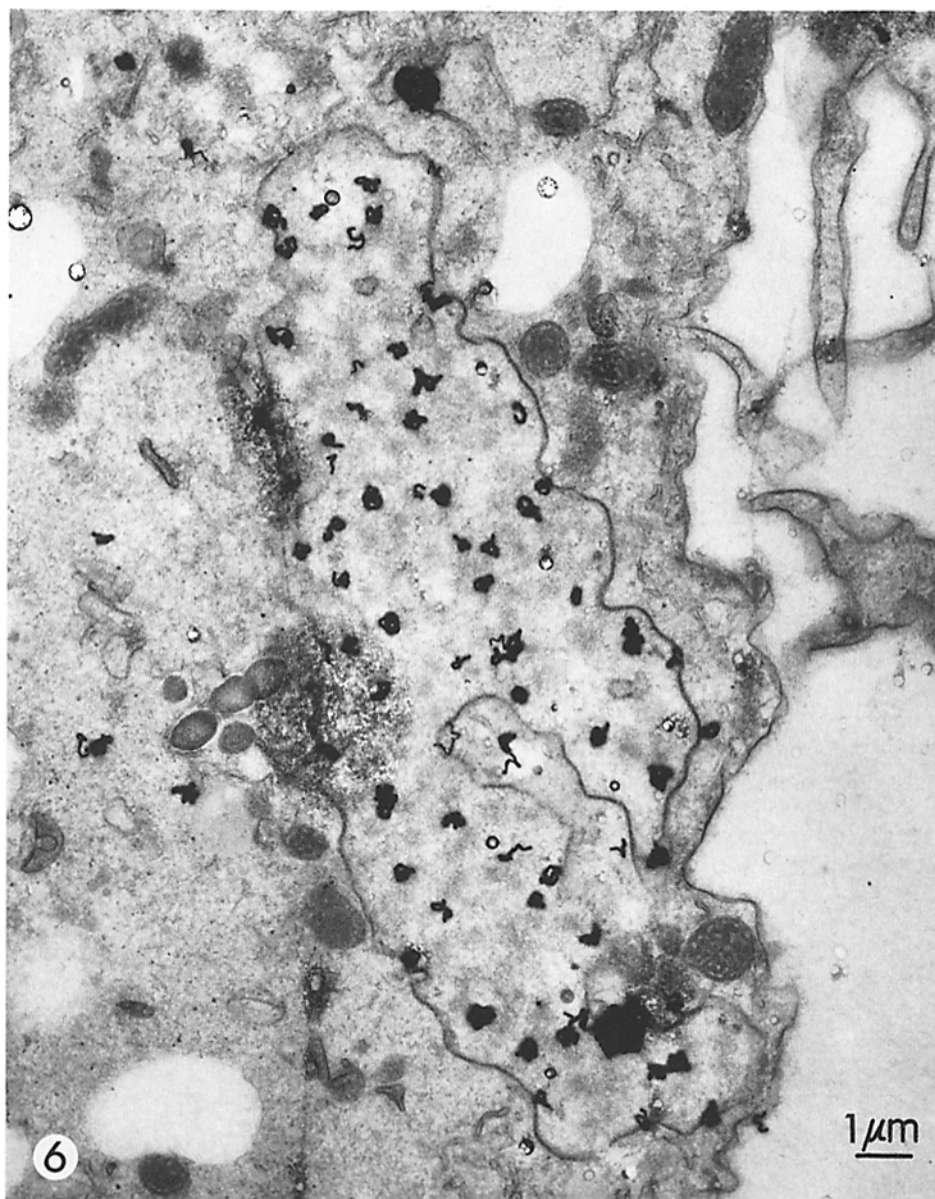
FIGURE 6   EM autoradiogram of [³H]snRNA localization at telophase. Almost all of the radioactivity is back in the nuclei, but the intranuclear localization is difficult to resolve. × 7,500.

Although we have not followed the post-division stages carefully, we know that by 3 h post-mitosis the ³H label is almost exclusively nucleoplasmic and that only a relatively small proportion of that is associated with chromatin.

DISCUSSION

Our primary concern in this paper is the relation of snRNAs to chromosomes. (The interesting associations of snRNAs with nucleoli will be taken up in another paper, and we cannot even speculate on the significance of a relatively high concentration of snRNAs adjacent to the nuclear envelope at certain mitotic stages.)

The major finding here clearly is that at metaphase, when the chromosomes are maximally con-

densed, essentially no snRNA is chromosomal, but that shortly before (during prophase) and shortly after (during anaphase) metaphase large amounts of snRNA are associated with chromosomes. The latter two stages are the times of highest chromosomal concentration of snRNAs, since during interphase the amount of snRNA found over chromosomes is relatively low (18). To summarize these findings another way: when chromosomes are maximally decondensed, a small proportion of the cellular snRNA is chromosomal; when the chromosomes are partially condensed (as during prophase and anaphase), a great proportion of cellular snRNA is chromosomal; and when chromosomes are maximally condensed, no snRNA is chromosomal.

A somewhat similar pattern of chromosomal events has been reported for human bone marrow cells. With these cells, a probe for "template activity" has a minimum affinity for metaphase chromosomes and a maximum affinity for telophase chromosomes, after which the affinity falls somewhat and remains level until the next prophase when it begins to fall again (10).

We should note here that we have recently demonstrated (Goldstein, Wise, Stephenson, and Ko, unpublished observations) that when ameba interphase chromosomes are caused to condense by treatment with actinomycin-D, the snRNAs become highly concentrated in chromatin.

Two technical points must be dealt with to make our general conclusion sound. First is the question of whether chromosomes begin to decondense before telophase, the stage at which (according to textbooks) post-mitotic chromosome decondensation has been considered to occur. At the risk of inviting accusations of circular reasoning, we propose that the high degree of snRNA association with anaphase chromosomes is evidence that those chromosomes are decondensing. Surely some change in the chromosomes must be occurring, and the onset of chromosome decondensation seems most plausible. More importantly, our interpretation of the electron micrographs is that the chromosomes of late anaphase are less compact than those of metaphase (compare Figs. 4 and 5) and that the amount of snRNA in chromosomes increases as anaphase progresses.

The other technical point relates to the question of autoradiographic resolution. The localization of radioactivity in most of the autoradiograms is clear enough, and our interpretations of them seem unimpeachable, but whether the metaphase chro-

mosomes are totally devoid of radioactivity in these experiments is somewhat uncertain. (That metaphase chromosomes have fewer snRNA's than prophase or anaphase chromosomes is unambiguous, however, and thus the general conclusions seem sound.) We used HVEMAR primarily because we could then utilize thicker-than-normal sections and thereby markedly shorten the autoradiographic film exposure time, a great advantage when using weakly radioactive samples. The disadvantage with this methodology is that thicker sections and emulsions result in poorer resolution than the 0.1–0.15 μm resolution achievable with thin sections and monolayer emulsions. However, Salpeter (16) demonstrated that the probability of a tritium β particle reaching the upper layers of a multilayered emulsion is low because the particle becomes severely retarded within the distance of one silver halide crystal of the size found in Ilford L4 emulsion. This we confirmed by taking stereo pair pictures that show that the vast majority of silver grains are in a plane adjacent to the section. The major limitation in resolution results, therefore, from the thickness (0.3 μm) of the sections we used. Although a majority of the β particles that strike the emulsion emanate from the top 0.1 μm of the section, a significant amount of β's reach the emulsion from the lower 0.2 μm of the section (1), and this may mean that some of the AR grains we see derive from sources 0.2–0.3 μm laterally from the grain. This is a large distance with respect to the dimensions of the ameba chromosomes. It is important to note in Fig. 4, however, that there is a virtual absence of AR grains over the middle of the metaphase plate. To claim that any snRNAs are associated with such chromosomes, one would have to invoke some rather strained arguments about snRNAs being restricted to only special parts of metaphase chromosomes. It probably is more reasonable to assume that the occasional AR grain seen over metaphase chromosomes results from the scatter of radioactivity originating from nonchromosomal sources.

What is the meaning of this pattern of snRNA localization? One of us has already argued (5) that this may well reflect the involvement of snRNAs in the programming of chromosomal transcriptions. Thus, it was proposed that snRNAs have an affinity for certain chromosomal sites whose activity they influence and that they are specifically displaced from these sites by acidic chromosomal proteins. In prophase, as the chromosomes are

condensing, these proteins are cast off and the snRNAs presumably have an opportunity to greatly increase their associations with the chromosomes. At metaphase, however, the chromosomes are so extensively condensed that the snRNAs too are cast off. During anaphase, the decondensing chromosomes presumably are again able to accept large amounts of snRNAs, which later may be displaced from the chromosomes by slower returning proteins. That the acidic nuclear proteins return to post-mitotic nuclei more slowly than do the RNAs has been demonstrated (13, 14). The mitotic displacement of chromosomal proteins may allow different proteins to associate with the post-division chromosomes and thereby "reprogram" genetic expression.

Another possibility is that the snRNAs are somehow involved in the profound changes that occur in chromosome structure during mitosis. The involvement of RNA in the folding of chromosomes has been well established for prokaryotes (19, 11), suggesting the possibility of similar roles in eukaryotes during post-metaphase stages.

Since several species of snRNAs are known to exist, it may be that snRNAs function in several different roles.

REFERENCES

1. CARO, L. G. 1964. High resolution autoradiography. *Methods Cell Physiol.* **1**:327-363.
2. CHALKLEY, H. W., and G. E. DANIEL. 1933. The relation between the form of the living cell and the nuclear phases of division in *Amoeba proteus* (Leidy). *Physiol. Zool.* **6**:592-619.
3. ELLIOTT, A. M., L. E. BROWNELL, and J. A. GROSS. 1954. The use of *Tetrahymena* to evaluate the effects of gamma radiation essential nutrilites. *J. Protozool.* **1**:193-199.
4. FLICKINGER, C. J. 1966. Methods for handling small numbers of cells for electron microscopy. *Methods Cell Physiol.* **2**:311-321.
5. GOLDSTEIN, L. 1976. Role for small nuclear RNA's in "programming" chromosomal information? *Nature (Lond.).* **261**:519-521.
6. GOLDSTEIN, L., and W. JELINEK. 1966. A comparison of the movement of enucleate fragments from interphase and mitotic amebae. *Exp. Cell Res.* **43**:51-55.
7. GOLDSTEIN, L., and C. KO. 1974. Electrophoretic characterization of shuttling and nonshuttling small nuclear RNAs. *Cell.* **2**:259-269.
8. JEON, K. W. 1970. Micromanipulation of ameba nuclei. *Methods Cell Physiol.* **4**:179-194.
9. KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27**:137 a (Abstr.).
10. NAKATSU, S. L., M. A. MASEK, S. LANDRUM, and J. H. FRENSTER. 1974. Activity of DNA templates during cell division and cell differentiation. *Nature (Lond.).* **248**:334-335.
11. PETTIJOHN, D. E., and R. HECHT. 1974. RNA molecules bound to the folded bacterial genome stabilize DNA folds and segregate domains of supercoiling. *Cold Spring Harbor Symp. Quant. Biol.* **38**:31-41.
12. PRESCOTT, D. M., and R. F. CARRIER. 1964. Experimental procedures and cultural methods for *Euplotes eurystomus* and *Amoeba proteus. Methods Cell Physiol.* **1**:85-95.
13. PRESCOTT, D. M., and L. GOLDSTEIN. 1968. Proteins in nucleocytoplasmic interactions. III. Redistributions of nuclear proteins during and following mitosis in *Amoeba proteus. J. Cell Biol.* **39**:404-414.
14. RAO, M. V. N., and D. M. PRESCOTT. 1970. Inclusion of predivision labeled nuclear RNA in post-division nuclei in *Amoeba proteus. Exp. Cell Res.* **62**:286-292.
15. ROTH, L. E., S. W. OBETZ, and E. W. DANIELS. 1960. Electron microscope studies of mitosis in amoebae. I. *Amoeba proteus. J. Biophys. Biochem. Cytol.* **8**:207-220.
16. SALPETER, M. M. 1966. General area of autoradiography at the electron microscope level. *Methods Cell Physiol.* **2**:229-253.
17. STEVENS, A. R. 1966. High resolution autoradiography. *Methods Cell Physiol.* **2**:255-310.
18. WISE, G. E., and L. GOLDSTEIN. 1973. Electron microscope localization of nuclear RNA's that shuttle between nucleus and cytoplasm and nuclear RNA's that do not. *J. Cell Biol.* **56**:129-138.
19. WORCEL, A., E. BURGI, J. ROBINTON, and C. L. CARLSON. 1974. Studies on the folded chromosome of *Escherichia coli. Cold Spring Harbor Symp. Quant. Biol.* **38**:43-51.