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# The Nucleus of HeLa Cell Contains Tubular Structures for $Ca^{2+}$ Signalling

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**It has long been assumed that $Ca^{2+}$ are translocated from the cytosol to the cell nucleus by a long distance to activate transcription machinery buried deep in the nucleoplasm. However, this model has been recently challenged. When HeLa cells were loaded with fluo-3, highly fluorescent spots of ~2 $\mu$m in diameter were observed in the cell nucleus while the fluo-3 signals were low in their neighbouring nucleoplasm as determined by confocal microscopy. These fluorescent spots were devoid of but usually associated with chromatin on their boundary. When cells were stimulated by ionomycin (1 $\mu$M), the fluo-3 fluorescence in these spots increased faster than that in their neighbouring nucleoplasm. In another experiment, optical sections with hot spot(s) were used to construct 3-D images to study the morphology of the hot spots. Views of reconstruction from different angles indicated that the hot spots formed a tubular structure with a connection to the nucleocytoplasmic interface. Moreover, injection of calcium green-dextran (70 kDa), a $Ca^{2+}$-sensitive indicator conjugated with an inert molecule of large molecular size, into the cytosol leads to a formation of signals also in a tubular shape inside the nucleoplasm. This suggests that the 'channels' are real inside the nucleus and they are derived from an invagination of the double-membraned nuclear envelope. Taken together, our results indicate (1) tubular structures are found inside the cell nucleus; (2) they are extended from the cytosol into the nucleus through the invagination of the double membraned nuclear envelope; (3) molecules of molecular size up to 70 kDa could penetrate into these 'tunnels'; (4) $Ca^{2+}$ can be released or transported into the cell nucleus through these tubular structures after ionomycin stimulation; and (5) the structures are usually associated with chromatin.** 

***Key Words:*** **nuclear $Ca^{2+}$; nuclear envelope; nuclear tubular structure; confocal microscopy.**

Many cellular functions including the shuttling of transcription factors and gene expression are regulated by changes in the concentration of intracellular free $Ca^{2+}$ (1-3). It is well established that the global $Ca^{2+}$ signals are evoked as a result of a recruitment of many subcellular $Ca^{2+}$ releasing units in a highly coordinated manner (4, 5). After stimulation, $Ca^{2+}$ signals are terminated by the re-uptaken of $Ca^{2+}$ into the endoplasmic reticulum (ER), which is regulated by the free [$Ca^{2+}$] in the store lumen (6).

In eukaryotic cells, the nucleoplasm is separated from the cytoplasm by the nuclear envelope (NE), a double-membraned structure contiguous with the ER and with an intermediate lamina network. On the NE, there are many channel-like nuclear pore complexes (NPC) with a diameter of ~9 nm and a length of 15 nm, which penetrate and fuse the double layer of NE. These 'pores' are thought to allow simple diffusion of molecules up to a molecular size of 50 kDa in and out of the nucleus (7, 8). It is therefore widely assumed that $Ca^{2+}$ is able to equilibrate rapidly in the cytosolic and nuclear compartment by simple diffusion (9). This passive and rapid diffusion of free $Ca^{2+}$ ions through the NPC was then accepted as a mechanism that cells produce synchronous $Ca^{2+}$ signals in the cytosol and cell nucleus upon stimulation (9).

However, data from electrophysiological studies indicate that the NPC are not freely permeable to ions (10). Moreover, it has been found that the NPC become 'plugged', when nuclear $Ca^{2+}$ stores are depleted, to block the movements of macromolecules across the nucleo-cytoplasmic interface (11,12). Recent data also indicate that depletion of NE/ER $Ca^{2+}$ inhibited the translocation of adenovirus DNA into the nucleus (13). Recently, it has been shown in isolated nuclei that the NE is a $Ca^{2+}$ store (14). Our results in living C6 cells also demonstrated that the NE is able to release and uptake $Ca^{2+}$ (15). Still, the nature and the source of $Ca^{2+}$ in the cell nucleus remain unclear (16-19).

In the present study, we attempted to use HeLa cells and confocal microscopy to investigate the source of $Ca^{2+}$ and the site of its release in the nucleus. Our

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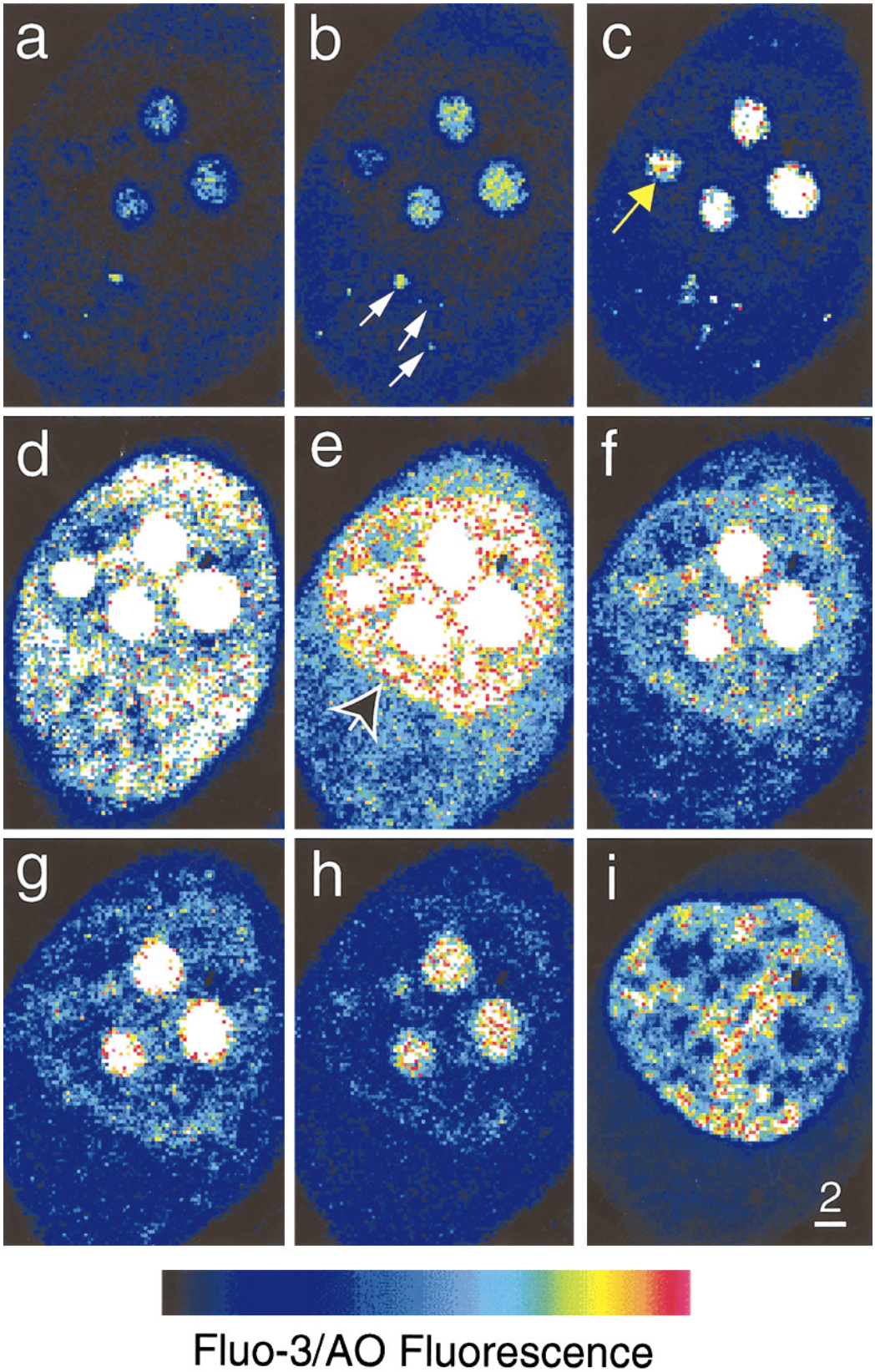



**FIG. 1.** $Ca^{2+}$ released from the nuclear hot spots. HeLa cells loaded with fluo-3 were stimulated with ionomycin at room temperature. Panels (**a - h**) show the spatial changes of fluo-3 fluorescence in the same optical section at −2.5, 10, 62.5, 82.5, 747.5, 757.5, 765, 855 sec respectively. Ionomycin (1 $\mu$M) was added at 0 sec. The cytosolic and nuclear region of the same optical section were defined by the acridine orange (AO) staining at the end of the experiment (**i**). The scale bar represents the cell dimension in $\mu$m and the colour palette represents the fluorescence intensity.

results indicate (1) tubular structures are found inside the cell nucleus; (2) they are extended from the cytosol into the nucleus through the invagination of the nuclear envelope; (3) $Ca^{2+}$ can be released or transported into the cell nucleus through these intranuclear extensions; and (4) these structures are usually associated with chromatin.

## MATERIALS AND METHODS

*Materials.* Fluo-3/AM, calcium green-dextran (70 kDa) were purchased from Molecular Probes. Other chemicals were from Sigma.

*Cell culture.* HeLa cells (purchased from American Type Culture Collection) were maintained in modified Eagle's medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco). The cells were passaged twice weekly. For $Ca^{2+}$ imaging studies, cells were seeded on coverslips in 35-mm diameter culture dishes at a cell density of $10^4$/ml and incubated at 37°C, under an atmosphere of 5% $CO_2$. Subconfluent monolayer cells were used for experiments at day 3 after passage.

*Determination of the intracellular $Ca^{2+}$ level by confocal laser scanning microscopy.* Fluo-3, a $Ca^{2+}$-sensitive fluorescent indicator compatible with laser excitation, was used to monitor changes in intracellular $Ca^{2+}$. HeLa cells grown on a coverslip were loaded with fluo-3/AM (2 $\mu$g/ml) at room temperature for 30 min to 1 h in $Na^+$-N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) buffer (concentration in mM: 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES, final pH 7.2). After washing, cells were incubated in 0.5 ml HEPES buffer and the changes in intracellular $Ca^{2+}$ distribution were measured using a confocal imaging system (Molecular Dynamics, USA) with Nikon objective ×60, n.a. 1.4. For microinjection, calcium green-dextran was dissolved in $Ca^{2+}$-free buffer and the injection volume was kept at 0.9 nl. Microinjections were performed with femotips, microinjector and micromanipulator (Eppendorf). For fluorescence measurements, excitation and emission were 488 nm and above 510 nm respectively. Fluorescence images were collected at room temperature. At the end of the experiment, the voltage of the photomultiplier detector was tuned down until no fluorescent image was obtained, then acridine orange (final concentration 0.1 $\mu$g/ml) was added to locate the nucleus. In some experiments, propidium iodide (PI) was used to labelled DNA. Excitation and emission for PI experiments were 488 nm and above 630 nm respectively. Excitation and emission for PI experiments were 488 nm and above 630 nm respectively. Images were processed by the ImageSpace software and the fluorescence intensities in the nucleus and in the cytosol calculated. For the pseudocolour images, cool colours represent low fluorescence while warm colours correspond to high fluorescence.

## RESULTS

To investigate the site of $Ca^{2+}$ rise in the nucleus, HeLa Cells loaded with fluo-3 were challenged with a low dose of ionomycin (1 $\mu$M) and the changes of fluo-3 fluorescence in the nucleus and cytosol were determined by a laser confocal microscope. For the location of cell nucleus, cells were stained with a membrane permeable nuclear dye, acridine orange (AO), at the end of the experiment. It is well documented that ionomycin is an agent that releases internal $Ca^{2+}$ and induces external $Ca^{2+}$ influx (20). Fig. 1 is the pseudocolour images of the same optical section that indicates spatial changes of $[Ca^{2+}]$ in the two compartments before and after ionomycin stimulation. Before stimulation, it is clear that several hot spots (with a diameter about 2 $\mu$m) with a high level of fluorescence were found in the nuclear region. The number of such hot spots in the nucleus, usually from 1 to 4, varied from cell to cell. Apart from that, the fluo-3 fluorescence in the nucleoplasm was basically lower than that in the cytoplasm. As expected, $[Ca^{2+}]$ increased in the cytosol after stimulation (arrows in Fig.1b). In the nucleus, a delay in $[Ca^{2+}]$ increase was found in the nucleoplasm (Fig.1b). On the contrary, the fluo-3 signals increased almost immediately in the centre of the hot spots (Fig.1b-c). Interestingly, a new hot spot appeared in the nucleus after ionomycin stimulation (Fig. 1b-d, yellow arrow). Moreover, $Ca^{2+}$ was confined in the hot spots for a while and then diffused outwards (Fig.1c-e). Subsequently, the cytosolic and nuclear $Ca^{2+}$ were uptaken into the nucleo-cytoplasmic interface, presumably the nuclear envelope (arrow head, Fig 1e-h) and the hot spots (Fig.1f-h). In particular, the fluorescence in the area of the newly formed hot spot returned to a low level (Fig.1h). In the AO image, the areas with nuclear hot spots were devoid of but associated with chromatin on their boundary (Fig.1i). It seems likely that some structures inside the nucleus for the release or transport of $Ca^{2+}$.

Next, HeLa cells were loaded with fluo-3 again and optical sections with a vertical displacement of 0.5 $\mu$m were acquired to study the morphology of the hot spots. As shown in Fig.2a, a hot spot was found in the section near the growth substrate (arrow in plate 1). This hot spot then branched out horizontally to be two spots in the upper section (plate 6, Fig.2a). In the AO images, the branched hot spot was again surrounded by chromatin. The dataset in Fig.2a, consisting of 11 XY planes of fluo-3 signals was used to construct 3-D images of different rotation angles (Fig.2b). It is clear in the reconstruction that the nucleus of HeLa cell contained delicate infoldings derived from the nucleo-cytoplasmic interface. It is therefore highly possible that the structures are invaginations of the NE.

To confirm this, we injected calcium green-dextran (70 kDa) into the cytosol of a HeLa cell. We reasoned that if both the inner and outer nuclear membranes are invaginated into the central region of the nucleoplasm, we could observe the calcium green signals inside cell nucleus. This thinking is based on the fact that the indicator is conjugated with an inert dextran of a large molecular size and the dextran will prevent the indicator from migrating into the cell nucleus through the NPC. After injection and equilibration, the distribution of the fluorescence signals was then determined. In the confocal images, we can see the calcium green signals both in the cytosol and the nucleus (Fig.3a). Similar to the observations in Fig.1 and 2, the calcium green signals in the nucleus were concentrated as a hot spot (the lower 2 panels, Fig.3a) and were

in a tubular shape which extended inwards from the nucleo-cytoplasmic interface (the first two panels, Fig.3a). Moreover, the fluorescence intensity was higher in the nuclear hot spot than the one in cytosol. No signal was found in other nuclear regions suggesting that the indicator could not cross the NE through the NPC. Collectively, the HeLa cell in Fig.3a showed one single intranuclear tubular structure extended deep into the nucleoplasm, oriented horizontally and then vertically to the growth substrate. The association of the intranuclear tubule with chromatin was then verified by propidium iodide (PI) (Fig.3b). In this experiment, a 630 nm long pass filter was used to capture the PI signals but eliminate the calcium green fluorescence. It is clear from the section nearest to the growth substrate that the shape of the calcium green signals was different from the one given by chromatin staining (the 1st panel, Fig.3b). In the upper sections (3rd & 4th panel in Fig.3b), chromatin formed a ring outside the tubular structure.

Taken together, Our results indicate (1) tubular structures are found inside the cell nucleus; (2) they are extended from the cytosol into the nucleus through the invagination of the nuclear membranes; (3) molecules of molecular size up to 70 kDa could penetrate into these 'tunnels'; (4) $Ca^{2+}$ can be released or transported into the cell nucleus through the tubular structures after stimulation and (5) these structures are usually associated with chromatin.

## DISCUSSION

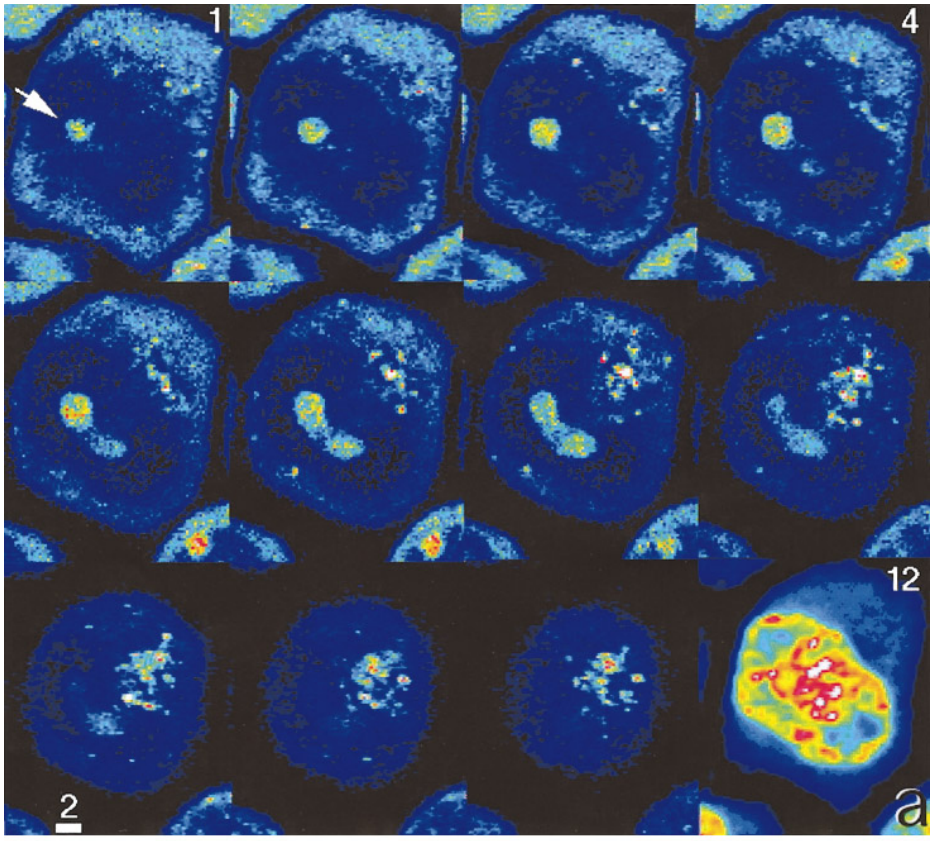
In the past decade, the mechanism(s) leading to $Ca^{2+}$ changes in the cell nucleus provoked intense debate. It has been claimed that the rise of $Ca^{2+}$ can be faster and greater in the cell nucleus and chromatin may contain releasable $Ca^{2+}$ pools (21). On the other hand, data also indicate that the behaviour of the $Ca^{2+}$-sensitive fluorescent indicators in the nucleus and cytosol is different (22). In particular, fluo-3 emits stronger fluorescence in the nuclear homogenates than the one in cytosol even [$Ca^{2+}$] was clamped (22). Therefore, the suggestion for an existence of $Ca^{2+}$ store(s) or transport channels inside the cell nucleus was not universally accepted.

In the present study, we found many hot spots in the nucleus of fluo-3 loaded HeLa cells. These hot spots are usually associate with chromatin and the number of hot spots varied from cell to cell (Fig.1-3). Detailed examination of the hot spots and chromatin suggests that they were closely associated but not overlapped (Fig.1 & 3). The possibility that these hot spots and their ability to release $Ca^{2+}$ were artifacts was eliminated by the following observations and reasons: (1) If the hot spots was only an association of fluo-3 to chromatin and the hot spots was not the $Ca^{2+}$ releasing site; increase in fluo-3 signals should have started from the boundary and then spread inwards to the centre of the hot spots when $Ca^{2+}$ travelled across the nucleus. Now, signals increased first in the center of the hot spots and a delay in the increase of signals was observed in their neighbouring nucleoplasm (Fig.1). (2) The hot spots were devoid of chromatin materials and at the same time chromatin concentrated outside the boundary of the hot spots (Fig.1 & 3). (3) A new hot spot was formed gradually inside the nucleus after ionomycin stimulation and the fluo-3 signals therein subsided significantly afterwards. (4) Injection of calcium green-dextran (70 kDa) into the cytosol can generate a hot spot inside the nucleus. Thus, the hot spots are not an artifact and they are part of the channel-like structures in the nucleus which are near-orthogonal to the growth substrate. We believe that these structures are derived from the invaginations of the NE. Invaginations of the NE can be single- or double-membraned. The nuclear signals in the deep nucleoplasm after injecting calcium green-dextran in the cytosol suggesting that these intranuclear infoldings are bounded by double membrane.

These tubular membrane-bound invaginations have been demonstrated in a number of cell types including HeLa cells (23). With an aid of electron and confocal microscopy, it has been demonstrated that these 'infoldings' contain NPC and are continuous with the ER (23). The association of the chromatin with the intranuclear double-membraned invaginations was reported twenty years ago (24). However, the physiological function of these structure is not known.

Another interesting result in our study is that these tubular structures contain a high level of fluo-3 or calcium green fluorescence. At present, we do not know the mechanism for this phenomenon. It is possible that the basal $Ca^{2+}$ activity in these areas is very high. Experimental evidence is accumulated that IP3 receptors are located on the inner membrane (14,25). Since these extensions are the double-membraned invaginations of the NE, we believe that IP3 receptors are located on these tubular structures. After ionomycin stimulation, [$Ca^{2+}$] increased almost immediately in the hot spots but no immediate $Ca^{2+}$ diffusion from these tubular structures to the nucleoplasm suggests that a gating system exists in these invaginations. To explain the appearance and the disappearance of the newly formed hot spot (Fig. 1),

**FIG. 2.** Intranuclear structure in HeLa cell labelled with fluo-3. (**a**) Serial optical sections of HeLa cell loaded with fluo-3 were collected at 0.5 $\mu$m intervals by confocal microscopy from the base of cell (plate 1-12). After AO staining, the optical section corresponding to plate (4) was also collected (plate 12). The scale bar represents the cell dimension in $\mu$m and the colour palette represents the fluorescence intensity. (**b**) To visualize the morphology of the hot spots, confocal sections in (a, plate 1-12) were used to construct 3-D images with different angles viewed from the top (upper panel) and from the bottom (lower panel).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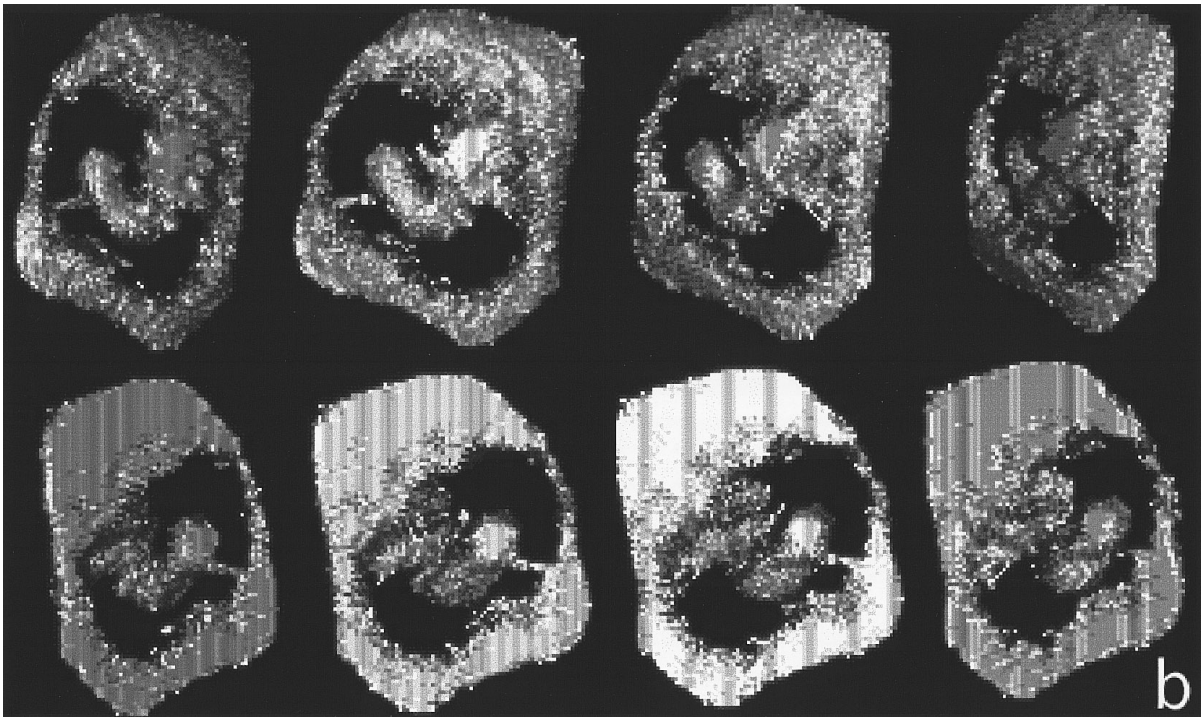
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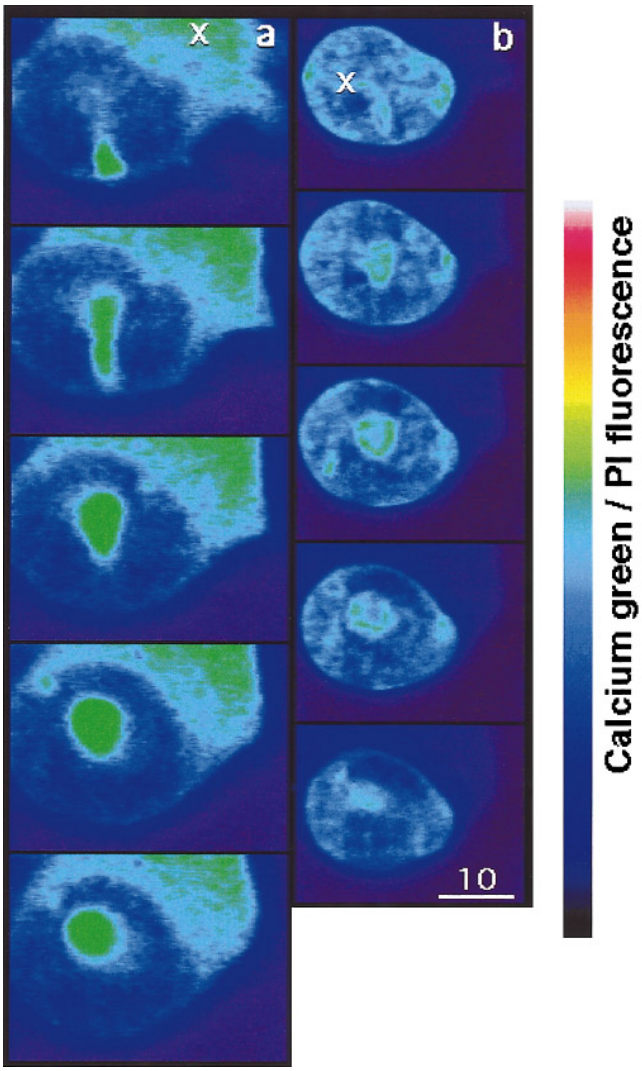


**FIG. 3.** Nuclear tubular structure is derived from the invagination of double-membraned nuclear envelope. (**a**) A resting HeLa cell was injected with calcium green-dextran (70 kDa) (5 mg/ml in pipette) in the cytosol at the site labelled 'x'. After equilibration, calcium green signals was determined by confocal microscopy. Panel (a) shows 5 consecutive optical sections with a vertical displacement of 2 $\mu$m. Note the near-orthogonal tubular structure in the cell nucleus. (**b**) Subsequently, the cells received propidium iodide (PI) injection in the cell nucleus at the site labelled 'x' to stain the nucleic acid. Panel (b) is the optical sections corresponding to the one in (a) and fluorescence was obtained with a 630 nm long-pass filter. Note the chromatin on the boundary of the nuclear 'channel'. Bar, 10 $\mu$m. Number of experiment $> 3$.

it is possible that this tubular structure is a dynamic system which can extend to a particular site to release $Ca^{2+}$. Alternatively, they are the result of $Ca^{2+}$ activity (release and uptake) at the tip of the tubular structure in which the regulation of $Ca^{2+}$ may be different from the one on the trunk of the tubular structures.

For the role of these extensions, it is conceivable by the association of the tubules with the chromatin on one hand and the generation of localized $Ca^{2+}$ signals on the other. As the spatial properties of $Ca^{2+}$ signals are important for determining the type of gene transcription (26), it seems therefore likely that these intranuclear tubular structures provide a fast and close contact of the signal molecules to the transcription machinery. Now, the site of transcription is no longer deep in the nucleoplasm but lies close to the nuclear envelope and even to the signal molecules generated at the cell surface.

## ACKNOWLEDGMENT

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