Dynamics of nuclear pore density and distribution patterns within developing pollen: implications for a functional relationship between the vegetative nucleus and the generative cell

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Summary

Quantitative transmission electron microscopy was used to study pore density (mean pore number±standard deviation/nuclear envelope area) on developing pollen nuclei of alfalfa. We found that pore density is essentially uniform around the vegetative nucleus (VN) at an early developmental stage (29±6 pores μm^{-2}), but later, when the VN forms a close physical association with the generative cell (GC), nuclear pore density is 69% higher on the surface of the VN facing the GC (27±12 pores μm^{-2}) compared to the surface away from the GC (16±9 pores μm^{-2}). The surface area of the VN does not change significantly during the stages of this study. Pore density is nearly equal on vegetative and generative nuclei in young pollen, but at pollen maturity the VN has a mean pore density 3.5 times greater than that of the generative nucleus. Our results are consistent with those of other studies comparing pore densities on developing and mature pollen nuclei. However, this is the first study, to our knowledge, that has followed vegetative nuclear pore density and distribution as it relates to the formation of a close physical association between the VN and the GC. Taken together with biochemical studies on RNA and protein synthesis during pollen development, and studies on nuclear pore function, these results support the notion that even though mean pollen nuclear activity may decrease during pollen maturation, the potential for nucleocytoplasmic exchange is not diminished appreciably in the area of the VN–GC association. This suggests that there is a direct functional relationship between the VN and the GC, and that gene expression may be not only temporally but also spatially separated within the VN during pollen development.

Key words: alfalfa, *Medicago sativa*, pollen gene activity, male germ unit, male gametophyte.

Introduction

The typical pollen grain of flowering plants consists of two haploid cells: a larger vegetative cell and a smaller generative cell. After pollination, the vegetative cell grows down the style of the female portion of the flower, forming a long tube through which two sperm cells travel. The sperm cells result from division of the generative cell. The vegetative and generative cells are formed from the same mother cell (microspore) through an unequal cell division. When first formed, the generative cell is appressed to the pollen wall, but soon moves inward and becomes entirely encompassed by the vegetative cell. Early in pollen development the vegetative nucleus and generative cell are widely separated, but later the vegetative nucleus, which is typically larger than the entire generative cell, partially surrounds the generative cell over a large surface area. This intimate association is commonly maintained during pollen tube growth and, after generative cell division, one or both sperm cells remain associated with the vegetative nucleus at least until their discharge from the pollen tube.

Although this striking physical association between the vegetative nucleus and the generative cell has been described in several species (e.g. see Cresti *et al.* 1985; Kaul *et al.* 1987; Ciampolini *et al.* 1988; Hu and Yu, 1988; Wagner and Mogensen, 1988; Zhu *et al.* 1990), only one study to date, to our knowledge, has considered this phenomenon from a functional standpoint. Tang (1988) followed ATPase activity in pollen and pollen tubes of *Amaryllis vittata* and *Clivia nobilis*, and concluded that an increased level of ATPase activity occurred at the vegetative nuclear envelope in the area of its association with the generative cell.

Experimental evidence supports the belief that nuclear pores are a major route for exchange of macromolecules between the nucleus and cytoplasm (Newport and Forbes, 1987). Since numerous studies have shown a positive

correlation between nuclear pore density and nuclear synthetic activity (LaFountain and LaFountain, 1973; Lott and Vollmer, 1975; Crevecoeur *et al.* 1982; Newport and Forbes, 1987; Wagner *et al.* 1990), we measured nuclear pore densities on vegetative and generative nuclei during pollen maturation, paying special attention to pore density within specific domains on the vegetative nucleus. Our finding in mature pollen of a significantly higher (69% higher) density of pores on the surface of the vegetative nucleus in close proximity to the generative cell, compared to that away from the generative cell, provides additional evidence for a direct functional relationship between the vegetative nucleus and generative cell.

Materials and methods

Plants of *Medicago sativa* L. (alfalfa), growing conditions, and tissue processing for this study were the same as those used for a previous study on alfalfa generative cells (Zhu *et al.* 1990). Of the fixation schedules used, including five variations of glutaraldehyde/osmium tetroxide (Zhu *et al.* 1990), only $KMnO_4$ (2% aqueous for 15 min) offered the clarity of nuclear envelope and pores necessary for the present study (Fig. 5).

Measurements for the estimation of nuclear pore density (number of pores μm^{-2} of nuclear envelope) were made from electron micrographic prints having a final magnification of ×7500 to ×16 000. Nuclear pore diameter was determined from the largest pore profile in sections from early (Figs 1, 3; pore diameter=70 nm) and mature (Figs 2, 4; pore diameter=50 nm)

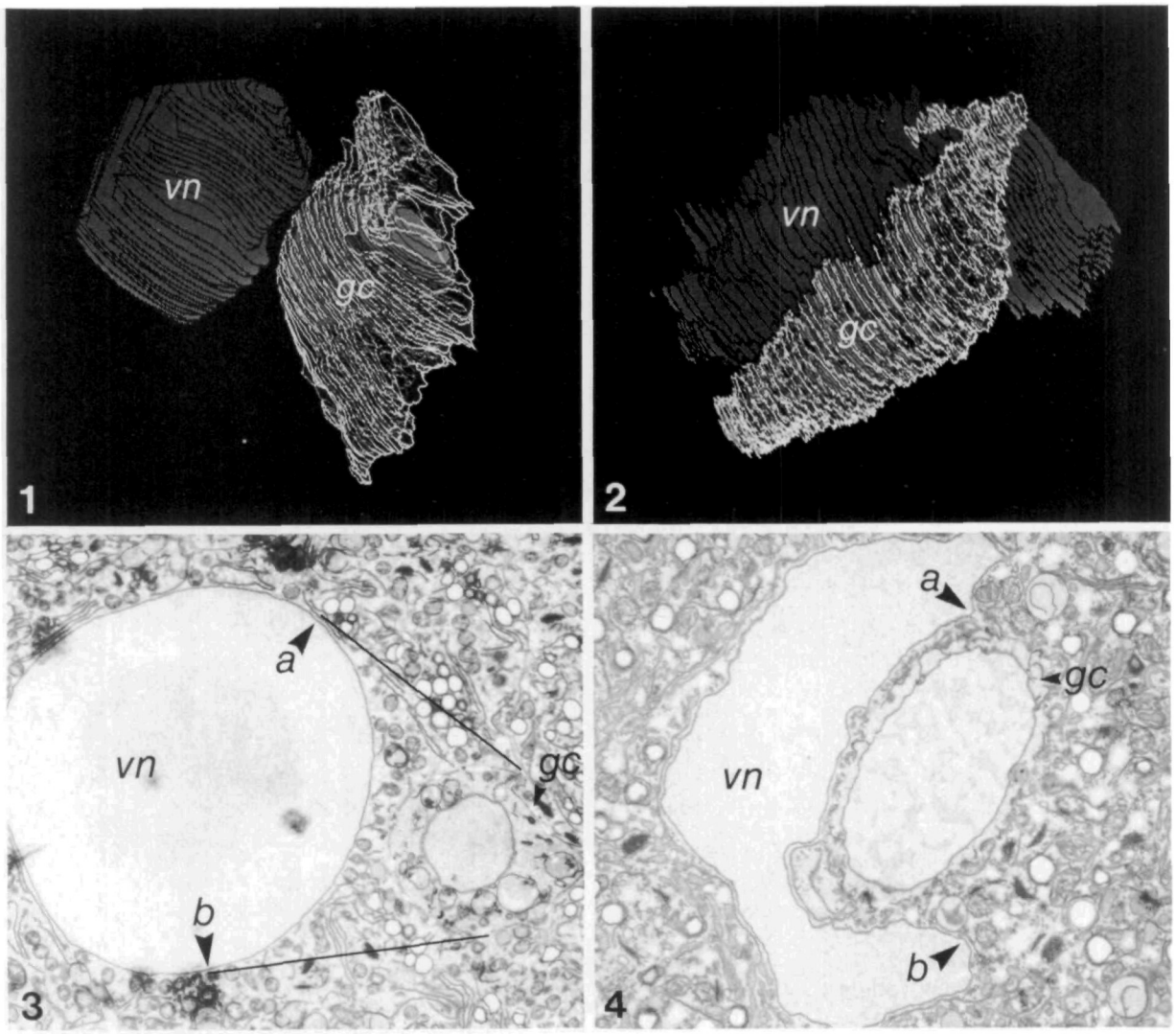

Fig. 1. Computer-generated reconstruction of the generative cell (gc) and a portion of the vegetative nucleus (vn) at an early stage of pollen maturation in alfalfa. Note that the vn and gc are in close proximity, but not yet intimately associated as in Fig. 2. ×3000.

Fig. 2. Computer-generated reconstruction of generative cell (gc) and vegetative nucleus (vn) at pollen maturity in alfalfa. Note that the vn partially surrounds the gc. ×5000.

Fig. 3. Transmission electron micrograph showing an early stage of pollen maturation, as in Fig. 1. That portion of the vegetative nuclear envelope designated as 'toward the generative cell (gc)' is included within the lines indicating points *a* and *b*. The rest of the vegetative nuclear envelope is designated as 'away from the generative cell'. vn, vegetative nucleus. ×4100.

Fig. 4. Transmission electron micrograph showing the association of the vegetative nucleus (vn) and generative cell (gc) at pollen maturity, as in Fig. 2. The portion of the vn envelope 'toward the gc' is included between points *a* and *b*. The rest of the vn envelope is designated as 'away from the gc'. ×5400.

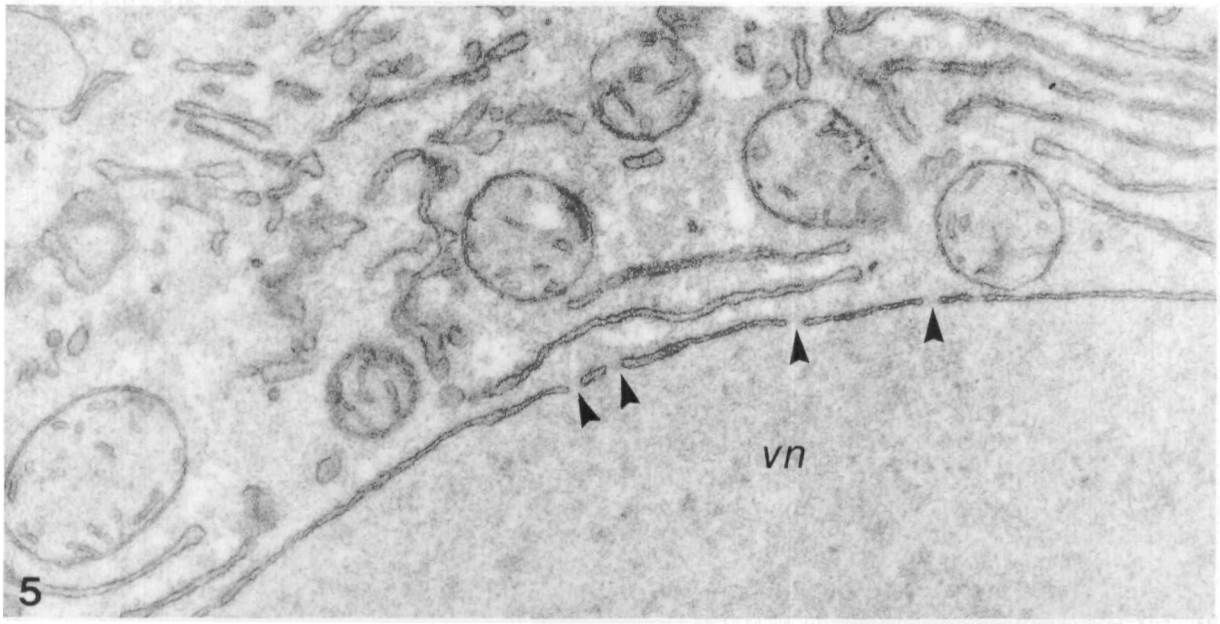

Fig. 5. Transmission electron micrograph showing the vegetative nuclear envelope and pores (unlabeled arrowheads). vn, vegetative nucleus. ×28 500.

stages of pollen development. The early stage was designated as that stage when the vegetative nucleus and generative cell are close enough to be able to determine the side of the vegetative nucleus toward and away from the generative cell, but before the vegetative nucleus begins to surround the generative cell. The mature stage was designated as that stage when the vegetative nucleus is intimately associated with and partially encompasses the generative cell. For a given stage, pore diameter was very consistent. Pore density (number of pores per μm^2) was calculated according to a method described by Steer (1981). The proportion of the nuclear surface occupied by pores was estimated by measuring the proportion of the sectioned envelope profile (P) occupied by pores, i.e. total pore diameter (μm)/total length of envelope (μm). Then the number of pores per μm^2 equals P/pore area in μm^2. The length (μm) of nuclear envelope in a given section was measured using a digitizing tablet and the computer program of Young *et al.* (1987). The validity of Steer's method for estimating pore density was tested using actual pore counts from a series of ultrathin sections of an early vegetative nucleus for which the surface area had been determined. The surface area was determined by entering a tracing of the nuclear envelope in each section, as recorded on photographic prints, into an IBM-AT computer *via* a digitizing tablet. The surface area was then calculated by a combination of the computer program of Young *et al.* (1987), and one written by M. L. Rusche (unpublished). Steer's method proved to be very accurate and, moreover, the pore densities of this study fall within the expected range of pollen nuclear pore densities, on the basis of those found in the literature.

For the early stage, the two portions of the vegetative nuclear envelope, i.e. toward and away from the generative cell, were delimited as shown in Fig. 3. For the mature stage, the two nuclear envelope portions were delimited as shown in Fig. 4. Data were collected within the mid region of the nucleus from 12 vegetative nuclei at the early stage, and from 45 vegetative nuclei at the mature stage (Fig. 6).

For comparisons of nuclear pore densities between vegetative and generative nuclei, measurements were taken at random from 12 nuclei of each type at the early stage. For the mature stage, 45 vegetative nuclei and 28 generative nuclei were measured (Fig. 7).

Surface areas and volumes of 20 vegetative nuclei (ten each from the early and mature stages) were estimated from light micrographic prints of serial semithin sections (1 μm; Table 1)

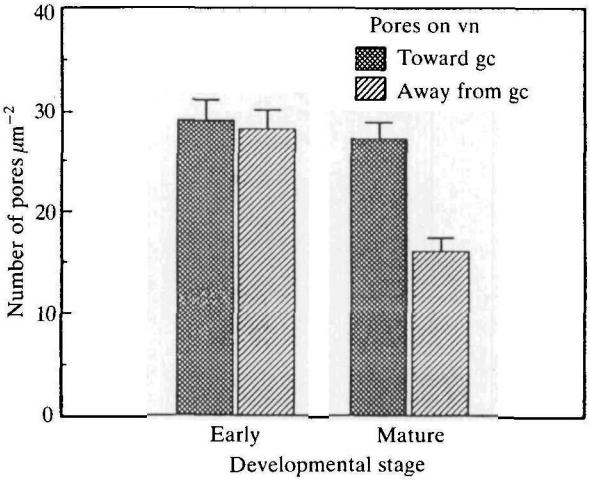

Fig. 6. Comparison of vegetative nucleus (vn) pore densities on the surface toward and away from the generative cell (gc) at early and late stages of pollen maturation in alfalfa. Values represent means plus standard error of the means. For the early stage, 12 nuclei were measured; for the mature stage, 45 nuclei were measured. The means are not significantly different at the early stage ($P>0.05$). At the mature stage, the means are significantly different ($P<0.001$).

using the computer programs of Young *et al.* (1987) and Rusche (unpublished) as described above.

Serial ultrathin sections of the vegetative nucleus and generative cell were used to produce the three-dimensional reconstructions of Figs 1 and 2. After making photographic prints of each section, tracings of the desired structures were digitized as above, and the three-dimensional images were produced by the program of Young *et al.* (1987). Photographs were then taken directly from the screen of the computer monitor.

A two-factor analysis of variance and t-tests were conducted for comparisons of differences between mean nuclear pore densities. The t-test was used to compare mean vegetative nuclear surface areas and volumes between early and late pollen stages.

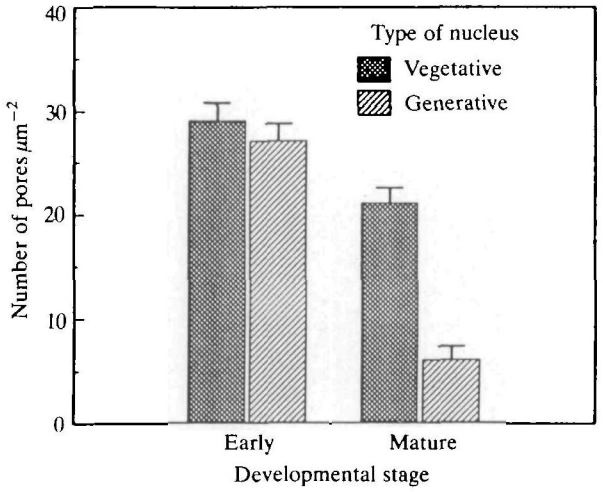

Fig. 7. Comparison of nuclear pore densities on vegetative and generative nuclei at early and mature stages of pollen development in alfalfa. Values represent means plus standard error of the means. For the early stage, 12 nuclei of each type were measured; for the mature stage, 45 vegetative nuclei and 28 generative nuclei were measured. Means are not significantly different at the early stage ($P>0.05$). At the mature stage, the means are significantly different ($P<0.001$).

Results

At an early stage, before the vegetative nucleus and the generative cell have formed a close physical association (Figs 1, 3), pore density (mean pore number±standard deviation of the mean μm^{-2} of nuclear envelope) does not differ significantly between the side toward (29±7; range 20 to 41; $P>0.05$) and the side away (28±7; range 17 to 37) from the generative cell (Fig. 6). However, in mature pollen, when the vegetative nucleus partially surrounds the generative cell (Figs 2, 4), mean vegetative nuclear pore density is significantly less on the side away from the generative cell (16±9; range 3 to 32; $P<0.001$), compared to that toward the generative cell (27±12; range 4 to 53; Fig. 6).

When comparing the side of the vegetative nucleus away from the generative cell in early and mature stages (Fig. 6), mean nuclear pore density is significantly reduced at the mature stage ($P<0.001$). Mean vegetative nuclear pore density is not significantly different between early and mature stages on the side toward the generative cell (Fig. 6; $P>0.05$). Mean nuclear pore density of the entire vegetative nucleus is significantly less at pollen maturity (21±10; $P<0.02$) than at the early stage (29±6; Fig. 7).

Mean pore density on the generative nucleus at the early stage is 27±6 (range 20 to 43). At pollen maturity, mean pore density on the generative nucleus is significantly less (6±7; range 0 to 31; $P<0.001$) than at the early stage (Fig. 7).

Although there is a significant decrease in vegetative nuclear volume ($P<0.001$), the surface area of the vegetative nucleus does not differ significantly between the early and late stages of this study (Table 1; $P>0.05$).

Discussion

Since the existence of nuclear pores was first reported (Callan *et al.* 1949; Callan and Tomlin, 1950), they have been thought to be a major passageway for materials between the nucleus and the cytoplasm. Experimental studies have since demonstrated that macromolecular traffic into and out of the nucleus occurs through the pores and is regulated by an active transport system requiring ATP and involving specific signal sequences (Newport and Forbes, 1987; Newmeyer and Forbes, 1988; Richardson *et al.* 1988). In addition to functioning as a site of exchange of materials between the nucleus and cytoplasm, nuclear pores are reported as the likely sites of ribosome assembly (Mepham and Lane, 1969; Hanzely and Olah, 1973), and the sites of initiation of DNA replication (Maul *et al.* 1972).

The density of pores μm^{-2} of nuclear envelope, as well as the total number of pores/nucleus, are known to change with developmental stage and/or with physiological states in both plant and animal systems (Newport and Forbes, 1987; Lott and Vollmer, 1975; Crevecoeur *et al.* 1982). Within angiosperm pollen the larger, more active vegetative nucleus has been observed to have a much higher pore density than that of the generative nucleus (Mepham and Lane, 1969; LaFountain and LaFountain, 1973). During the first 25 min after germination of *Tradescantia paludosa* pollen, the vegetative nucleus synthesizes about twice as much RNA as does the generative nucleus (LaFountain and Mascarenhas, 1971). During this same period, nuclear pore density on the vegetative nucleus is approximately twice that of the generative nucleus (LaFountain and LaFountain, 1973). In germinating pollen of *Hordeum vulgare*, nuclear pore density is approximately four times greater on the vegetative nucleus than on sperm nuclei (Mogensen and Wagner, 1987). The density of nuclear pores on the vegetative nucleus decreases during pollen activation in both *Tradescantia paludosa* (from 14 μm^{-2} to 11 μm^{-2}; LaFountain and LaFountain, 1973) and *Nicotiana tabacum* (from 30 μm^{-2} to 15 μm^{-2}; Wagner *et al.* 1990). In the latter case, the decrease in pore density was correlated with results from biochemical studies showing a decrease in RNA production.

Results of the present study on alfalfa also show a marked difference in pore density between the nuclei of mature pollen, the vegetative nucleus having 3.5 times more pores μm^{-2} than the generative nucleus (Fig. 7). A significant decrease in overall pore density on the vegetative nucleus was found to occur during pollen maturation (Fig. 7) as also reported by Mepham and Lane (1969) for *Tradescantia bracteata*, but not quantified. In

Table 1. *Comparison of surface areas and volumes of vegetative nuclei (vn) at early and mature stages of pollen development in alfalfa*

	Early vn ($n=10$)			Mature vn ($n=10$)		
	Mean	S.D.	Range	Mean	S.D.	Range
Surface area (μm^2)	306.6	61.9	249.8–453.5	292.9	34.3	250.9–354.2
Volume (μm^3)	528.5	112.2	420.4–799.9	364.1*	74.0	256.2–488.8

*Significantly less than early vn ($P<0.001$).

our study, however, we have shown that pore density is nearly the same on the vegetative and generative nuclei at an early stage. Moreover, we have demonstrated that the observed reduction in pore density on the vegetative nucleus is primarily due to a decrease in pore density specifically on the surface of the nucleus that is not physically associated with the generative cell (Fig. 6).

Since the surface area of the vegetative nucleus does not change significantly from the early to mature stages of this study (Table 1), the observed decrease in nuclear pore density on the surface away from the generative cell is apparently due to a decrease in actual pore number. The maintenance of a high nuclear pore density throughout pollen maturation on the surface facing the generative cell may be indicative of nuclear synthetic activity that could influence generative cell development. Since the physical association between the vegetative nucleus and generative cell is sustained during pollen tube growth in alfalfa (Zhu *et al.* 1990), the vegetative nucleus may also play a role in postpollination generative cell differentiation and sperm cell morphogenesis.

Recent studies, which used probes from cDNA libraries constructed to poly(A) RNA isolated from mature pollen of *Tradescantia* and *Zea*, have determined more precisely the stages at which transcription of mRNA occurs during pollen development. On the basis of the timing of their activation, there are at least two sets of genes being expressed. One set becomes active shortly after microspore mitosis, and mRNAs accumulate increasingly throughout the rest of pollen development. These genes are specific to pollen. Another group of genes, which are active in sporophytic tissues as well as in pollen, become active during early microspore interphase. The mRNAs reach their highest concentration at late microspore interphase, then decrease significantly in mature pollen (Mascarenhas, 1989). Another recent study (Detchepare *et al.* 1989), on *Brassica oleracea*, has demonstrated two periods of protein synthetic activity that correspond to the mid and later stages of pollen development.

It seems reasonable that localized distribution of nuclear pores could act to direct nucleocytoplasmic transport to rather restricted areas of the cell. Our results do not provide information on the cause and effect relationship between nuclear pore concentration and the location of gene activity within the nucleus. However, conceivably, the pattern of nuclear pore density and distribution observed in the present study reflects not only temporal, but also spatial, differences in gene activation during pollen development. Genes active only at the early stage may be transcribing throughout the vegetative nucleus, whereas those involved with generative cell differentiation may remain active up to pollen maturity and be specifically positioned near the surface of the nucleus in close proximity to the generative cell. Such a hypothesis may be testable using the technique of *in situ* hybridization with probes for specific genes or gene products, similar to what has been done to detect rDNA in cultured rat kidney nuclei (Jimenez-Garcia *et al.* 1989), and to locate specific chromosomes within interphase nuclei of human lymphocytes (Pinkel *et al.* 1988). This hypothesis is consistent with results from studies on mammalian cells that indicate that interphase chromosomes occupy distinct domains within the nucleus (Cremer *et al.* 1982; Hens *et al.* 1983); and with the statement of Schwarzacher *et al.* (1989) that 'The spatial positioning of the chromosomes at interphase may influence major cell functions, such as aspects of gene expression'.

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