

Quantitative Digital Analysis of Diffuse and Concentrated Nuclear Distributions of Nascent Transcripts, SC35 and Poly(A)

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Digital imaging microscopy was used to analyze the spatial distribution and levels of newly synthesized RNA in relation to steady-state poly(A) RNA and to the splicing factor SC35. Transcription was monitored over time after microinjection of BrUTP and was detected using antibodies. Poly(A) RNA was detected with probes directly conjugated to fluorochromes, allowing direct detection of the hybrids. Objective methods were used to determine genuine signal. A defined threshold level to separate signal from noise was established for each nucleus. The nucleolus was used to determine poly(A) and SC35 background and the juxtannuclear cytoplasm was used for the BrUTP background. The remaining signal was segmented into high (concentrated) and low (diffuse) levels. Surprisingly, for all probes examined, most of the signal was not in concentrated areas, but rather was diffusely spread throughout the nucleoplasm. A minority (20–30%) of the SC35 signal was in concentrated areas (“speckles”) and the rest was dispersed throughout the nucleoplasm. In addition, the concentrated areas had a mean intensity only twice the average. The amount and significance of the colocalization of the diffuse, or concentrated, areas of SC35 [or poly(A)] with BrUTP incorporation were analyzed. The image from one probe was translated with respect to the other in three dimensions to compare colocalization with random alignments. Both poly(A) and SC35 were found to have low colocalization with the total BrU signal. Sites of transcription were determined using an algorithm to find maxima of BrUTP signal within clusters. From 849 to as many as 3888 sites per nucleus were detected. A rim of hybridization to poly(A) coinciding with the nuclear envelope was eliminated by actinomycin treatment, suggesting that these transcripts were exiting from the nucleus. These results emphasize the importance

of utilizing the full dynamic range of the image before drawing conclusions as to the distribution of nuclear components. © 1997 Academic Press

INTRODUCTION

The spatial relationship of newly transcribed RNA to other components in the nucleus is currently unresolved [see for reviews, 1, 2]. Some evidence has suggested that the nucleus is compartmentalized with respect to gene expression: for instance that RNA transcription and subsequent processing may each occur at or near sites of concentrations of splicing factors [3–7] or poly(A) [8]. Other evidence suggests that the nucleoplasm is homogeneous with respect to pre-mRNA synthesis and processing, irrespective of the proximity of highly concentrated factors [9]. The incorporation of BrUTP *in vivo* has been used to investigate the appearance of nascent transcripts by use of an antibody to the incorporated analog [10, 11]. A punctate appearance of incorporated BrUTP was dispersed seemingly randomly throughout the nucleus. Its distribution was similar to those seen for early replication when BrdU was incorporated [12, 13]. These sites of active transcription appeared not to show significant overlap with the “speckles” [14] seen using antibodies to the splicing factor, SC-35 [15]. The “speckles” appear to be mainly storage and recycling structures, in equilibrium with the transcription activity throughout the nucleus, but not always the site of the activity (see 16). The work presented here proposes that high concentrations of factors, such as the speckles, are a minor part of a continuum of nuclear signal.

Criteria used to determine the various nuclear compartments have focused on visualizing the brightest signals, corresponding to the highest concentrations of the probe. Operationally, these concentrations are visualized by determining a threshold which is then used to separate them from low levels of signal. In digital images, these thresholds may be used for subtraction of “background.” In analog images, the same result can be achieved by time of photographic exposure. Cur-

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rently, there is no objective method for establishing this threshold and evaluating how much genuine signal is removed. Likewise, colocalization of images is used to correlate the overlap between two components, yet quantitative methods have not yet been used to evaluate the significance of this colocalization or what percent of the total signal the colocalization represents. In this work, we have developed objective methods for determination of total signal and colocalization of two images.

New reagents and digital analysis methods were employed in order to assess the exact relationship of nascent transcripts, with SC35 and poly(A). BrUTP was microinjected to assay the general transcriptional activity of the nucleus. Fluorochrome-conjugated probes [17] provided for improved detection of poly(A). An accurate background level of the sample was determined in order to set objective thresholds which remove background but minimize loss of low levels of signal. Imaging algorithms which allow the rigorous assessment of the significance of spatial coincidence of pairs of spectrally distinguishable signals were developed. In order to determine whether transcripts increased or decreased their association with SC35 or poly(A), colocalization of BrUTP incorporation with these factors was monitored with time after microinjection. This approach determined that a minor percentage of total SC35 and poly(A) signal colocalized with the new transcripts.

MATERIALS AND METHODS

Cells and Microinjection

Human diploid fibroblasts were cultured on gridded coverslips containing 100 boxes, each $100 \mu\text{m}^2$ and individually numbered (Klarman Ruling, NH). Cells were prepared for microinjection by placing the coverslip in a chamber maintained at 37°C on an inverted microscope. Cells were microinjected with 144 mM BrUTP (Sigma) in buffer [18] using a picospritzer (e.g., 250 ms, 60 psi). Cells were transferred to an incubator after injection, when appropriate. The time and position of each cell was recorded, so that following fixation the exact time interval between BrU injection and fixation for each cell could be accurately determined. Cells were fixed with 4% paraformaldehyde solution in PBS and stored in 70% ethanol until analyzed. A short time of labeling, $<10 \text{ min}$, would be expected to result in mostly nascent transcripts [10, 11]. Later times might be expected to indicate predominantly finished transcripts. Fifty-two cells from six separate experiments were used for detailed analysis.

In Situ Hybridization and Immunofluorescence

Cells were first used for *in situ* hybridization for poly(A) RNA using a 43-nt probe conjugated directly to five fluorochromes [19] and then for immunofluorescence after extensive washing. Hybridization was for 3 h at 37°C in a solution containing 15% formamide in hybridization buffer (0.3 M NaCl, 1% BSA, tRNA and salmon sperm, $500 \mu\text{g}/\mu\text{l}$). The antibody for BrdUr (Jackson Immunoresearch, Bar Harbor, ME) was used at a dilution of 1:1000 in PBS. A fluorescein-conjugated anti-rat antibody was used for the immunofluorescence. A monoclonal antibody for SC35 was obtained from Sigma. Nuclear pore

monoclonal antibody was obtained from P. Bangs [20]. Antibody cross-reactivity was tested independently and found to be negligible. Cells were viewed under a microscope equipped for epifluorescence (Nikon).

Image Analysis

A series of images at focal planes separated by $0.25 \mu\text{m}$ were digitized using a Photometrics CCD camera (Tucson, AZ). Images were taken using a $60\times$ planapo 1.4 NA objective and a $5\times$ eyepiece. Captured images were processed using a constrained deconvolution algorithm [21] to reassign light to the position in 3D where it originated, using a point spread function obtained from a bead added to the sample before mounting. Deconvolved images were realigned for each wavelength using the beads as fiducial markers.

The dynamic range of the genuine signal may be inadvertently truncated by choosing too high a threshold. Low-intensity levels of signal may be removed from the image as "background" when background level is obtained by averaging control cells, since there is considerable variation from cell to cell. This low-intensity signal may contain important information. We developed objective criteria which could distinguish genuine background from low levels of signal. Biological criteria were used for this procedure. Intensity inside the nucleolus was used to determine a "noise threshold" for the poly(A)/SC35 data (since they are excluded from the nucleolus). The mean + 2 standard deviations was used as this threshold. In most cases, this resulted in removal of 95% of the nucleolar "noise." Similarly, the BrU data was thresholded at the mean + 2 standard deviation of the intensity in the cytoplasm. The nucleolus provided a realistic background for poly(A) since pol I transcripts are not polyadenylated. The cytoplasmic area just outside the nucleus was chosen for background measurements of BrUTP incorporation since no transcription occurred there. This calculation was done on each cell, to determine the true background for that cell. The important result of this approach was to determine the *total* signal throughout the nucleus. It allowed the portion of the image which contained regions of higher concentration (speckles) to be quantitated relative to the total.

Registration of images was performed using beads containing multiple fluorochromes as fiducial markers to spatially align images. In order to evaluate the significance of their colocalization, one image was translated with respect to the other and the extent of colocalization of the signals from the two probes in each of the resulting image pairs was assessed in 75 trials (see below). The colocalizations were ranked with respect to how many translations provided better colocalization values. It would be expected that colocalization of random noise would provide a random ranking order. Segmentation of the voxels within an image into above the mean and below the mean brightness levels distinguished between the dimmer and brighter components of the image. Colocalization analysis was done with the brighter and dimmer signals for each probe; these represented concentrated versus diffuse signal.

The images shown in the figures may not always visually appear to agree with the statistics stated (e.g., percent colocalization observed). This is typically due to two factors. First, a projection is displayed through a three-dimensional volume. Thus voxels in the back or interior of the nucleus tend to get obscured by voxels in the front. Sometimes the top sections are removed so that the interior can be viewed better, but even then an inaccurate visual impression may be formed since only a subset of the volume is actually being observed. Second, bright voxels are much easier to see against the dark background. Therefore, the visual impression formed of the image will deviate from reality since it tends to be guided mostly by the characteristics of the bright voxels. It is this point which this work emphasizes: that the visual data are not the statistical data. For statistical purposes, all voxels above the noise threshold are counted.

BrU/Poly(A) or SC35 colocalization. We calculated the extent of BrU "overlap" with either poly(A) or SC35 and its significance. Both images were "masked" to limit all analysis to the nucleoplasm. To

do this, the nuclear membrane and nucleolus were manually identified. The colocalization ("overlap") of the BrU with the poly(A)/SC35 was taken to be the percentage of BrU voxels (i.e., $\text{BrU} > \text{threshold}$ and within the nucleoplasm) which also had poly(A) present (i.e., poly(A) above its threshold).

Perhaps more important than the colocalization itself is whether the observed colocalization represents a statistically significant departure from what would occur due to "random chance." This type of analysis depends upon the data characteristics and the class of situations which "random" must include. If the data in one voxel are taken to be independent of neighboring voxels, the observed colocalization can be compared to that predicted from a binomial distribution. If the objects exceed one voxel in size, this assumption may deviate significantly from reality. One method for analysis of our data would be to identify "objects" (connected groups of voxels) in the BrU image. Random images could then be created by randomly moving these objects around within the nucleoplasm. Unfortunately, most of the BrU data is not amenable to the isolation of separate objects, as much of the image is interconnected (especially at later time points).

The best method of creating random images which still contained the important spatial correlations was merely to translate the images "randomly." Images were translated over a range of ± 10 voxels (± 1870 nm) in x and y and the ± 5 voxels in z (along the optical axis). To save computational time translations at 5-pixel increments (e.g., $-10, -5, 0, 5, 10$) were examined; this yielded 75 translations (5×3). The true colocalization percentage (0 translation) was ranked relative to the other 74. If the rank was, for instance, 5, this indicated that the observed colocalization was significantly greater than that due to chance, with 90% confidence (since 5 is in the top 10% of 75). A ranking of 0 indicated that there was no better colocalization possible with the set examined. A ranking of 74 indicated an exclusion, i.e., that all possibilities were better. Some of the data were examined using small translations (integer translations within a ± 3 -voxel range) to ensure that correlations over short distances were not missed. These were sometimes slightly less significant (possibly picking up tiny misregistrations between the image pairs). Cressie [22] discusses the general approach to statistical analysis of bivariate spatial point processes. A similar analysis to the one described here has recently been reported by Van Steensel *et al.* [23].

BrU sites. We determined the number of transcription sites in the nucleus. Prior to analysis the mean intensity (and its standard deviation) of the cytoplasm was determined. Since incorporation of BrUTP does not occur in the cytoplasm, this was taken as a measure of the noise level in the cell. The nuclear envelope and nucleolar boundaries were defined manually so that only the nucleoplasm was examined (other voxels were "masked out"). This masked image was then thresholded at the mean + 2 standard deviations of the noise level. Since we expect the BrU sites to be small ($< 0.5 \mu\text{m}$) bright spots, [11, 12] we cross-correlated the image with the image of a $.25\text{-}\mu\text{m}$ sphere (with a blurred boundary). Signal detection theory indicates that a matched filter is the optimum detector when the signal sought is known exactly and noise in the system is white [24]. Cross-correlation is equivalent to applying a matched filter. After cross-correlation, we then identified those voxels which were a local intensity maximum (in all three dimensions). This is necessary since cross-correlation tends to produce broad responses, so it would be inappropriate only to threshold the resulting image and count voxels.

The same analysis was applied to the control cells. This controls for labeling "noise" as well as system noise (Poisson noise, read-out noise, etc.).

In order to determine the significance of the observed colocalization of BrU sites with SC35 or poly(A), we generated 99 random distributions of BrU for each cell. This was straightforward since the sites were represented as single isolated voxels. The same number of sites was used as was observed in each individual cell, for random placement. The colocalization with poly(A) or SC35 was calculated, with 99 random repetitions. The real colocalization was compared to the random distributions and ranked accordingly. If the BrU sites colocalized better than chance, it would be the best of the 100 trials (ranking = 0), and if it was significantly excluded it would be the worst out of 100 trials (ranking = 100).

Speckles. SC35 data were analyzed to determine the ratio of fluorescence within the speckles to that within the entire nucleus (excluding the nucleolus). The threshold appropriate to visualize "speckles" required a subjective decision. Therefore, the analysis was done for three different thresholds; one clearly a little too low (speckles too large), one which seemed best (mimicked published pictures of speckles), and one clearly a little too high (speckles too small). The nuclear and nucleolar borders were identified manually. Noise was eliminated in the following way: the average intensity within the nucleolus was determined. Since SC35 was not present in the nucleolus, this was used as a mean "noise" level per voxel (N) for the image. Total light within the speckles (T_s) was determined by thresholding the image such that only "speckles" remained. We then calculated

$$\frac{T_s - V_s \times N}{T - V \times N},$$

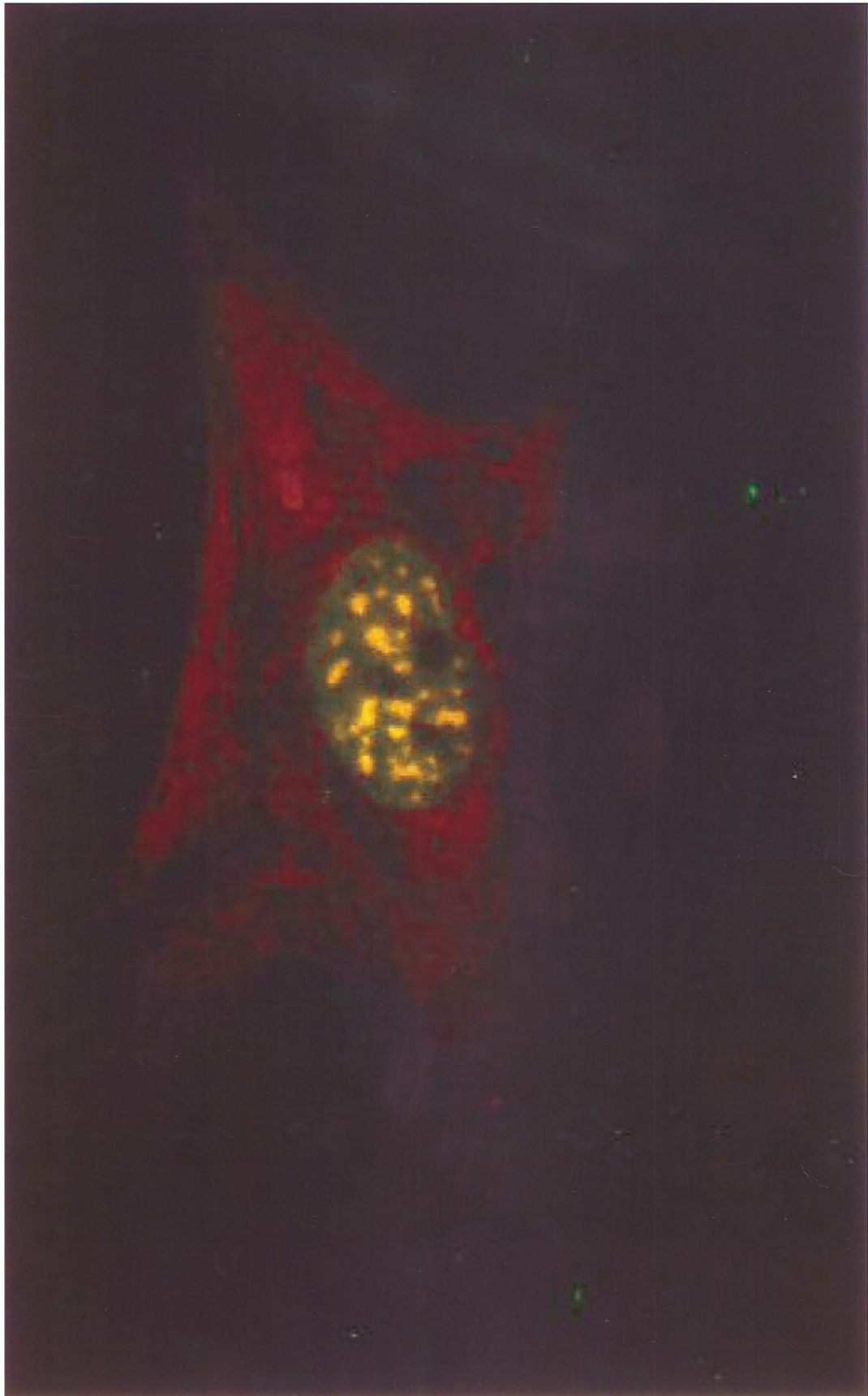
where V_s was the volume of the "speckles," T was the total light in the nucleus (excluding nucleolus), and V was the volume (in number) of the non-zero voxels after thresholding the image at a level of the mean + 2 SD of the noise. This was necessary since even after the background noise was thresholded from the signal, voxels above threshold still had a noise component which we subtracted out ($V_s \times N$ or $V \times N$).

RESULTS

Directly Conjugated Probes Penetrate the Nucleoplasm

The directly conjugated probes were assessed in comparison with probes which were detected by secondary reagents, such as antibodies or avidin. The poly dT probe was labeled with either biotin or directly with fluorochromes. Equimolar quantities of each probe were used for *in situ* hybridization, and the resulting hybrids viewed by fluorescence. The directly labeled probe was visible in green throughout the nucleus, whereas the biotin-labeled probe was detectable in structures previously described as speckles or patches

FIG. 1. Poly(A) hybridization detected by a directly conjugated or biotin-labeled oligo(dT) probe. Cells were hybridized with either biotin-labeled or fluorescein-conjugated poly(dT). The probes were identical in size and were equimolar in concentration. Detection of the biotin was with streptavidin-Texas red and the cell was viewed by simultaneous dual-color epifluorescence using a double filter. An analog photograph is presented. Green areas are the directly conjugated probe only, red areas are the stronger avidin detection of the biotin-labeled probe. Yellow indicates overlap of the two probes. Note that in the cytoplasm, the red signal is stronger. In contrast, the green signal predominates in the nucleoplasm, presumably where avidin doesn't penetrate.



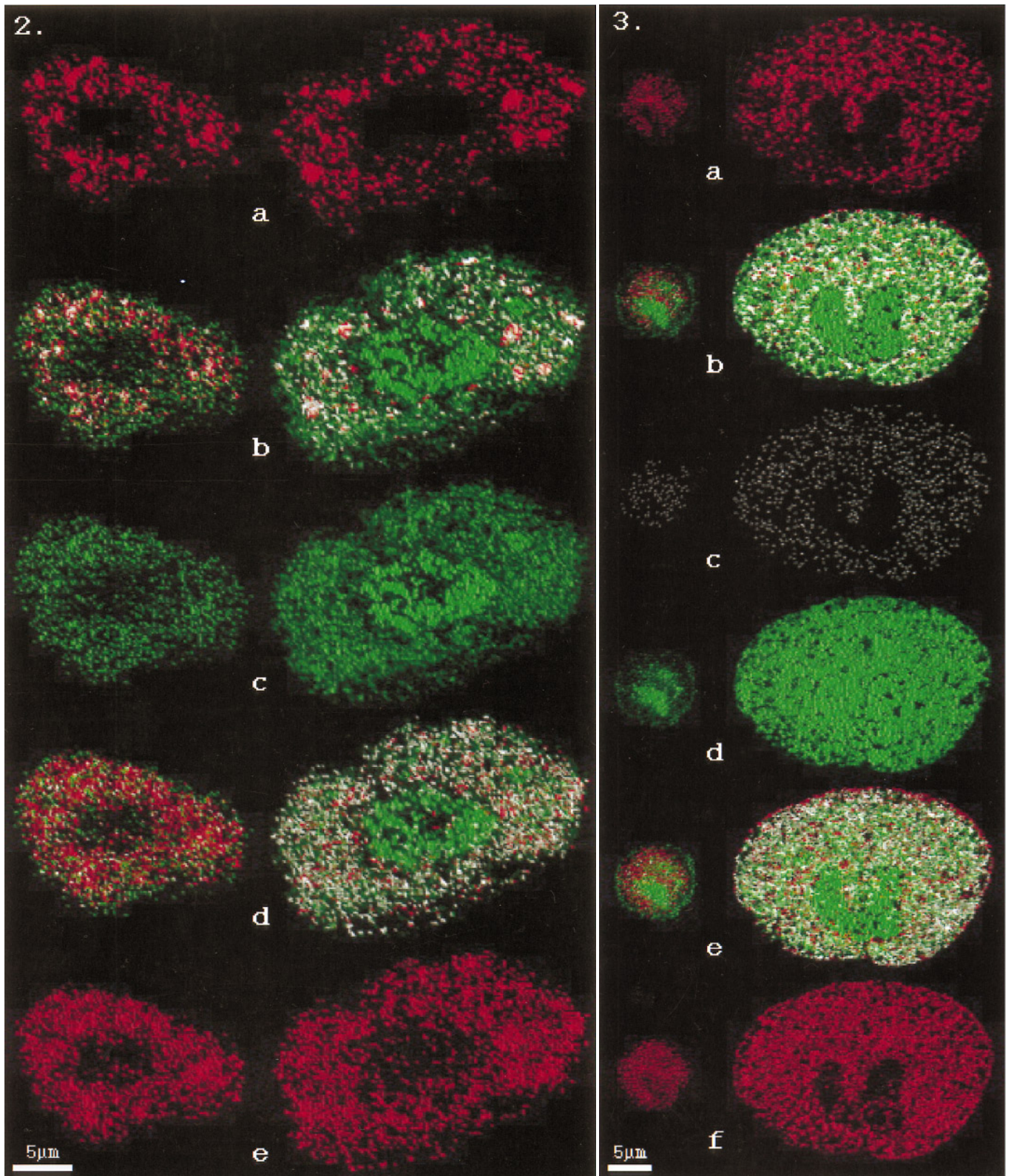


FIG. 2. BrUTP incorporation colocalized with antibodies to SC35. (Left column) Cells were microinjected with BrUTP and after 9.5 min, the cells were fixed and stained for immunofluorescence using an anti-BrdUTP antibody (green) and SC35 (red). The image was then captured and the SC35 signal above (a) and below (e) of the mean value was displayed. Zero voxels after thresholding were displayed as

(Fig. 1). Both probes colocalized in these structures, indicating that it was a region of higher relative concentration of poly(A), but only the directly labeled probe appeared to label throughout the nucleoplasm. This suggests that the small size of this probe confers an ability to penetrate the nucleoplasmic volume and report the hybridization directly without the need for accessibility to a second detector. In the cytoplasm, the streptavidin signal was stronger, since accessibility was apparently not an issue and the secondary reporter could amplify the hybridization signal.

The "Speckles" Represent a Minor Part of the Total Signal

An example of the importance of including low-level signal in the analysis is illustrated by the SC35 image. The genuine background was determined as described under Materials and Methods. The total signal in the nucleoplasm was determined by summing all voxels above this level. A number of thresholds were chosen for the distinction of the speckles; these showed that when the speckles were quantitated, they represented as low as 11% or, with a less stringent threshold, no more than 37% of the total SC35 signal in the nucleus, the average being 25% (see Fig. 2 as example). Cells varied with respect to the percent of SC35 signal in speckles. However, in all cases, the regions of high concentrations of SC35 represented the *minority* of the total brightness due to detection of this antigen in the nucleus. This indicated that the SC35 speckles were actually peaks of signal superimposed on a basal level of lower concentrations. The mean intensity of the signal in the speckles is approximately twice the mean signal of the total SC35 signal. The speckles, therefore, are regions of slightly higher relative concentration of SC35 than the surrounding nucleoplasm. This emphasizes that visual inspection of a sample emphasizes higher concentrations of signal at the expense of lower concentrations.

Most of the BrUTP Incorporation Does Not Colocalize with SC35 Signal

We segmented the SC35 image (Fig. 2) into those voxels below the mean intensity, after thresholding for noise (Fig. 2a; left, short times, and right, longer times of incorporation) or above the mean intensity (Fig. 2e; left and right), in order to analyze spatially how the brighter and dimmer signal from BrUTP or SC35 colocalized. The BrU signal, with 19.5 min of labeling (right column) did not colocalize to a large extent with the high-intensity (speckled) SC35 signal (<10%), although that which did was significant (ranking = 0). At early times of incorporation (9.5 min, left column) the BrU colocalization with speckles was somewhat higher (21%), with a high significance of colocalization (ranking = 0). The low-intensity, diffuse SC35 signals were also colocalized better with BrUTP at early times of labeling (15%) than at later times (<10%). The level of colocalization of all pairs, albeit low, was highly significant (ranking = 0), indicating that despite the low colocalization, it was higher than would be expected due to random chance. Therefore, we concluded that the majority of the sites of transcription were not preferentially colocalized with the SC35 signal.

Numbers of Sites of Transcription Vary per Nucleus

Cells were microinjected with BrUTP in order to view the incorporation of the analog into newly synthesized transcripts (e.g., Fig. 2). Signal was distributed throughout the entire nucleus up to the nuclear envelope (e.g., Fig. 5). Both the nucleolus and the nucleoplasm were labeled, indicating that at least polymerases I and II incorporated the analog into transcripts. There was no label detected in cells treated with high levels of actinomycin before microinjection (Fig. 4) or in cells microinjected with the analog BrATP (not shown). The density of the label in the nucleolus was greater than that in the nucleoplasm, presumably because the genes are much more concentrated. Analysis of the labeling showed that the BrU signal occupied significant

black. Colocalization (white pixels) with BrU was 23.0% (b) and 15.4% (d), respectively, above and below mean. Both represented the best colocalization of all possible translations (ranking = 0). The BrU alone is presented in (c). (Right column). The same as above, except that BrU labeling was for 19.5 min. (b) Colocalization of high SC35 signals is 5.1% of the BrU signal; ranking = 0. (e) Colocalization of low SC35 signal is 15.0% of the BrU signal; ranking = 0. The "speckles" (a) represent 31% of the total SC35 signal.

FIG. 3. Colocalization of BrUTP with poly(dT) probe. Cells microinjected with BrUTP were fixed and then hybridized with fluorescein-poly(dT) for poly(A) distribution. Cells were subjected to analysis as in Fig. 2 to test for the extent of association between the two labels. (Left column) BrU labeling was for 9.25 min. (a) Above mean poly(A) signal. (b) Colocalization of the high signal was 3.3% of the BrU signal in the nucleoplasm. Colocalization was the worst of all possibilities (ranking = 74). The sites of transcription as described under Materials and Methods were determined and represented (c). The number of sites in this nucleus thresholded at noise was 1029, among the lowest nuclei examined in transcriptional activity. (d) BrU alone. (e) Colocalization of the low signal was 4.9% of the BrU signal. (f) Below mean, dispersed poly(A) signal. (Right column) The same as above except that BrU labeling was for 43 min. (a) Above mean signal. (b) Colocalization with above mean signal was 11.0%, the best possible (ranking = 0). The number of sites of transcription (c) was 3888, the highest transcriptional activity of any nucleus examined. (d) BrU only. (e) Colocalization with below mean signal was 16.9%, the best possible (ranking = 0). (f) Below mean poly(A) signal.

volume within the nucleus; particularly with later times of labeling (Fig. 2C, right). Occasionally the labeling of both nucleolus and nucleoplasm was weak (Fig. 2, left). The BrU signal was restricted to objects defined by an algorithm that isolated foci of high intensity and surrounding voxels (see Materials and Methods). Using this analysis, it was found that the nuclei in various cells contained from a low of 849 sites (left) to a high of 3888 sites (right) of transcription (Fig. 3C). We interpret these results to indicate a range of transcriptional activities in cells. These average considerably less than the estimated number of transcribing genes in a fibroblast (about 20,000), and this suggests that either transcription units are clusters of genes [25] or that only about 10% of the genes are active over this period. The number of these sites changed somewhat with increased times of labeling, indicating that some new sites were being detected with increased labeling times. However, the number increased only about two- to threefold, not enough to account for the number of genes. These results tend to support the former hypothesis, that transcription sites represent many genes. There was no obvious indication that these transcripts were clustered into preferential areas of the nucleoplasm. There was no change in the number of sites with dilutions of the BrU to 15% with UTP. In contrast, within the nucleolus, some structure was evident (Fig. 3e, right). The dense core of the nucleolus showed a fibrillar pattern of transcription which has been suggested to be the sites of transcription of the ribosomal RNAs [26].

When the BrU sites were analyzed for their colocalization with SC35, the brightest 10% of these sites colocalized significantly (from 37 to 65%) with any SC35 signal, whereas the dimmest 10% of BrU sites did not colocalize with any SC35 to a significant degree (0–6.6%). The significance of these colocalizations was assessed by randomly distributing the same number of sites on the nucleus and measuring the colocalization with 100 random trials. The rankings indicated that the brightest BrU was the best possible colocalization of the 100 and the dimmest were the lowest possible colocalizations of this set.

Transcription Sites Do Not Colocalize Significantly with Poly(A)

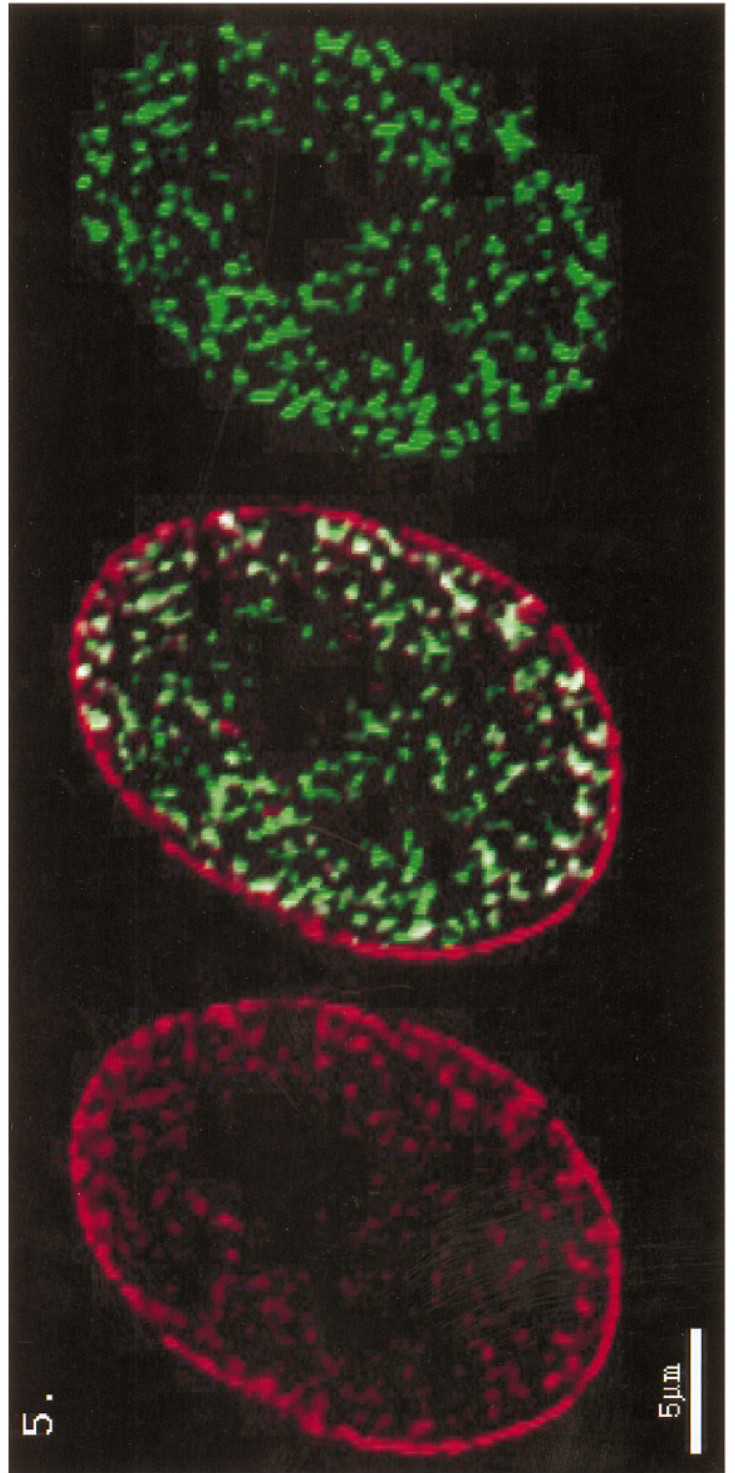
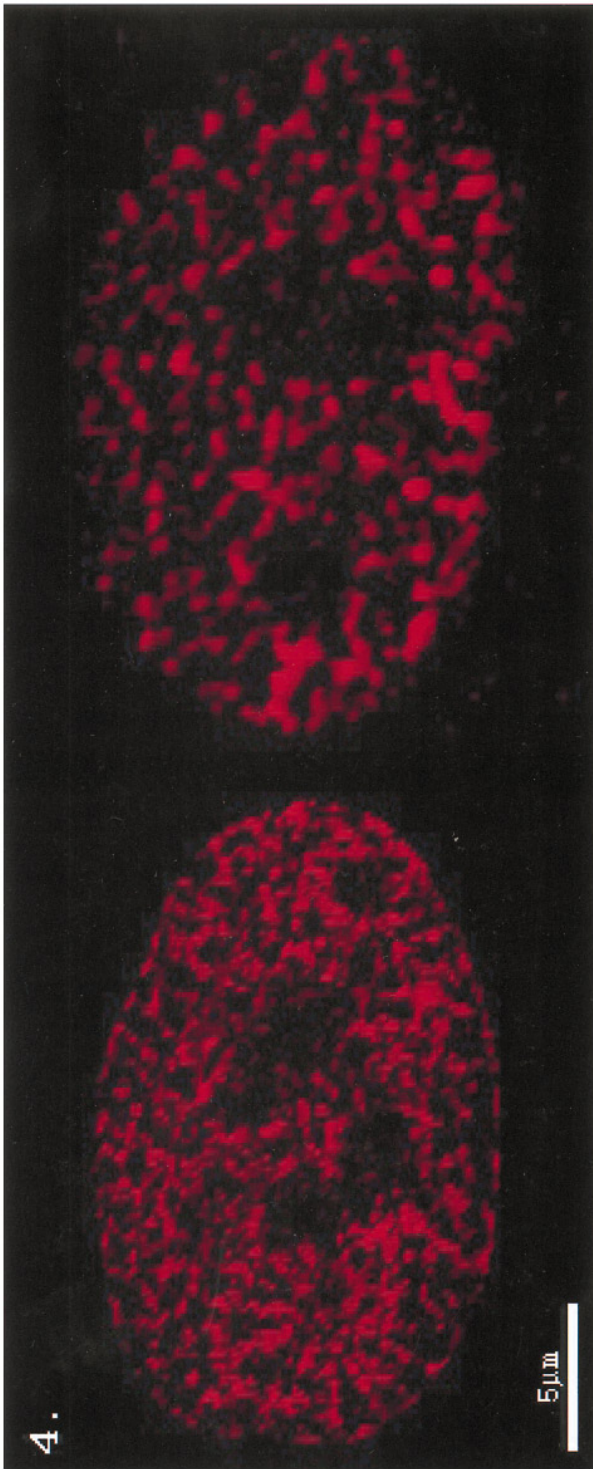
We investigated whether sites of transcription could be colocalized with sites of poly(A) concentrations. Therefore, BrUTP was microinjected into cells and its incorporation into newly synthesized transcripts was evaluated in cells where the poly(A) was simultaneously detected by hybridization to a fluorochrome-conjugated poly(dT). Figure 3 demonstrates the incorporation into the nucleoplasm and the nucleolus of the BrUTP and the steady-state distribution of poly(A).

Since the poly(A) was not present in the nucleolus, the pol I labeling in the nucleolus was readily evident when the images were superimposed. This allowed a threshold to be determined, as was done with SC35, and likewise a segmentation of low- and high-level signal. The BrU signal colocalized poorly with poly(A) over short times of labeling (9.25 min). The colocalization was slightly greater with the low-level signal (4.9%) than the high-level signal (3.3%). Over time, the BrU increased in colocalization to as high as 17% of the low-level signal after 18 min and 14% of the high-level signal. At all time points, the low-level signal localized better than the high-level signal. The colocalization ranking was very significant at both times, but it was the best possible of the translations at later times and the worst possible at early times (<18 min). The minimal ranking suggests a specific exclusion of nascent transcripts from all poly(A) locations. This exclusion may reflect the spatial separation of the polyadenylation from the transcription at early times, when most labeled transcripts have not yet been terminated. There was not a significant “chase” into the poly(A) pool (high or low) with time after microinjection. Like the SC35, the analysis indicates that a minority of the BrU colocalizes with either the diffuse or the higher level “patches” of poly(A). A rim of poly(A) at the nuclear envelope surrounded the signal from BrUTP.

Poly(A) Signal Nearest the Nuclear Envelope Appears to Be Exiting

A rim of poly(A) was evident when the BrU and poly(A) images were superimposed, as described by Huang *et al.* using electron microscopic methods [27]. This signal presumably represents a rate-limiting step in the transit of polyadenylated transcripts through the nuclear pores. In order to test this the cells were exposed to actinomycin for 2 h. In all cells, the rim of poly(A) signal disappeared from the nucleus, suggesting that these molecules had moved out of the cell (Fig. 4). The remainder of the signal derived from the hybridization to poly(A) appeared to coalesce. The amount of the signal did not change appreciably. This is in agreement with work using electron microscopy showing that α -amanatin- and DRB-treated cells retained poly(A) in nuclear substructures, but the signal was lost around the envelope. [27]. In addition, the effect of transcriptional inhibitors has been shown to cause a coalescence of SC35 signal [4].

Some of the BrU signal after labeling for an hour colocalized with nuclear pore antibodies near the periphery. The distribution of the rim of poly(A) (Fig. 4) resembled that of the nuclear pore antibodies and further suggested that this signal was in the process of export. Some of the transcripts near the rim colocalized with these antibodies; possibly they were in the export process (Fig. 5).



DISCUSSION

This work reports the use of objective criteria for the analysis of *in situ* hybridization and immunocytochemical data obtained from digital images. The important feature of this approach was to establish the *total signal* above background rather than focus on the brightest voxels in the image. The voxels of low light level contain considerable information, but because they are less bright, they are easily ignored when using less objective criteria. However, for the SC35 and, to some extent, the poly(A) probes, it was this low-level diffuse portion of the signal which colocalized better with the newly synthesized transcripts. The brighter, more concentrated speckles were not coincident, to a large extent, with transcription (<10%), and this low level of coincidence could not be improved by translating the images in three dimensions; hence, it is not simply a random association. Therefore, this evidence appears to support the conclusion that the bulk of transcription does not occur in the presence of detectable concentrations of these factors. In the case of SC35, it is consistent with the view that the speckles mainly represent storage or recycling sites for the splicing machinery, in equilibrium with its usage throughout the nucleoplasm [16]. In the case of poly(A) it is consistent with the view that polyadenylation takes place throughout the nucleoplasm, at diffuse sites where pol II [28] and factors involved in polyadenylation [29, 30] are found. No bulk movement of transcribed RNA was detected relative to any of the other factors.

Compartmentalization of RNA transcription and processing has been a subject of considerable interest. It has been suggested that the nucleus may be spatially organized in a way which suggests a functional organization. Structural organization in the nucleus is best exemplified by the nucleolus, where specific transcription of ribosomal RNAs via pol I is sequestered and where assembly and transport of pre-ribosomal particles occurs. In contrast, pol II transcription is much more diffuse throughout the nucleoplasm, and specialized structures, if they exist, for mRNA transcription, assembly with splicing factors, hnRNPs, or transport complexes are much less obvious microscopically. Therefore, examination of pol II transcription has been

correlated with factors known to be involved in this process; the splicing factor, SC35, and the poly(A). However, the transcription of the whole class of active pol II genes does not appear to be correlated with high concentrations of either of these markers. Consistent with this, previous work has shown that the *entire* nucleoplasm, not specifically areas near speckles, was capable of supporting both splicing and transcription of adenovirus which infected the nucleus in a spatially random distribution. Nor were the actin genes spatially correlated with high concentrations of SC35. [9]. However, other evidence suggests a correlation of active genes with speckles [6, 7]. There may be a quantitative explanation for these disparate observations. Specific genes which have exceptionally high levels of splicing, or many exons (e.g., collagen, [6, 7]), which have high transcriptional activity, such as occurs during viral infection, or transfections of multiple copies of plasmids would be expected to recruit proportionately higher levels of factors involved in these activities [9, 16, 30, 31]. The higher concentrations of (e.g.) SC35 at active transcription and processing sites could make them brighter by immunofluorescence and hence create the appearance of speckles. We emphasize that the areas of high concentrations of SC35, i.e., the speckles, average only *twice the intensity* of the mean signal. Therefore, an increase in only twofold of SC35 concentration could result in a region being perceived as a speckle.

The colocalization of BrUTP with SC35 appeared to decrease with time. The newly labeled and brightest transcripts (above the mean intensity) had a higher colocalization with high concentrations of SC35 which decreased after 18 min of transcription. This indicated that, at early times, higher levels of transcription were more likely to be associated with higher levels of SC35, supporting the above argument that high levels of transcription or processing would be more likely to result in detection. Conversely, lower levels of transcription (i.e., voxels with less BrU intensity) were associated with lower SC35 signal. When BrU sites were isolated and treated as single point sources, the correlation was even more dramatic; sites with high BrU intensity were much more likely to be associated with SC35 than sites with low BrU intensity. This further supports, mathe-

FIG. 4. Poly(A) distribution with or without actinomycin treatment. Cells were microinjected with BrUTP without (left) or with (right) actinomycin treatment, and the BrU was detected with antibodies and poly(A) by *in situ* hybridization. Since there was no incorporation of BrUTP (right), because transcription was suppressed, only the steady-state poly(A) is represented here. Note the disappearance of poly(A) from the outer rim of the nucleus and the coalescence of the remainder of poly(A) in the nucleoplasm.

FIG. 5. BrUTP incorporation colocalized with nuclear envelope antibodies. Cells were labeled for 60 min after microinjection of BrU (right) and then an antibody (left) was used to delineate the nuclear envelope. The transcripts showed little colocalization with nuclear pore antibodies (middle).

matically, the correlation of activity of transcription with the *detectable* presence of the splicing factor.

This relationship of intensities did not appear to exist with poly(A). In fact, it appeared to be the reverse; high levels of BrU signal were more highly colocalized with low-level poly(A) (22%, ranking = 0), consistent with the view that some of this signal may represent polyadenylation. However, most of the BrU signal didn't colocalize with poly(A). In fact, the bulk of the poly(A) did not even appear to represent transcripts destined for the cytoplasm, since an actinomycin "chase" did not eliminate much signal [see also 27]. However, a small amount of poly(A) was seen to disappear from the nuclear periphery, presumably representing the population of molecules in the process of export. The significance of the bulk of the poly(A) in the nucleus is unclear, but it may represent the slow turning-over population of nuclear RNA reported in a number of works [32–34].

Longer times of labeling using BrUTP resulted in the death of the cells after 12–24 h. Interestingly, the use of BrATP did not kill the cell, suggesting that brominated poly(A) could be tolerated by the cell. When cells were labeled for several hours, the labeled RNA could not be seen moving into the cytoplasm. This would suggest that BrUTP incorporated into the RNA resulted in inhibition of some aspect of processing and/or export. One possibility may be the polypyrimidine tracts necessary for splicing, which contain polyuridylylate oligomeric sequences which substantially increase the probability of incorporating at least one BrUTP, and this may be sufficient for inhibition [35]. It is likely, therefore, that the transcripts labeled with BrUTP cannot be processed properly. Previous work has shown that correct processing is required for export [36]. This could explain why the BrUTP-labeled transcripts were not exported. However, dilution to 15% with UTP restored the ability of the transcripts to splice [35], but did not result in the export of the RNA. Likewise, the lack of colocalization with SC35 and/or poly(A) could have resulted from inhibition of the splicing reaction, but this lack of colocalization was unchanged with dilution of the BrU described above (data not shown).

The fact the BrUTP incorporation did not colocalize with concentrated areas containing poly(A) and SC35 would support the conclusion that processing of RNA occurred throughout the nucleoplasm at the site of transcription [37]. This suggests further that a system, possibly diffusion, may operate to move factors necessary for processing of RNA to the sites of transcription of specific genes. Other examples exist for this in the case of splicing snRNPs [38], microinjected RNA [39], an intron containing RNA in *Drosophila* [40], a large transcript in *Chironomus* [41], and mRNAs preceding from the site of transcription [42].

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