

Reduced Mobility of the Alternate Splicing Factor (ASF) through the Nucleoplasm and Steady State Speckle Compartments[Ⓞ]

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Abstract. Compartmentalization of the nucleus is now recognized as an important level of regulation influencing specific nuclear processes. The mechanism of factor organization and the movement of factors in nuclear space have not been fully determined. Splicing factors, for example, have been shown to move in a directed manner as large intact structures from sites of concentration to sites of active transcription, but splicing factors are also thought to exist in a freely diffusible state. In this study, we examined the movement of a splicing factor, ASF, green fluorescent fusion protein (ASF-GFP) using time-lapse microscopy and the technique fluorescence recovery after photobleaching (FRAP). We find that ASF-GFP moves at rates up to 100 times

slower than free diffusion when it is associated with speckles and, surprisingly, also when it is dispersed in the nucleoplasm. The mobility of ASF is consistent with frequent but transient interactions with relatively immobile nuclear binding sites. This mobility is slightly increased in the presence of an RNA polymerase II transcription inhibitor and the ASF molecules further enrich in speckles. We propose that the nonrandom organization of splicing factors reflects spatial differences in the concentration of relatively immobile binding sites.

Key words: ASF/SF2 • IGCs • FRAP • cell nucleus • nuclear matrix

Introduction

It is well established that the cell nucleus is spatially organized through compartmentalization of functionally related biomolecules (Carmo-Fonseca et al., 1996; de Jong et al., 1996; Jackson, 1997; Lamond and Earnshaw, 1998; Misteli and Spector, 1998; Sleeman and Lamond, 1999; Wei et al., 1999; Misteli, 2000). Perhaps the most striking example of this organization is the distribution of splicing factors that participate in pre-mRNA metabolism (Spector, 1993). Splicing factors are highly enriched in nuclear domains referred to as speckles. The basis for compartmentalization of splicing factors into nuclear domains, such as speckles, has not been determined. One possibility is that specific nuclear structures contain binding sites for factors and that these structures are physically immobilized by a structural framework or karyoskeleton.

Although a subject of widespread investigation, the acceptance of such a framework has suffered from the inability of years of ultrastructural research to reveal this nuclear component without prior elution of chromatin (Pederson, 1998). Recently, we used elemental imaging to detect a protein-based framework that has an organization consistent with an architectural role in the cell nucleus (Hendzel et al., 1999). This karyoskeleton was particularly evident within nuclear speckles (Hendzel et al., 1998, 1999).

The potential implications of a structural basis to nuclear compartmentalization have arisen in recent studies. The observation that small portions of speckles appear to break away from the larger domain and move to sites of newly transcribing genes (Misteli et al., 1997, 1998) implies that the karyoskeleton can be dynamically remodeled and provides a basis for directed transport of macromolecular assemblies. This movement of splicing factor assemblies was found to be dependent on the phosphorylation state and transcriptional activity of the nucleus (Misteli et al., 1997), suggesting that the movement of at least a subpopulation of splicing factors is regulated. These observations

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were made using alternative splicing factor (ASF)¹-green fluorescence protein (GFP) fusion proteins as a probe to monitor the dynamics of ASF-enriched structures. Direct microscopical visualization of GFP fusion proteins in living cells is useful for studying the movement of proteins on a macro scale. In addition to directed movement of intact factor-rich structures, it has been speculated that a substantial pool of splicing factors, observed as a dispersed nuclear population outside of speckles (Fay et al., 1997), is freely mobile within the nucleoplasm (Singer and Green, 1997). Fluorescence recovery after photobleaching (FRAP)-based studies are well suited to directly determine how splicing factors move through the cell nucleus.

We set out to determine whether the movement of splicing factors throughout the nucleus occurs mainly due to a directed transport of macromolecular assemblies, or whether significant quantities are able to freely diffuse. In this study, we used three-dimensional (3-D) time-lapse (4-D) fluorescence deconvolution microscopy to confirm the movement of ASF-enriched structures described by Misteli et al. (1997). ASF-GFP (Sleeman et al., 1998) displays the well-characterized speckled nuclear organization observed for other splicing factors, such as SC-35. We compared the movement of ASF-enriched domains to domains formed by the overexpression of histone deacetylase 4 (HDAC4). We demonstrate that the movement of ASF-enriched domains, unlike HDAC4, does not conform to structures that are diffusing but confined to an interchromatin channel network (Cremer et al., 1993; Zirbel et al., 1993).

After the determination of structure mobility, we then evaluated molecular mobility. We used the GFP protein to define the mobility of a protein capable of moving relatively freely throughout the nuclear space. Surprisingly, the mobility of ASF into and out of speckles is virtually identical to the mobility of ASF in the nucleoplasm. Importantly, we find the mobility of ASF to be many times slower than free diffusion. The redistribution of ASF into photobleached areas is not dependent upon the reported translocation of ASF-enriched domains to and from nuclear speckles. We found that under conditions where the translocation of microdomains enriched in ASF has ceased, the ASF-GFP recovers with similar kinetics. A slight increase in mobility in both the nucleoplasm and in nuclear speckles was observed under conditions of RNA polymerase II transcriptional inhibition.

Materials and Methods

Cell Culture and Transfections

Mouse 10T1/2 cells, human intestinal smooth muscle cells, human lung cancer cells, human neuroblastoma cells, or Indian muntjac fibroblasts were plated onto glass coverslips and cultured 1–2 d in growth medium until 50% confluent. At this point, complete medium was removed and cells were transfected using lipofectamine (GIBCO BRL) and an ASF-

GFP fusion protein construct described previously (Sleeman et al., 1998). After transfection, cells were cultured in complete medium for ~24 h before imaging. In some cases, staurosporine (Sigma-Aldrich) or 5,6-dichlorobenzimidazole riboside (DRB; Sigma-Aldrich) were added at 100 µg/ml and 75 µg/ml, respectively, between 2 and 4 h before imaging.

Live Cell Imaging

Coverslips were placed on glass slides containing several drops of media surrounded by vacuum grease. The vacuum grease allows an airtight seal to form. Cells are capable of growing in these conditions for at least 24 h at 22°C. For 4-D imaging, a Zeiss AxioPlan II microscope, a 100× 1.4 NA lens, and a 12-bit cooled CCD (Cooke SensiCam) were used to collect images. Images were further processed by digital deconvolution using AutoQuant Autodeblur. For FRAP, the laser scanning microscope (Zeiss LSM 510) was set to laser scanning mode and the initial imaging conditions were determined. A 25× 0.8 NA lens was used for these experiments and pixel sampling was set between 90 and 120 nm/pixel. The argon laser spectral line at a wavelength of 488 nm was set to an intensity of no greater than 1.25% of its total power (15 mW) for image collection. A mask, which typically covered half of the cell nucleus, was then photobleached using 100 iterations at 3.75 mW laser power. 12-bit images were collected before, immediately after, and at defined intervals after bleaching. Our total experiment time was set to 500 s, which was sufficient for complete recovery from photobleaching.

For quantitative imaging, a 3-µm wide strip of the cell was photobleached and then scanned during recovery. Photobleaching was completed in ~2 s. Similar proportions of GFP (~25%) and ASF-GFP (~30%) total nuclear fluorescence were lost during photobleaching. Because the GFP protein recovered very rapidly, it was necessary to only scan the photobleached region in order to adequately sample GFP recovery. For simplicity in the graphical presentation of the data, the initial post-bleach value of the photobleached region was normalized to zero by subtraction and the maximal value obtained during recovery was normalized to 100. The $t_{1/2}$ of recovery can then be directly read off of the graph as the 50% value. Identical results were obtained when 2-µm spots, rather than strips, were bleached. To determine the immobile population of ASF-GFP, the maximal recovered value was divided by the starting value of the photobleached region after correction for total nuclear fluorescence lost during photobleaching. This correction factor was determined by measuring the total nuclear fluorescence immediately after photobleaching and dividing this value by the total nuclear fluorescence immediately before bleaching. The laser intensity during recovery was set so that no measurable loss of fluorescence was observed during the monitoring period.

To track the mobility of HDAC4-GFP domains and small foci enriched in ASF-GFP we used the track points function in Universal Imaging's MetaMorph 4.0 imaging software. The image sequences were viewed on screen at 200% magnification and the center of each focus was identified with a mouse. For HDAC4 domains, the structures sometimes moved out of the plane of focus. In this instance, the foci could still be followed by identifying slightly elevated regions of signal intensity. Although difficult to identify in single frames, these could be easily identified by rapidly moving between the several frames where the domains move into and out of focus. This occurred for two of the five foci tracked. These foci, however, remained in focus for most of the time course.

Photobleaching and Cell Viability

Photobleaching experiments have an undeserved reputation of being photodestructive to living cells. Excessive photodamage, if it occurred, would complicate collection and interpretation of FRAP-based experimental data. However, bleaching GFP using the monochromatic laser at low intensities needed for bleaching and image acquisition, does not noticeably perturb live cells (reviewed White and Stelzer, 1999). After repeated bleaching, cells expressing GFP-tagged trafficking proteins (Nakata et al., 1998) and centrosome proteins (Khodjakov and Rieder, 1999) have remained viable for periods of hours, even to continue through mitosis, an event noted for its sensitivity to cell toxicity. As for cells remaining viable for periods long enough to complete photobleaching experiments, the conditions we have used are sufficient for cells to grow for >24 h and to undergo mitosis (our unpublished observations). To assess that cells remain viable during the initial photobleaching and recovery period, we monitored cells by differential interference contrast (DIC) optics for changes in cellular morphology as well as the maintenance of cytoplasmic organelle transport and the absence of detectable changes in mitochondrial structure (e.g.,

¹Abbreviations used in this paper: 2-, 3-, and 4-D, two-, three-, and four-dimensional; ASF, alternative splicing factor; DIC, differential interference contrast; FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent fusion protein; HDAC4, histone deacetylase 4.

swelling). Therefore, we are confident that our experimental conditions and/or design does not erroneously influence the collection of FRAP or fluorescence loss in photobleaching (FLIP)-based data.

Online Supplemental Material

Video 1 further depicts Fig. 1. A cell expressing ASF-GFP is shown in 3-D red/green stereo time-lapse. Individual frames were captured at 2-min intervals.

Videos 2 and 3 further depict Fig. 2. A cell expressing an HDAC4-GFP fusion protein (video 2) or ASF-GFP (video 3) was captured as a 2-D time-lapse experiment at 1-s intervals for 60 s. These videos are available at <http://www.jcb.org/cgi/content/full/150/1/41/DC1>.

Results

4-D Dynamics of the ASF Splicing Factor in Living Cells

Previous observations using the ASF-GFP fusion protein clearly indicate that nuclear speckles are structurally dynamic subnuclear domains (Misteli and Spector, 1996; Misteli et al., 1997, 1998). We used this probe and 4-D microscopy to examine more closely the spatiotemporal dynamics of nuclear speckles. Consistent with Misteli et al. (1997), we find that speckles change shape over time and subdomains are capable of both blebbing and fusing with individual speckles (Fig. 1). Examples of smaller domains budding from speckles (small arrows) and speckles changing shape (large arrows) are shown in red/green stereo pairs. Numerous other examples can be seen by closely examining video 1 (available at <http://www.jcb.org/cgi/content/full/150/1/41/DC1>).

[org/cgi/content/full/150/1/41/DC1](http://www.jcb.org/cgi/content/full/150/1/41/DC1)). We confirm that nuclear speckles undergo considerable structural dynamics during the course of an intermediate time frame of 60 min, but remain relatively positionally stable within the nuclear space.

Large Nuclear Domains Are Capable of Random Motion within the Cell Nucleus

The movement of nuclear structures as large as those that originate and migrate away from nuclear speckles could be defined by macromolecular crowding in the nucleoplasm. In particular, chromosomal territories have been demonstrated to occupy, collectively, a considerable portion of the nuclear volume (Cremer et al., 1993; Zirbel et al., 1993). Therefore, it has been proposed that chromatin barriers may prevent the diffusion of large complexes (Cremer et al., 1993). This model of nuclear compartmentalization is referred to as the interchromosomal channel network and is postulated to be an environment that is enriched in pre-mRNA processing factors due to an exclusion from chromosomal domains. If this model held, larger structures would not be capable of significant intranuclear mobility, which would explain the slow and apparently directed movement of splicing factors in structures derived from nuclear speckles. To differentiate between passive diffusion through a restricted space and a more directed process, it is necessary to have a control for the potential mobility of large structures in the nucleoplasm. During the course of unrelated experiments, we have been examining

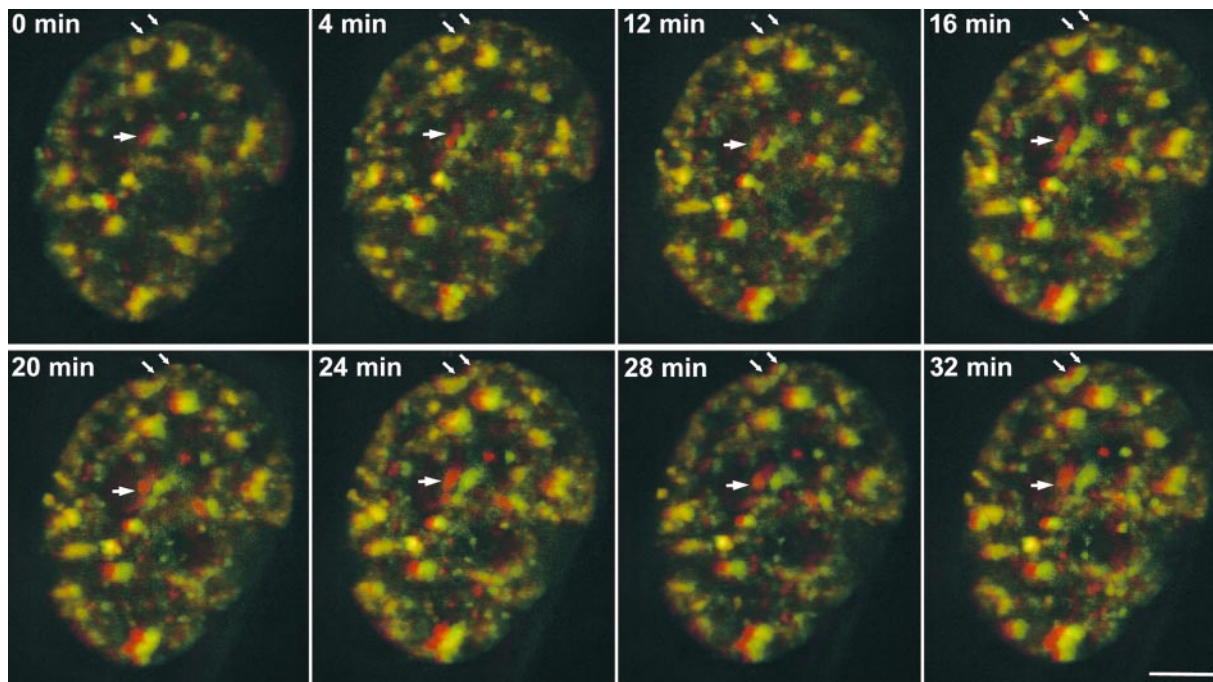


Figure 1. 3-D organization of ASF within living cells. A mouse 10T1/2 fibroblast cell line was transfected with an ASF-GFP fusion protein. An expressing cell was examined by deconvolution microscopy and 3-D reconstruction through time. Z series were collected at 2-min intervals for 60 min. Individual time frames are shown as red and green stereo pairs. The small arrow indicates the position of a smaller ASF-enriched particle budding and moving away from a speckle. The large arrow indicates the position of a speckle observed to unfold into a fiber and then refold back into a speckle. Supplemental material available at <http://www.jcb.org/cgi/content/full/150/1/41/DC1>. Bar, 5 μ m.

the localization of histone deacetylase 4 (HDAC4). Like many GFP- and FLAG-tagged constructs (e.g., Htun et al., 1996; Fejes-Toth et al., 1998; Fischle et al., 1999) containing components of the transcriptional machinery, FLAG-tagged HDAC4 localizes to several hundred small nuclear foci located throughout the nucleoplasm. HDAC4-GFP, when transiently expressed in cells, does not show evidence of this focal organization. In most cells, HDAC4-GFP is diffusely distributed. In a small number of cells, however, spherical structures highly enriched in HDAC4-GFP are formed. They are present in either the cytoplasm or the nucleus and, in some cells, are found in both compartments. These structures are also variable in size, particularly between individual cells. They are visible using DIC optics and are not evident in nontransfected cells. Most importantly, movement of these structures, regardless of size, was evident by direct observation in the microscope. This suggested that these structures, although probably nonphysiological, could serve as a reference for the potential mobility of larger complexes within the cell nucleus.

A complete characterization of the relationship between the size of the structure and its mobility characteristics will be presented elsewhere. At this point, we wished to simply address the issue as to whether structures in the size range of the buds derived from nuclear speckles are capable of rapid and random motion in the cell. To address this, cells expressing nuclear HDAC4-GFP complexes of ~ 500 nm diameter were imaged by time-lapse microscopy. Exposures were taken every second for 60 s. The results are shown in Fig. 2 and are also presented in supplemental materials online as video 2 (available at <http://www.jcb.org/cgi/content/full/150/1/41/DC1>). Fig. 2 shows an individual frame that demonstrates the presence of small spherical domains in a cell transiently expressing HDAC4-GFP (top left panel). The top right panel shows the tracks followed by five individual domains over a 60-s time course. The middle panel illustrates the individual domains tracked. This is also illustrated in video 2. The largest distance traveled by HDAC4 in this cell was ~ 1.0 μm in 1 s. This occurred in the right uppermost nuclear focus between frames 3 and 4 (video 2). Some foci move in

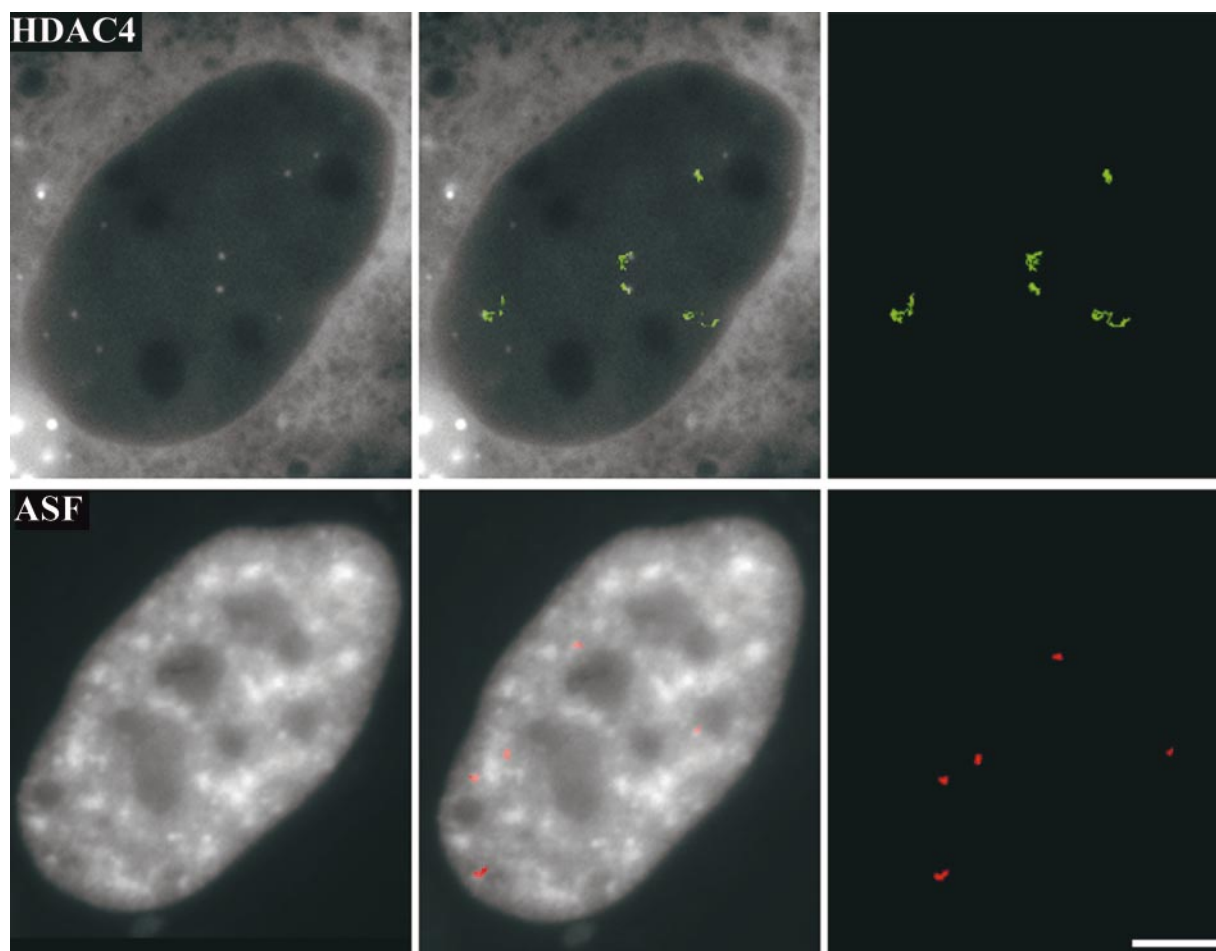


Figure 2. Mobility of an HDAC4-GFP-enriched structure. (Top) A mouse 10T1/2 cell was transfected with a hybrid protein containing an NH₂-terminal fusion of GFP to HDAC4. A cell expressing nuclear HDAC4-enriched domains was analyzed by time-lapse microscopy. The first panel shows a single time point. The middle panel shows a superimposition of the movement track onto the image. The third panel shows the movement track over the 60-s time course. (Bottom) A mouse 10T1/2 cell was transfected with the ASF-GFP fusion protein. The first panel shows a single time point. The middle panel shows a superimposition of the movement track onto the image. The third panel shows the movement track over the 60-s time course. Supplemental material available at <http://www.jcb.org/cgi/content/full/150/1/41/DC1>. Bar, 5 μm .

and out of the plane of focus during the time course. We suspect that the observed movement reflects the probability of a collision, although the establishment of large compartments with physical barriers to movement of large structures cannot be ruled out. For comparison, an identical time course and process was carried out with a cell expressing ASF-GFP (Fig. 2, video 3). The motion observed for individual ASF foci is considerably more restricted over this very short time course than is observed for HDAC4 domains of similar size. This indicates that the slow movement of ASF foci in the longer time course shown in Fig. 1 and video 1 does not represent spatial averaging of undersampled more rapid movement. In addition, the movement of ASF subdomains is distinct from the rapid motion potential realized by HDAC4 domains of similar size.

FRAP Analysis of ASF-GFP Movement

We have confirmed the dynamic properties of nuclear speckles and splicing factor-enriched structures that fuse or bleb from these domains. By using an HDAC4 construct with novel expression properties, we have clearly shown that the movement of the speckle domain buds is distinct from random motion. Although structures as large as speckles are physically limited in their abilities to migrate through the nucleoplasm (Hendzel, M.J., and D. MacDonald, manuscript in preparation), structures in the size range of the smaller splicing factor-enriched domains (e.g., HDAC4 foci) are capable of rapid and random motion. The mobility of these structures, when compared with ASF, indicates that the smaller ASF-enriched domains are not moving freely through nuclear space and slowed only by their high molecular mass. Since it has been demonstrated that these splicing factor subdomains can be recruited to sites of transcription, and, hence, may function in the efficient processing of pre-mRNAs, it is important to understand the mobility properties of the pools of molecules associated with these structures.

To do this, we characterized the movement of the ASF protein, which is present both within and outside of nuclear speckles, using FRAP-based experiments. As a control, we characterized the fluorescence recovery rate of GFP in the absence of the ASF protein. The top two panels of Fig. 3 show a photobleaching experiment performed on an Indian muntjac fibroblast cell expressing GFP. The first image was taken before photobleaching and the second image was taken within 1 s of the completion of photobleaching. Under conditions in which a photobleached region is easily generated in the ASF-GFP expressing cell (bottom panels), the 3- μm photobleached strip through the cell was not evident in the GFP expressing cell. This is because the image scan time, ~ 1 s, is large relative to the rapid mobility of the freely diffusing GFP protein. The partial equilibration of the GFP protein during scanning results in the observed decrease in fluorescence intensity throughout the entire cell. This is particularly evident in the nucleus and is consistent with the free mobility of the bulk of the pool of GFP within the cell. The bottom panels show the photobleaching of a 3- μm strip through an ASF-GFP expressing cell. The photobleached region remains quite evident even at the 10-s time point. Complete recovery

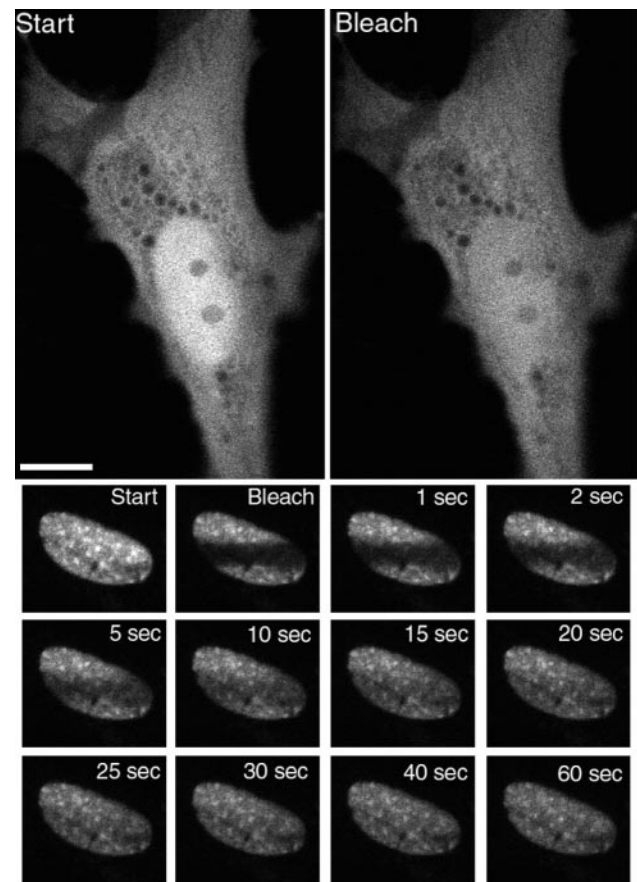


Figure 3. Fluorescence recovery after photobleaching of the ASF-GFP factor in living cells. Indian muntjac fibroblast cells were transfected with a GFP expression vector (top panels) or an ASF-GFP expression vector. A 3- μm line was bleached across the width of the cell nucleus and images were collected during the course of recovery. Bar, 5 μm .

is not observed until at least 30 s. The immobile fraction of ASF-GFP was calculated for both human intestinal smooth muscle and mouse 10T1/2 fibroblasts. There were no differences between cell types. The mean immobile population was calculated to be $6.7 \pm 4.7\%$. Control experiments, in which the entire nuclear fluorescence was bleached, demonstrated that de novo synthesis, import of a cytoplasmic pool, or refolding of the GFP molecule was not a significant contributor to fluorescence recovery (data not shown).

To quantitatively assess how much slower the ASF protein migrated through the cell nucleus compared with the freely mobile GFP protein, we performed experiments in which a 3- μm strip extending through the cell was scanned at rapid intervals. This enabled us to collect images every 50 ms. This was necessary because under conditions in which a clear bleach zone is observed with ASF-GFP, the GFP protein infiltrates the photobleached region sufficiently fast that only a gradient of intensity is observed and recovery is nearly complete during the time required to acquire a single frame (~ 1 -s scan time). A plot of the recovery of the GFP protein is shown in Fig. 4. The estimated $t_{1/2}$ of recovery is <300 ms. Fig. 4 also shows the same experi-

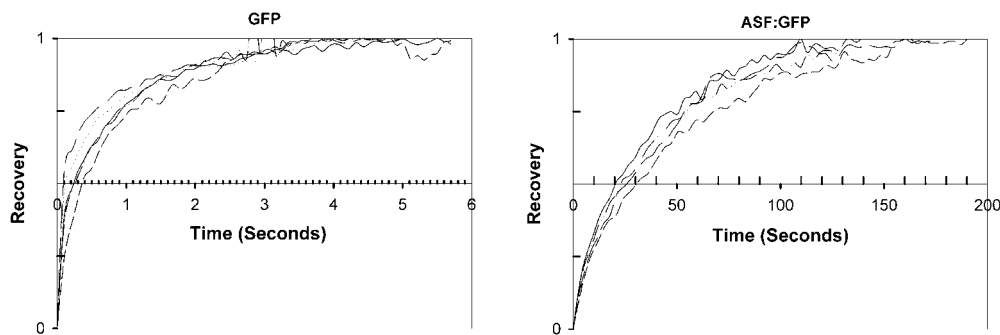


Figure 4. A comparison of recovery rates of GFP and ASF-GFP during FRAP. Human A549 cells were transfected with either a GFP expression vector or an ASF-GFP expression vector. A 3- μ m line was bleached across the width of the cell nucleus and images were collected during the course of recovery. The first image collected after photobleaching

was measured for intensity in the photobleached region and this value was normalized to zero and the maximal intensity during recovery was normalized to 1. The individual time points were then plotted.

ment performed on an ASF-GFP expressing cell. The estimated $t_{1/2}$ is ~ 20 s. The mean recovery time of several cells from four different cell lines is summarized in Table I. Differences were observed between cell lines in the mobilities of both GFP and ASF-GFP and there was a significant amount of variability in the recovery rates between individual cells. This is reflected in the high standard deviation and may reflect variability in expression levels. Cells expressing very low levels of ASF-GFP are difficult to image and so the data set was biased towards cells expressing intermediate or higher levels of ASF-GFP. However, only cells expressing the normal speckled ASF distribution were imaged and, although there was a tendency for the highest expressing cells to recover faster than the lower expressing cells, this relationship was not absolute. Additionally, the level of expression could not be used to predict the amount of free ASF-GFP. If we use the amount of fluorescence lost during the photobleach period from nucleoplasmic regions distant from the bleaching site as an estimate of the amount of free ASF-GFP, fluorescence loss was measured at between 0 and 25.5% with a mean of 10.9% and a standard deviation of 8.4%. This value did not correlate with expression level of the transiently expressed protein. Out of the entire data set, only one cell showed a recovery time < 10 -fold slower than GFP. It is clear from these results that the majority of the ASF-GFP population does not behave as a freely mobile molecule.

Movement of Splicing Factors During Transcription or Kinase Inhibition

Transcriptional inhibition causes speckles to reorganize,

Table I. Quantitative Analysis of Recovery Times of ASF-GFP vs. GFP in Different Cell Lines

Cell type	Protein	$t_{1/2}$ recovery	Fold difference vs. GFP
A549 (human)	ASF-GFP	25.0 ± 4.4 ($n = 4$)	119
	GFP	0.21 ± 0.11 ($n = 5$)	1.0
HeLa (human)	ASF-GFP	18.7 ± 3.1 ($n = 5$)	62.3
	GFP	0.30 ± 0.1 ($n = 5$)	1.0
HISM (human)	ASF-GFP	10.3 ± 4.2 ($n = 22$)	29.4
	GFP	0.35 ± 0.1 ($n = 5$)	1.0
10T1/2 (mouse)	ASF-GFP	14.7 ± 7.5 ($n = 26$)	39.7
	GFP	0.37 ± 0.1 ($n = 5$)	1.0

becoming both larger and more spherical (Henzel and Bazett-Jones, 1995). This correlates with a decrease in the structural dynamics of speckles and the cessation of budding and transport of smaller domains derived from speckles (Misteli et al., 1997). This reorganization may be dependent upon protein phosphorylation since treatment of cells with the protein kinase inhibitor staurosporine produces a similar pattern and inhibits speckle dynamics (Misteli et al., 1997). Therefore, we asked whether inhibiting transcription or ongoing phosphorylation would affect the mobility of the ASF. To address this, we performed FRAP experiments on cells that had been treated with the RNA polymerase II transcription inhibitor, DRB, or the kinase inhibitor staurosporine. As expected, the transcriptionally inhibited cells show a reorganization of the nuclear speckles and ASF-GFP distribution (Fig. 5 C). Speckles become considerably larger, fewer in number, and more spherical. We observed the fluorescence signal recovery reached equilibrium ~ 5 min after bleaching, which was similar to results obtained with cells treated with staurosporine (Fig. 5 D). We found that the ASF-GFP moves into and out of speckles and between individual speckles with kinetics that are similar to untreated control cells expressing ASF-GFP (Fig. 5 B), but is considerably slower than GFP itself (Fig. 5 A). These drugs do, however, affect the mobility of some nuclear proteins. Histone H1 mobility, for example, is dramatically reduced by treatment with either drug (Lever, M.A., J.P.H. Th'ng, X. Sun, and M.J. Henzel, manuscript in preparation). Indicating that although the structural dynamics of speckles and the subnuclear distribution of ASF are dependent upon protein phosphorylation or ongoing RNA polymerase II transcription, the overall mobility of ASF is not.

Quantitative Analysis of Fluorescence Recovery in Nuclear Speckles and the Nucleoplasm

Although we cannot resolve individual molecules of ASF, we can detect and measure their mean mobility. If ASF maintained high-affinity associations in speckles and the population of dispersed ASF molecules was freely diffusible, then we would expect a rapid uniform recovery of the nucleoplasm followed by the slower recovery of speckles. Instead, we observe a progressive front of GFP signal recovery that moves gradually toward the pole of the bleached half of the nucleus (Fig. 5 B) with signal recovery

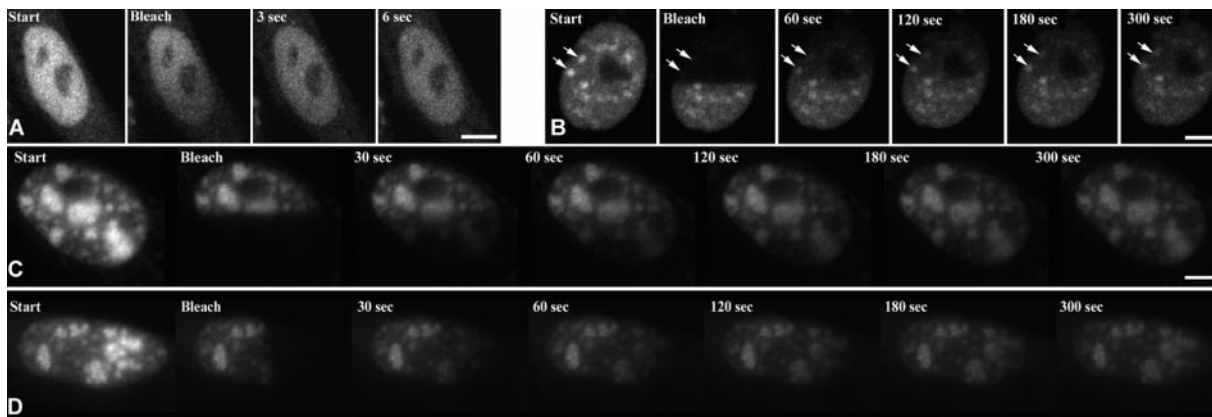


Figure 5. Effect of RNA polymerase II and kinase inhibitors on ASF-GFP movement. Mouse 10T1/2 fibroblasts expressing the GFP protein (A) or the ASF-GFP (B, C, and D) chimeric protein were incubated in the presence of the transcriptional inhibitor DRB (C) or in the presence of the kinase inhibitor staurosporine (D) for 2–4 h before imaging. Arrows in A denote two individual speckle domains. Cells were photobleached and the recovery of fluorescence monitored by time-lapse microscopy. Bars, 5 μm .

in bleached speckles occurring concomitantly with signal recovery in the surrounding nucleoplasm. This indicates that the dispersed population of ASF molecules does not behave differently, with respect to mobility, than sites of enrichment.

We quantitatively analyzed recovery from nuclear speckles and nucleoplasmic regions by simultaneously bleaching two 1.5- μm spots aligned in the y axis of the image scan. Bleaching time was ~ 200 ms. Because Phair and Misteli (2000) had demonstrated a slight increase in the mobility of ASF upon inhibition of RNA polymerase II transcription, we also quantitatively analyzed the recovery of ASF under conditions of transcriptional inhibition. The mean of five cells for each experiment was plotted (Fig. 6). Standard deviations ranged from 5 to 8% at the 0.5 recovery value. The $t_{1/2}$ of recovery under untreated conditions was not significantly different for control speckles versus control nucleoplasm. Consistent with the results of Phair and Misteli (2000), recovery times are slightly decreased during transcriptional inhibition. A slight increase in mobility is observed for nuclear speckles but recovery in the nucleoplasm was measured to be $1.6\times$ faster during recovery than the control nucleoplasm. A smaller increase in mobility was observed for speckles. Nonetheless, even under conditions where nascent transcripts are not present in the nucleoplasm, nucleoplasmic ASF moves substantially slower than a freely mobile protein.

Second, after photobleaching half of the cell nucleus, we compared the rate of fluorescence draining by monitoring the relative intensities of speckles in an unbleached region of the nucleus. To do this, in unbleached regions of the nucleus, the ratio of the speckle maximum to the neighboring nucleoplasmic minimum was normalized to one and plotted over time. If a higher affinity binding site existed in nuclear speckles, we would expect an initial increase in the ratio of speckle signal to dispersed nucleoplasmic signal (ratio >1.0), reflecting the loss of the more mobile nucleoplasmic factor into bleached regions. We have observed this behavior with other GFP fusion proteins that are currently under investigation (unpublished observations). Fig. 7 shows the ratio of speckles relative to adjacent nu-

cleoplasmic regions in an unbleached region during recovery. Consistent with the FRAP results, this experiment shows that the relative intensity remains at ~ 1.0 , indicating that the change in speckle intensity equals that measured for the nucleoplasm. Thus, even though the spatial organization of ASF is not uniform, the mobility of ASF is not significantly different between the speckle compartment and the nucleoplasm. Both of these experiments support the conclusion that the association between ASF and binding sites in nuclear speckles and the nucleoplasm is qualitatively similar.

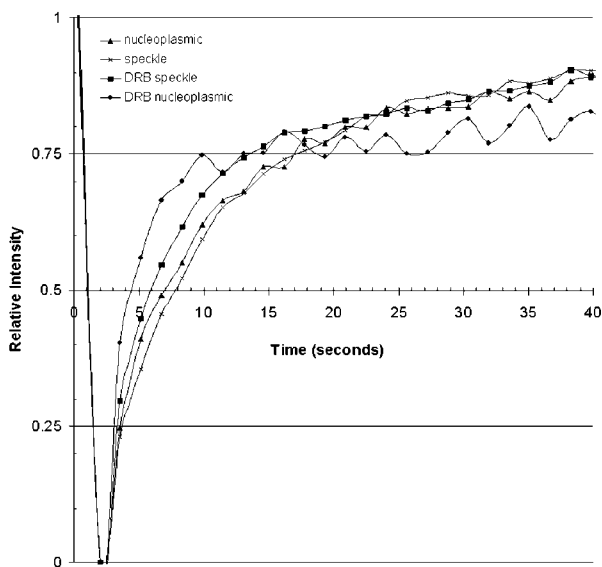


Figure 6. Quantitative analysis of FRAP after spot-bleaching of a nuclear speckle and a nucleoplasmic region of the same cell. Two spots of $\sim 1.5\text{-}\mu\text{m}$ diameter were photobleached in HeLa cell nuclei, one in a speckle domain and the other in the nucleoplasm. These spots were in perfect register horizontally within the image scan and were bleached simultaneously. The initial intensity of the photobleached regions were normalized to 0 and the maximal intensity obtained during recovery was normalized to 1.0. The recovery of each region was then plotted versus time.

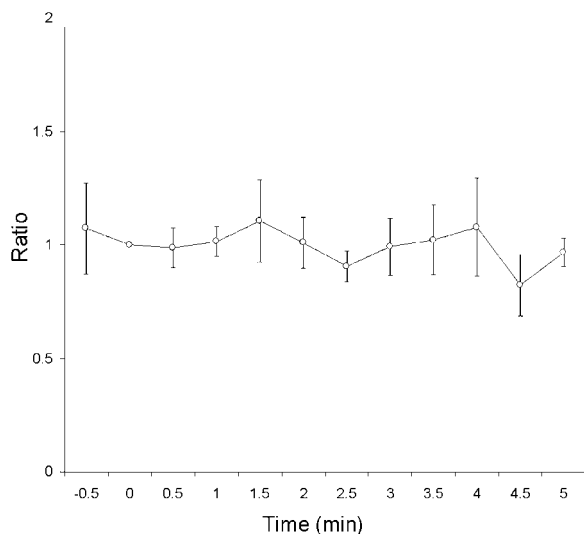


Figure 7. Quantitative analysis of the relative ASF-GFP distribution in nuclear speckles and the surrounding nucleoplasm during recovery after photobleaching. A line scan three pixels wide was drawn through the nucleus to include both individual speckles and surrounding nucleoplasm. The relative concentration between the individual speckles and the surrounding nucleoplasm was normalized to one in the first image collected immediately after photobleaching. The ratio between fluorescence signal present within the speckles and the surrounding nucleoplasm is plotted over time as a mean of five separate speckles from individual experiments. The negative time point represents the image collected immediately before photobleaching.

Connectivity between Pools of ASF Associated with Immobile Binding Sites in Speckles and the Nucleoplasm

FLIP experiments can be used to demonstrate the connectivity between compartments. Our observations of FRAP recovery, particularly those where the large half-nucleus regions are bleached, suggest that the nucleoplasmic and speckle compartments constantly exchange molecules with each other. This was confirmed by FLIP analysis. A region of the cell nucleus was cyclically bleached and allowed to recover until the fluorescence intensity of the cell nucleus was near the detection limit in the experiment. If a significant population of molecules remained bound in nuclear speckles, we would expect that it would not be possible to drain the fluorescence within these regions upon repeated photobleaching of a nucleoplasmic region. Fig. 8 shows that repeated photobleaching of a region within the nucleus results in overall loss of nuclear fluorescence. Thus, the ASF molecules, regardless of their nuclear location during the initial line scan, are in constant flux and move throughout the entire nucleus.

Discussion

ASF-enriched Domains Do Not Behave as Structures that Are Free within the Nucleoplasm

The observation that ASF-enriched domains are able to

move in a directed manner from nuclear speckles to sites of transcription (Misteli et al., 1997, 1998) potentially reflects a controlled and targeted migration of processing machinery to locations in the cell nucleus where this factor is required. It is formally possible, however, that such movement represents a passive movement of domains that are confined in their motion by a restriction of the nuclear space available for diffusion. Using HDAC4-GFP, which forms nonphysiological domains of similar size in overexpressing cells, we were able to demonstrate that chromatin density or other potential impediments to domain movement do not restrict the mobility of relatively large structures in the cell nucleus. In contrast to the random movement of HDAC4-GFP-containing domains, the movement of ASF-GFP-enriched domains is nonrandom and considerably slower. Moreover, when the HDAC4 domains approach or exceed the size of the speckles themselves, they still undergo rapid, but confined motion. They appear to vibrate within the nucleoplasmic space on 1-s time scale intervals (unpublished observations). Thus, ASF-containing structures behave like structures that are physically confined through associations with other immobile elements of the nucleoplasm rather than structures whose mobility is impeded solely by size but that are otherwise freely mobile. Therefore, it is possible that the movement of ASF-enriched domains to sites of transcription reflects an active and directed process.

The Movement of the ASF-splicing Factor through Nucleoplasmic Space

An implication of the mobility properties of speckles and smaller domains enriched in splicing factors is that splicing factor availability is tightly controlled in a spatiotemporal fashion. This could be mediated by association with putative architectural elements of the nucleus and tracking along this karyoskeleton as large multicopy domains. It has also been reported that there are significant quantities of splicing factors that are dispersed throughout the nucleoplasm (Fay et al., 1997). This has fueled speculation that splicing factors move very rapidly throughout the cell nucleus by free diffusion (Singer and Green, 1997). If the population of freely diffusing molecules is very high, then spatiotemporal regulation would be much more difficult to establish. We have been able to estimate the amount of freely mobile ASF by assuming that the loss of fluorescence in a region distant from the site of bleaching (as is observed with the freely mobile GFP protein during photobleaching) reflects a freely mobile population. In many cells examined, this population is very low or undetectable. For 4 of 11 cells we estimated this at <5%. Although our method may underestimate this population, we are examining overexpressing cells. This should also be considered in the kinetic analysis of movement. The observed enrichment of ASF in nuclear speckles (two- to threefold) is considerably lower than endogenous splicing factors observed in immunofluorescent studies (unpublished observations). Because all cells examined are transiently overexpressing the ASF protein, it is quite possible, if not likely, that the measured mobility of ASF is overestimated as a consequence of saturating specific nuclear binding sites.

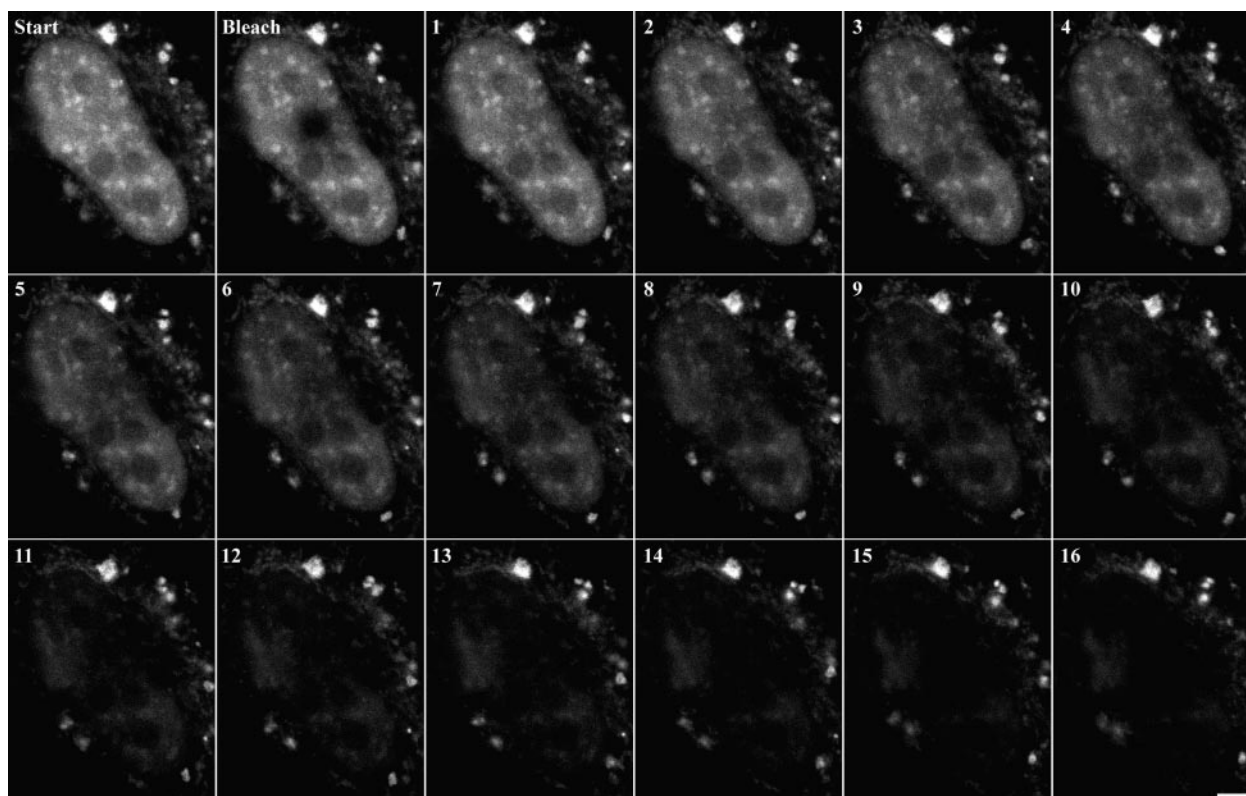


Figure 8. Fluorescence loss in photobleaching demonstrates connectivity between speckles and the nucleoplasm. A single spot 2 μm in diameter was repeatedly photobleached and allowed to recover for 30 s. The experiment was performed in a human intestinal smooth muscle cell expressing ASF-GFP. The initial image, collected after photobleaching, and the images collected 30 s after photobleaching are shown. Bar, 5 μm .

In this study, we demonstrate that in the nucleoplasm splicing factors display a similar overall mobility to the speckle-associated ASF. It is possible that higher resolution techniques, such as fluorescence correlation spectroscopy, will reveal multiple populations that produce an average mobility that is reflected in our experiments. Certainly, over very short distances, this is probably the case. Interestingly, in one cell line, HISM, staining of the fibrillar centers or the dense fibrillar components of the nucleolus was occasionally observed. When cells containing this population were examined and the nucleoplasmic or speckle recovery was compared with nucleolar recovery, the nucleolar population recovered approximately twice as slowly as the extranucleolar populations. Because we suspected that nucleolar association was an artefact of overexpression, such cells were not included in our primary FRAP analysis. However, this observation, and the results of Phair and Misteli (2000), comparing the mobility of ASF to nucleolar and chromatin-associated proteins, indicates that this method is capable of resolving populations with distinct mobilities.

An important observation in this study is that the movement of the population of ASF molecules occurs much more quickly than the time required for ASF-enriched structures, or speckles themselves, to move through nuclear space. This is evident in the comparison of rates of movement of structures in the 3-D time-lapse experiments

versus the movement of molecules in the FRAP experiments. It is further evidenced in experiments where cells have been treated with transcription or kinase inhibitors. Under these conditions, the translocation of ASF-enriched structures from speckles is minimized (Misteli et al., 1997), but the overall mobility of ASF is not reduced (Phair and Misteli, 2000; this study). We conclude that the mobility of ASF is not due mainly to a mechanism involving the directed transport of speckle subdomains through the nucleus. The spatial organization of ASF, however, is modulated by processes such as transcription and ongoing phosphorylation.

Although splicing factors are capable of movement through the nucleus independently of the shuttling of factor-rich structures, this mobility is not representative of a freely mobile state. We observe both the dispersed and speckle-associated ASF to move at a rate significantly slower than other proteins that exhibit free mobility, such as HDAC4-GFP (unpublished observations) and GFP itself. Essentially identical mobility results have recently been reported by Phair and Misteli (2000). In their study, they determined the diffusion coefficient of ASF-GFP to be $0.24 \mu\text{m}^2/\text{s}$, a value $\sim 1\%$ of that of a freely mobile protein in the nucleoplasm and slower than the measured mobility of diffusing poly(A) RNA (Politz et al., 1998). One possibility is that this reflects association with RNAs cotranscriptionally. Phair and Misteli (2000) detected a

slight increase in the mobility of ASF in cells that had been treated with transcriptional inhibitors.

The concept of a large pool of molecules or complexes that move freely through the cell nucleus until they associate with their substrate does not accurately reflect the behavior of splicing factors. Instead, our results are consistent with the nucleus containing a large number of relatively immobile sites capable of binding ASF, thereby significantly reducing its overall mobility compared with freely mobile proteins. The reduced mobility of ASF, even under conditions where newly synthesized transcripts are blocked from forming, indicates that RNA contributes only a portion of the total nucleoplasmic binding sites. The significance of nascent RNA in retarding the mobility of ASF is difficult to assess, however, since transcriptional inhibition results in a redistribution of nuclear ASF molecules. Before transcriptional inhibition, HeLa speckles were enriched 1.8-fold over nucleoplasmic regions. After transcriptional inhibition, speckles were enriched 2.5-fold over nucleoplasmic regions. Thus, it may simply be that the increased mobility of ASF in the nucleoplasm reflects a redistribution of nonRNA-binding sites from the nucleoplasm to the speckles rather than the straightforward explanation that it is a direct consequence of the absence of cotranscriptional binding sites for ASF within the nucleoplasm.

We propose that a transient association of ASF with these putative immobile acceptor sites serves to retard the translational mobility of splicing factors within the cell nucleus and establishes a dynamic equilibrium between mobile and immobile populations (see Phair and Misteli, 2000, for a kinetic analysis). This does indicate that, as a consequence, ASF molecules move only small distances in solution before once again associating with relatively immobile acceptor sites. This is reflected in the relatively clean boundary between photobleached and unbleached regions of the nucleus, in comparison to the absence of discernible bleach zone boundaries when examining free GFP, and the slow migration of the fluorescent front during recovery. This is not simply due to the increased molecular weight of the chimeric protein. Seksek et al. (1997) have demonstrated that under conditions of free diffusion, the $t_{1/2}$ of a 2-mD molecule is only approximately eightfold greater than that of a 20-kD molecule. The kinetics of movement suggests that ASF molecules, regardless of their location, spend considerably more time in a bound and immobilized state than in a diffusible state. Since there is no difference in the mobility of ASF populations associated with nuclear speckles or dispersed throughout the nucleoplasm, these binding sites must be qualitatively similar but heterogeneous in concentration within the nucleus. The immobilization of these binding sites could be explained by association with the karyoskeleton, which has a high density in speckles but is also present throughout the nucleoplasm (Hendzel et al., 1998, 1999). Spector et al. (1991) previously demonstrated that the SC-35 splicing factor associates with nuclear speckles independent of RNA implying associations with non-RNP components of these structures. Thus, it may be more appropriate to think of translocating factor-rich structures as movable splicing factor steady state reservoirs rather than stably assembled complexes that are shuttled from sites of storage to sites of use.

A Model for Regulating the Distribution of Splicing Factors in the Cell Nucleus

We propose that heterogeneity in splicing factor concentration throughout the nuclear volume reflects differences in the concentration of immobile karyoskeleton-associated binding sites. These putative binding sites are present in lower abundance within the nucleoplasm and enriched in distinct morphological components of the nucleus, such as speckles. The cell nucleus is able to dynamically reconfigure the karyoskeleton, or the organization of binding sites within the karyoskeleton, to reposition domains. This reorganization occurs by blebbing, fusing, and translocating assemblies that contain high concentrations of these sites. This allows the cell to respond to changing needs for transcription-processing machinery in the vicinity of transcriptionally active genes. As suggested by Phair and Misteli (2000), it may also provide a mechanism that favors the assembly of multiprotein complexes. Overall, the constant dissociation of factors from these binding sites enables most of these factors to also be available for catalytic activity near their site of concentration while the association provides a means of spatiotemporal regulation of splicing factor availability. The ability of splicing factors to move throughout the entire nuclear volume over relatively short periods of time (min) indicates that although compartmentalization may increase the efficiency of splicing in the vicinity of nuclear speckles, the location of a gene distant from a speckle does not necessarily mean that it will not have access to splicing machinery. We suggest that compartmentalization of factors reflects the dynamic organization of karyoskeleton-based sites and that this organization is influenced by nuclear processes such as transcription (see Huang and Spector, 1996; Misteli et al., 1997).

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References

- Carmo-Fonseca, M., C. Cunha, N. Custodio, C. Carvalho, P. Jordan, J. Ferreira, and L. Parreira. 1996. The topography of chromosomes and genes in the nucleus. *Exp. Cell Res.* 229:247-252.
- Cremer, T., A. Kurz, R. Zirbel, S. Dietzel, B. Rinke, E. Schrock, M.R. Speicher, U. Mathieu, A. Jauch, P. Emmerich, et al. 1993. Role of chromosome territories in the functional compartmentalization of the cell nucleus. *Cold Spring Harb. Symp. Quant. Biol.* 58:777-792.
- de Jong, L., M.A. Grande, K.A. Mattern, W. Schul, and R. van Driel. 1996. Nuclear domains involved in RNA synthesis, RNA processing, and replication. *Crit. Rev. Eukaryot. Gene Expr.* 6:215-246.
- Fay, F.S., K.L. Taneja, S. Shenoy, L. Lifshitz, and R.H. Singer. 1997. Quantitative digital analysis of diffuse and concentrated nuclear distributions of nascent transcripts, SC35 and poly(A). *Exp. Cell Res.* 231:27-37.

- Fejes-Toth, G., D. Pearce, and A. Naray-Fejes-Toth. 1998. Subcellular localization of mineralocorticoid receptors in living cells: effects of receptor agonists and antagonists. *Proc. Natl. Acad. Sci. USA.* 95:2973–2978.
- Fischle, W., S. Emiliani, M.J. Hendzel, T. Nagase, N. Nomura, W. Voelter, and E. Verdin. 1999. A new family of human histone deacetylases related to *Saccharomyces cerevisiae* HDA1p. *J. Biol. Chem.* 274:11713–11720.
- Hendzel, M.J., and D.P. Bazett-Jones. 1995. RNA polymerase II transcription and the functional organization of the mammalian cell nucleus. *Chromosoma.* 103:509–516.
- Hendzel, M.J., M.J. Krhlak, and D.P. Bazett-Jones. 1998. Organization of highly acetylated chromatin around sites of heterogeneous nuclear RNA accumulation. *Mol. Biol. Cell.* 9:2491–2507.
- Hendzel, M.J., F. Boisvert, and D.P. Bazett-Jones. 1999. Direct visualization of a protein nuclear architecture. *Mol. Biol. Cell.* 10:2051–2062.
- Htun, H., J. Barsony, I. Renyi, D.L. Gould, and G.L. Hager. 1996. Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. *Proc. Natl. Acad. Sci. USA.* 93:4845–4850.
- Huang, S., and D.L. Spector. 1996. Intron-dependent recruitment of pre-mRNA splicing factors to sites of transcription. *J. Cell Biol.* 133:719–732.
- Jackson, D.A. 1997. Chromatin domains and nuclear compartments: establishing sites of gene expression in eukaryotic nuclei. *Mol. Biol. Rep.* 24:209–220.
- Khodjakov, A., and C.L. Rieder. 1999. The sudden recruitment of γ -tubulin to the centrosome at the onset of mitosis and its dynamic exchange throughout the cell cycle, do not require microtubules. *J. Cell Biol.* 146:585–596.
- Lamond, A.I., and W.C. Earnshaw. 1998. Structure and function in the nucleus. *Science.* 280:547–553.
- Misteli, T. 2000. Cell biology of transcription and pre-mRNA splicing: nuclear architecture meets nuclear function. *J. Cell Sci.* 113:1841–1849.
- Misteli, T., and D.L. Spector. 1996. Serine/threonine phosphatase 1 modulates the subnuclear distribution of pre-mRNA splicing factors. *Mol. Biol. Cell.* 7:1559–1572.
- Misteli, T., and D.L. Spector. 1998. The cellular organization of gene expression. *Curr. Opin. Cell Biol.* 10:323–331.
- Misteli, T., J.F. Caceres, and D.L. Spector. 1997. The dynamics of a pre-mRNA splicing factor in living cells. *Nature.* 387:523–527.
- Misteli, T., J.F. Caceres, J.Q. Clement, A.R. Krainer, M.F. Wilkinson, and D.L. Spector. 1998. Serine phosphorylation of SR proteins is required for their recruitment to sites of transcription in vivo. *J. Cell Biol.* 143:297–307.
- Nakata, T., S. Terada, and N. Hirokawa. 1998. Visualization of the dynamics of synaptic vesicle and plasma membrane proteins in living axons. *J. Cell Biol.* 140:659–674.
- Pederson, T. 1998. Thinking about a nuclear matrix. *J. Mol. Biol.* 277:147–159.
- Phair, R.D., and T. Misteli. 2000. High mobility of proteins in the cell nucleus. *Nature.* 404:604–609.
- Politz, J.C., E.S. Browne, D.E. Wolf, and T. Pederson. 1998. Intranuclear diffusion and hybridization state of oligonucleotides measured by fluorescence correlation spectroscopy in living cells. *Proc. Natl. Acad. Sci. USA.* 95:6043–6048.
- Seksek, O., J. Biwersi, and A.S. Verkman. 1997. Translational diffusion of macromolecule-sized solutes in the cytoplasm and nucleus. *J. Cell Biol.* 138:131–142.
- Singer, R.H., and M.R. Green. 1997. Compartmentalization of eukaryotic gene expression: causes and effects. *Cell.* 91:291–294.
- Sleeman, J.E., and A.I. Lamond. 1999. Nuclear organization of pre-mRNA splicing factors. *Curr. Opin. Cell Biol.* 11:372–377.
- Sleeman, J., C.E. Lyon, M. Platani, J.P. Kreivi, and A.I. Lamond. 1998. Dynamic interactions between splicing snRNPs, coiled bodies and nucleoli revealed using snRNP protein fusions to the green fluorescent protein. *Exp. Cell Res.* 243:290–304.
- Spector, D.L. 1993. Nuclear organization of pre-mRNA processing. *Curr. Opin. Cell Biol.* 5:442–447.
- Spector, D.L., X.D. Fu, and T. Maniatis. 1991. Association between distinct pre-mRNA splicing components and the cell nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3467–3481.
- Wei, X., S. Somanathan, J. Samarabandu, and R. Berezney. 1999. Three-dimensional visualization of transcription sites and their association with splicing factor-rich nuclear speckles. *J. Cell Biol.* 146:543–558.
- White, J., and E. Stelzer. 1999. Photobleaching GFP reveals protein dynamics inside living cells. *Trends Cell Biol.* 9:61–65.
- Zirbel, R.M., U.R. Mathieu, A. Kurz, T. Cremer, and P. Lichter. 1993. Evidence for a nuclear compartment of transcription and splicing located at chromosome domain boundaries. *Chromosome Res.* 1:93–106.