INTRODUCTION

In the past two decades in vitro studies have enormously amplified our knowledge of the molecular details of eukaryotic gene expression. However, the mechanisms by which the control of transcription and post-transcriptional processes are integrated on interphase chromosomes within nuclei in vivo are as yet unknown. Higher order chromatin structure and the nuclear architecture as a whole have a profound impact on gene expression. Correct initiation at rates approaching those found in vivo have never been accomplished in vitro with any of the soluble transcription systems, and the largest stimulatory effects observed to date in vitro are small when compared with the large differences in the rates of transcription that occur during development (Ivarie et al., 1983). Phenomena like position-effect variegation (Henikoff, 1990) and transvection (Wu and Goldberg, 1989), as well as numerous observations with transgenes, suggest that the placement of genes within nuclei is nonrandom and that the activity of genes is dependent on their spatial arrangement and chromosomal environment. It has even been hypothesized that a strictly regulated intranuclear positioning of genes, coupled with a directed nuclear export of their transcripts, may result in an asymmetric distribution of gene products, thereby creating cell polarity (Blobel, 1985). While this would suggest a comparatively inflexible nuclear architecture, in vitro studies have demonstrated considerable movement and rotation of chromatin inside nuclei (De Boni and Mintz, 1986; Park and De Boni, 1991).

What do we know about the placement of transcribed genes within nuclei? A nonrandom distribution of active genes with a bias towards the nuclear periphery has been reported by Hutchison and Weintraub (1985). Using electron microscopy and in situ hybridization methods, fiber tracks of specific mRNAs in transit from the site of synthesis to nuclear pores have been observed (Lawrence et al., 1989; Huang and Spector, 1991; Meier and Blobel, 1992; Xing et al., 1993; Carter et al., 1993), suggesting that the nuclear architecture may play a role in integrating transcriptional and post-transcriptional processes. The intranuclear distribution of hnRNP proteins, snRNP proteins, splicing factors and snRNA has been investigated in detail (e.g. Choi and Dreyfuss, 1984; Spector et al., 1991; Carmo-Fonseca et al., 1991; Huang and Spector, 1992). However, to our knowledge, these observations have been made only on fixed cells. Data derived from fixed material are readily susceptible to artifacts, since interphase chromosomes are...
highly complex and labile during any kind of manipulation. RNA, DNA and protein, each concentrated in the nucleus to about 0.1 g/ml, have an inherent tendency to precipitate upon changes in the ionic environment. During fixation, components may be extracted and chromatin structure may be rearranged drastically. For example, the conflicting results obtained by different groups attempting to localize the intranuclear sites of splicing may reflect variations in their fixation methods (Spector, 1990; Carmo-Fonseca et al., 1991; but see Huang and Spector, 1992). Fixed material, in addition, lacks the temporal dimension and only major changes with time may be inferred from the analysis of a large number of samples. Many proteins, already present within cells, are recruited to transcription sites during activation. One would like to know their temporal order of appearance, intracellular origin and dynamic behavior throughout the cell cycle. It follows that sensitive and nondisruptive methods are required for a four-dimensional analysis of such factors in vivo.

The distribution and fate of specific proteins in a developing and living organism has been studied most successfully in early embryos of Drosophila. The method involves microinjection of a fluorescently labeled probe into the embryo and the use of either confocal optical sectioning microscopy (reviewed by Brakenhoff et al., 1989) or deconvolution of wide-field microscope images (reviewed in Agard et al., 1989) for high temporal and spatial resolution of the fate of these molecules. The in vivo distribution of labeled nuclear proteins such as histones, the lamins, topoisomerase II and tubulin has been studied by fluorescence microscopy (Hiraoka et al., 1989; Kellogg et al., 1988; Minden et al., 1989; Sullivan et al., 1990; Swedlow et al., 1993). Microinjection of fluorescently labeled antibodies or antibody fragments can serve the same function, as has been demonstrated with anti-tubulin (Warn et al., 1987). In addition to their use for indirect “protein tagging”, neutralizing antibodies can serve to disrupt the function of a specific antigen in the embryo, as we have demonstrated recently with anti-topoisomerase II antibodies (Buchenau et al., 1993).

Here we describe for the first time the spatial arrangement of transcribing genes in interphase nuclei of living Drosophila embryos. The monoclonal antibody, Bj6, used to label these sites is specific for a chromatin-associated Drosophila protein (NONA) of Mr 82,000, encoded by the no-on transient A (nonA) gene, which shows homology to single-stranded nucleic acid binding proteins (Jones and Rubin, 1990; Besser et al., 1990; Stanewsky et al., 1993). On polytene chromosomes, the NONA protein is present in many puffs, the cytologically visible sites of intense RNA synthesis (Frasch, 1989). For the intemolt puff at 3C11-12, it was found that NONA protein binding depends on the presence of the enhancer region of the Sgs-4 gene located within this puff. This suggests that NONA may function in the initiation or maintenance of transcriptionally active chromatin structure (Saumweber et al., 1990). Microinjection of fluorescently labeled anti-NONA antibodies into early Drosophila embryos permitted us to follow the fate of this protein by confocal laser scanning microscopy throughout embryonal development and with high spatial resolution.

MATERIALS AND METHODS

Drosophila strains and cell lines

Drosophila melanogaster wild-type strain Oregon R-P2 (Allis et al., 1977) was used throughout. Flies were raised on a medium of cornmeal, agar, soy bean meal, malt extract, molasses, yeast, 0.5% (v/v) propionic acid and the mold inhibitor methyl p-hydroxy benzoate (Nipagin\textsuperscript{\textregistered}, Cesar and Lorentz, Hilden, Germany), at room temperature.

Antibodies

The generation and propagation of the NONA antibody-secreting mouse cell line Bj6 has been described (Frasch and Saumweber, 1989). The antibody Bj6 was purified over a protein G Sepharose column (Pharmacia, Freiburg, Germany) before further use. Fab fragments of Bj6 antibody and mouse IgG were prepared as described previously (Buchenau et al., 1993). Commercial antibodies were obtained from Jackson Immunotechnology, supplied by Dianova (Hamburg, Germany). The following antibodies were used: (1) affinity-purified rhodamine-coupled goat anti-mouse IgG (H+L chain specific) for indirect immunofluorescence; (2) affinity-purified fluorescein- and rhodamine-labeled goat anti-rabbit IgG (or Fab) for control injections; and (3) affinity-purified alkaline phosphatase (AP)-coupled F(ab)\textsubscript{2} fragment of goat anti-mouse IgG (H+L specific) for western analysis.

Bj6 and control antibodies were labeled with tetramethyl-rhodamine-isothiocyanate (TRITC; Molecular Probes, Eugene, OR) to a ratio of about two rhodamine residues per antibody molecule. Unbound dye was removed from the labeled antibodies by gel exclusion chromatography on Sephadex G50 in phosphate-buffered saline. All antibodies were concentrated before injection by centrifugation in Centricon 10 tubes (Amicon). The labeling ratio was determined photometrically using the molar absorption coefficients ε\textsubscript{550} 6×10\textasciitilde{4} mol\textasciitilde{1} cm\textasciitilde{1} for TRITC and ε\textsubscript{280} 2.1×10\textasciitilde{5} 1 mol\textasciitilde{1} cm\textasciitilde{1} for the coupled antibody.

Western analysis and immunostaining

Antibody-injected Drosophila embryos were processed directly or following incubation on coverslips for 1-4 hours under Voltalef oil (Atochem, Pierre-Bénite, France). The oil was removed and the embryos were homogenized in SDS-sample buffer (Laemmli, 1970). Embryo and marker proteins were run on a 9% to 17% gradient sodium dodecyl sulfate-polyacrylamide gel according to that of Campos-Ortega and Hartenstein (1985). Microinjected embryos were fixed in 95% ethanol for 20 min and dehydrated in a graded series of ethanol to 100%. Embryos were hand-dechorionated, glued onto a coverslip and desiccated overnight. They were then embedded in Voltalef oil, using glass needles with an opening of 2-4 μm and hand-injected as described previously (Buchenau et al., 1993). Commercial antibodies were concentrated before injection by centrifugation in Centricon 10 tubes (Amicon). The labeling ratio was determined photometrically using the molar absorption coefficients ε\textsubscript{550} 6×10\textasciitilde{4} mol\textasciitilde{1} cm\textasciitilde{1} for TRITC and ε\textsubscript{280} 2.1×10\textasciitilde{5} 1 mol\textasciitilde{1} cm\textasciitilde{1} for the coupled antibody.

Microinjection, microscopy and image analysis

Microinjection of Drosophila embryos was carried out as described previously (Buchenau et al., 1993). Briefly, embryos were hand-dechorionated, glued onto a coverslip and desiccated for 10-20 min. The injection was then performed under 10× Voltalef oil using glass needles with an opening of 2-4 μm. The injected volume was between 200 and 500 pl. The Bj6 concentration in the injection needle was 1.5 mg/ml. For high resolution studies the injected embryos were observed on a cooled microscope stage at 17°C. A confocal laser scanning microscope (CLSM; Zeiss model LSM10) was used for collecting digital images of optical sections through living as well as fixed embryos. Either a x25 NA 0.8 Plan-Neofluar or a x40 NA 0.9 Plan-Neo-
The distribution of the NONA protein in fixed embryos has been investigated with standard epifluorescence microscopy (Frasch and Saumweber, 1989; Frasch et al., 1986). To compare these data with our in vivo studies, we first documented the NONA distribution in fixed, whole-mount Drosophila embryos by CLSM. In brief, the results were found to be very similar to those described previously. The NONA protein is stored maternally in the egg. During the first 13 nuclear divisions it was localized in the cytoplasm as shown for an embryo in nuclear cycle 12 (Fig. 1A). The protein shifted into the nuclear compartment prior to gastrulation, at the onset of transcription in the embryo (Fig. 1B). This progression is seen more clearly at higher magnification (Fig. 1C-E). In interphase of nuclear cycle 11, NONA was excluded from the nuclei, which appeared as dark holes in a brightly stained cytoplasm (Fig. 1C). A small amount of NONA protein could be seen in interphase nuclei of cycle-13 embryos, distributed in a number of weakly staining intranuclear loci. The bulk of the NONA protein, however, remained in the cytoplasm at this stage (Fig. 1D). This same weak speckled pattern was observed in a few cycle-14 embryos, which were presumably in the early part of interphase. In contrast, the majority of cycle-14 embryos showed most of the NONA protein in the nuclear compartment, where it was differentially distributed on the chromatin (Fig. 1E; see below).

Low magnification images of fixed embryos from nuclear cycle 14 and the immediate subsequent stages demonstrated a pattern of NONA protein in all nuclei without obvious variations between different cell types (Fig. 1F). For comparison, we show a fixed embryo at a similar stage, documented by conventional epifluorescence microscopy (Fig. 1G). The only difference observed is a blurring of the conventional image due to the presence of out-of-focus information, which is largely removed by the confocal technique. We conclude that observations for the NONA distribution in fixed embryos made by CLSM are very similar to those made by conventional microscopy.

Studies in living embryos at low and medium resolution

Before exploring the temporal and spatial distribution of NONA protein in vivo by introducing rhodamine-labeled, anti-NONA antibodies (Bj6) into early embryos, we performed a number of control experiments to validate the methodology. The integrity of antibodies in the embryos was tested by western analysis at different time points following injection (see Materials and Methods). The injected antibodies did not change in amount, nor was degradation of Bj6 heavy and light chains observed for up to 4 hours post-injection (the longest time period tested; data not shown). The developmental stage of the embryo at the time of injection was found to be important. Since early cleavage stage embryos were more sensitive to manipulation (see below), most embryos were injected during nuclear cycle 8 or later. The position of the microinjection was not found to be critical. Injection at 50% egg length and 100% dorsal position was found to be most convenient and therefore used in most experiments. The antibodies were allowed to distribute within the embryos for approximately 20 minutes before the first data were collected. This allowed sufficient time for the antibody molecules to spread throughout the embryo, as has been measured following injections of fluorescently labeled antibodies, antibody fragments and dextrans of different molecular weight (Buchenau, 1991).

The distribution of fluorescently labeled nonspecific mouse IgG in living embryos was studied as a further control. If injected during early syncytial blastoderm, nonspecific antibodies were excluded from the nuclei and the latter appeared as dark holes in an otherwise brightly staining cytoplasm (Fig. 2A). This distribution remained over several nuclear cycles and into gastrulation (Fig. 2B). After more than 10 hours of embryo development, non-specific control antibodies stained both the nuclear and cytoplasmic compartments equally. In contrast, fluorescently labeled Fab fragments of non-specific mouse IgG, because of their smaller size, entered the nuclei already in the nuclear cycle immediately following injection and were homogeneously distributed in the embryo from this time on (Fig. 2C).

The distribution of microinjected Bj6 fluorescent anti-
body was clearly different from these controls and reflected the NONA protein pattern predicted from our studies of fixed embryos. Following injections early during nuclear migration, Bj6 antibody was localized exclusively in the cytoplasm up to nuclear cycle 13 (Figs 3A, 4A), a fact which held true for Bj6 Fab fragments as well (data not shown). As shown with the control antibodies, Fab fragments of this size were capable of entering the nuclei. Thus, Bj6 was actively excluded from the nuclei, showing that its distribution reflects that of the NONA protein.

Starting with interphase cycle 13, increasing amounts of Bj6 antibody accumulated within nuclei. As the NONA protein entered the nuclei in late blastoderm, the antibodies were also targeted to the nuclear compartment, indicating that they did not impair the redistribution of the associated protein. This nuclear accumulation was gradual with time, as shown in a series of higher magnification frames (Fig. 4) taken from a single living embryo as it progressed through cycles 12 to 14. In cycle 13 a few labeled loci were found inside nuclei, but the bulk of the NONA protein remained in the cytoplasm (Fig. 4C). Similarly, early in cycle 14 only a few weakly labeled loci were found within nuclei (Fig. 4E). Eight minutes later, in cycle 14, the NONA protein was distributed equally between both compartments and the embryo stained homogeneously (Fig. 4F). Thereafter a differential nuclear distribution was apparent in which a number of bright intranuclear loci could be discerned (Fig. 4G). Later in cycle 14, when the formation of cell membranes was in progress, this pattern became more prominent (Figs 3B, 4H); it was also characteristic for the NONA distribution at later stages (Figs 3C,D, 5).

At low resolution both immunofluorescence staining of fixed embryos and in vivo labeling gave similar results for early stages of embryo development (compare Fig. 1A,B to 3A,B). However, at later development stages we noted striking differences. Particular nuclei in late embryos labeled in vivo were considerably brighter than others (Fig. 3D). These nuclei were in tissues such as the amnioserosa and in a subset of cells from the ventral cord (not shown). In almost all embryos the nuclear relocation of NONA occurred during nuclear cycle 14 as a gradual process. However, we wish to emphasize that we noted individual differences in the timing of these events. As an extreme case we observed a single embryo that still contained the majority of NONA in the cytoplasm at the end of the gastrula stage. In this embryo the shift into the nuclear compartment
NONA distribution in vivo occurred in the immediate following stage (data not shown), and the embryo proceeded to develop normally.

During the mitotic phases of the nuclear cycles 12 and 13 the in vivo antibody staining was homogeneous, suggesting an even distribution of the NONA protein in the cytoplasm (Fig. 4B,D). The first embryonic regions to undergo postblastoderm cell division are the mitotic domains anterior to the cephalic furrow (Foe, 1989; Campos-Ortega and Hartenstein, 1985). Here it was especially easy to follow the fate of microinjected Bj6 antibodies throughout cell division. Fig. 5 shows a series of confocal images taken at a single focal plane, that cover the anterior part of an embryo during gastrulation. NONA was first present in all nuclei anterior to the cephalic furrow (Fig. 5A). Upon onset of cell division, the nuclear label became weaker in this region and most of the NONA protein appeared to be distributed homogeneously in the cells (Fig. 5B,C). Between the anaphase/telophase boundary and the first sign of differential nuclear staining in the following interphase we noted a gap of 7-10 minutes in which staining appeared homogeneous. Thereafter NONA began gradually to accumulate within nuclei again (Fig. 5D). The cytoplasmic dispersion of the NONA protein-antibody complex during mitosis and its relocalization to the nuclei took place repeatedly through subsequent mitoses (data not shown). The staining was apparent in all nuclei of developing embryos until at least stage 15, a time by which most cells have completed 3-4 mitoses following gastrulation (compare Fig. 3D). These observations suggest that injected Bj6 antibodies are biologically active in recognizing and binding the NONA protein for at least 24 hours post-injection.

The distribution of NONA in interphase embryonic nuclei at high magnification

The NONA protein is present in a series of puffs and minipuffs on polytene chromosomes that are known to be transcriptionally active (Frasch, 1989). A speckled staining had already been observed in nuclei of fixed Kc-cells and whole-mount embryos by conventional immunofluorescence microscopy using Bj6 antibodies (H.S., unpublished observations). The superior resolution of the CLSM enabled us to study the NONA distribution in interphase nuclei of fixed and living embryos in greater detail.

We examined the nuclei of the amnioserosa, an extraembryonic tissue, the cells of which do not divide following blastoderm formation but which develop large nuclei. In fixed embryos the NONA protein was present in a number of discrete sites in these nuclei. Fig. 6A shows a stereo pair reconstruction of a representative nucleus obtained in a fixed embryo. We count approximately 100 loci of different size and intensity that are arranged three-dimensionally in the nucleus; note that these loci are present throughout the nuclear volume. The NONA spots did not match the most intense regions of DNA staining by the fluorescent dye Hoechst 33342, indicating that they are not correlated with sites of condensed DNA. Interestingly, the high intensity NONA staining often appears to be arranged along fiber tracks which can be followed over considerable distances in the nucleus. These tracks bend and form loops reminiscent of the chromosome loops described in polytene nuclei (Hochstrasser et al., 1986). This spatial arrangement of the NONA protein, however, is not unique to amnioserosa nuclei. Fig. 6B shows a stereo view of a reconstructed diploid epidermal nucleus in a fixed embryo. About 90 distinctly stained sites of different size and intensity can be discerned here as well. The sites again are arranged along fiber tracks bending and looping within the nucleus.

Stereo pairs of an amnioserosa nucleus recorded from a living embryo are shown in Fig. 7A-D. In comparison with fixed embryos a number of differences were immediately apparent. In vivo a smaller number of loci, which on average were larger in size than those seen in fixed embryos, account for the bulk of NONA protein. The number of distinct loci was in the range of about 20-30 sites per nucleus. In contrast to fixed nuclei, the majority of the NONA loci in vivo lay towards the nuclear periphery and often several sites were found grouped together. A stereo pair reconstruction of a nucleus of a living ectodermal cell is shown in Fig. 7E,F. Here in particular, the above-mentioned clustering of the NONA containing loci is obvious. Again, sim-
ilar to the case of the amnioserosa nuclei, the NONA distribution in the in vivo ectodermal nucleus is more peripheral, clustered and shows larger intensity differences as compared with similarly situated, fixed nuclei.

The temporal distribution of NONA protein in vivo could be followed by recording stacks of optical sections from single nuclei at different time points and viewing the reconstructed nuclei, as shown in Fig. 7. For technical reasons the minimum time separating two image stacks is presently 2 minutes. The position of stained loci did change somewhat during this time (Fig. 7A,B). Observing amnioserosa nuclei over longer time intervals we could detect changes in pattern which were largely compatible with movements of single sites or groups of sites within the nuclei with respect to each other (Fig. 7B-D). Overall the number and staining intensity of most of the loci remained approximately constant during an observation period of 15 minutes, as shown here. However, one can discern sites at new locations which cannot be easily explained by simple movements of the chromatin (labelled by arrows in Fig. 7C,D). More dramatic and rapid changes in the NONA distribution were particularly evident in the case of in vivo temporal recordings of ectodermal nuclei (for example, see Fig. 7E,F, where the two image stacks are separated by only three minutes).

Fig. 3. NONA distribution in living embryos. Embryos were injected with rhodamine-labeled monoclonal antibody Bj6 (rh-Bj6, 1.5 mg/ml in PBS) to visualize the NONA protein in vivo (compare with Fig. 1). (A) Nuclear cycle 13. (B) Nuclear cycle 14. (C) Gastrulation (stage 6). (D) Retracted germ band (stage 13). A represents an overlay of 3 optical sections, all other panels show single sections. Bars, 50 µm.

Fig. 4. Time course of the NONA shift into the nuclei in late blastoderm. Each image shows a single optical section of the same living embryo that had been injected with rh-Bj6 during syncytial blastoderm (A-H). The nuclear cycle or the process of mitosis (M) is indicated at the top right of each panel. Times relative to panel A (0 min) are: 5, 23, 33, 41, 49, 60 and 67 minutes, respectively. Note the cytoplasmic signal at the beginning of interphase 14. Objective, ×40 NA 0.9. CLSM-zoom, ×50. Bar, 20 µm.
**Bj6 antibodies do not interfere with normal development**

As shown above, injected Bj6 antibodies recognized their antigen in vivo and were active in binding throughout embryogenesis. We did not detect abnormalities in the development of the injected embryos and many of them were followed to the larval stage. Binding of the antibody did not negatively influence development, in contrast to our observations made by injecting inhibitory antibodies to topoisomerase II (Buchenau et al., 1993). To quantify this effect we determined the hatching rates for non-injected, buffer-injected, mouse-IgG-injected and Bj6-IgG-injected embryos as a function of their developmental age at time of injection (Fig. 8). Variations due to the degree of desiccation were kept at a minimum (see Materials and Methods). The four curves obtained for the different treatments are almost superimposable. The embryos were most sensitive to the microinjection procedure before nuclear migration and fewer than 50% of these young embryos survived. The survival rates increased with the age of the embryos used, reaching 80-100% in stages > 5. There was no apparent difference in survival between the different treatments, i.e. injecting Bj6-IgG was not more detrimental than dechorionization and mounting alone. Concentrations of up to 2 mg/ml mouse-IgG and Bj6-IgG in the injection solution (40-100 µg/ml in the embryo) were tolerated without detectable effects on development.

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**Fig. 5.** In vivo localization of NONA during cell division. The panels show a single section of the anterior part of a rh-Bj6-injected embryo at subsequent time points (stages 6-8). (A) Stage 6, before beginning of cell division. All cells show nuclear localization of NONA. (B) Stage 7, loss of nuclear signal in the dorsal-most region marks the beginning of cell division anterior to the cephalic furrow (cf). (C) Stage 7, homogeneous distribution of NONA in most cells anterior to cf. (D) Stage 8, nuclear localization of NONA anterior to cf starts to reappear. Objective, ×40 NA 0.9. CLSM-zoom, ×30. Bar, 20 µm.

**Fig. 6.** High resolution stereo images of the NONA distribution in formaldehyde-fixed embryonic nuclei. (A) Amnioserosa nucleus. (B) Epidermal nucleus from a gnathal segment. Each stereo image was reconstructed from 20 sections 0.3 µm apart. Objective, ×63 NA 1.4. CLSM-zoom, ×100 for A and ×115 for B. Bars, 1 µm.
Fig. 7. Time course of the NONA distribution in nuclei recorded from living embryos.

(A-D) Stereo images of an amnioserosa nucleus reconstructed from 15 optical sections 0.5 µm apart. The individual time points are separated by 2, 7 and 3.3 minutes, respectively. The right column shows illustrations in which examples of labeled loci are identified at each time point (nos 1-4). Arrows mark loci that might have (dis)appeared during the time of recording.

(E and F) Stereo images of a live ectodermal nucleus from the ventral part of an extended germ band embryo. Each stereo image was reconstructed from 12 optical sections 0.8 µm apart. The 2 time points are separated by 3 minutes. Objective, ×40 NA 0.9. CLSM-zoom, ×60 for A-D and ×70 for E and F. Bars, 1 µm.
DISCUSSION

Microinjection of fluorescently labeled antibodies allowed us to study the in vivo distribution of the NONA protein in Drosophila embryos. The injected antibodies were not visibly degraded and were active in binding throughout embryo development. In fact, in first instar larvae hatched from injected embryos we observed specific nuclear staining of the NONA-Bj6 complex in the epidermis. Antibodies are too large to penetrate the nuclear envelope by diffusion through nuclear pores and indeed non-specific mouse IgG remained in the cytoplasm following injection (Fig. 2). The active nuclear accumulation of Bj6-IgG after nuclear cycle 13 demonstrated that we were observing the antibody bound to the NONA protein in vivo (Figs 3, 4). Similarly, Bj6 Fab fragments, which are small enough to cross the nuclear pores by passive diffusion, remained bound to the NONA in the cytoplasm in syncytial stage embryos, whereas non-immune Fab fragment controls were always evenly distributed between the nuclei and the cytoplasm (Fig. 2). We conclude that Bj6 antibody is a reliable indicator of the NONA protein distribution in vivo.

How does NONA enter the nucleus? Our time lapse studies suggest that NONA protein-antibody complexes are actively transported across the nuclear envelope, since the nuclear staining starts post-mitotically and increases as interphase progresses, as observed in nuclear cycle-14 blastoderm embryos (Fig. 4). A similar process occurs at later stages (see Fig. 5). We observed a gap between the end of mitosis and the first time at which NONA-antibody complex could be seen in daughter nuclei. In this case as well, staining increased later in interphase. The kinetics of transport may be different for the NONA-antibody complex compared to the NONA protein alone because of the size difference, but we believe that the gradual nature of this process, which has only been possible to observe by our in vivo studies, is biologically significant.

The observation, that up through nuclear cycle 12 NONA is excluded from nuclei, but actively accumulates in nuclei from cycle 14 onwards, may be explained by post-translational modification of NONA or by changes in the nuclear targets for NONA. At this time it is not known which of these explanations is correct. Unmasking mechanisms of nuclear targeting signals are known in the rel-family of proteins like dorsal or NFκB (Blank et al., 1992). However, nuclear targeting signals have not yet been identified in the NONA protein. The kinetics of nuclear accumulation seen following mitosis provide evidence for the creation of intranuclear binding sites, perhaps as the result of assembly of transcriptional complexes. The nuclei may attain their full transcriptional competence gradually during interphase, and this would be reflected by the increasing accumulation of NONA. Note that a gradual expansion of Drosophila nuclei has been observed during interphase (Foe and Alberts, 1983) and postulated to result from chromatin decondensation as a consequence of increasing transcriptional activity.

Injected embryos, even at the highest antibody concentrations used, were as viable as the mock-injected controls. Either antibody binding does not inhibit NONA function or the protein is not required in the period of embryo development we have studied here. Both propositions are tenable. Loss-of-function mutations of nona are temperature-sensitive lethal. The zygotic requirement for the protein, however, is at the late pupal stage. Removal of the maternal component shows that NONA is essential in the female germ line. Experiments in which the amount of the maternal NONA protein was depleted suggest that before activation of the zygotic genome small amounts of the protein are required for normal embryo development (Stanewsky et al., 1993). We showed that the binding of the antibody to the NONA protein under our conditions during early development has no detrimental consequences, a prerequisite for the in vivo labeling studies described here (Fig. 8). In contrast, we have shown previously that antibody injections in the embryo can be used to inhibit the function of proteins essential in early development, like topoisomerase II (Buchenau et al., 1993). By careful tailoring of monoclonal antibodies, or by adjusting the concentration of injected antibodies, either inhibition or monitoring of normal functions in vivo can be pursued.

The spatial resolution of the CLSM is 0.25 μm in the X-Y plane and about 0.7 μm along the Z axis. The latter dimension could be improved by image deconvolution. However, even without added image processing, about 100 loci can be resolved in fixed nuclei (Fig. 6), a number comparable to that for NONA-binding sites seen on squashed polytene chromosomes (Frasch and Saumweber, 1989). The NONA loci are arranged on looping and bending fiber tracks, presumably reflecting the arrangement of the interphase chromatin in these nuclei. However, we emphasize that these structures probably do not correspond to the localization or architecture within the in vivo nucleus (Fig. 7). There are clear differences in the number and size of NONA sites observed in fixed cells and in vivo. In living nuclei the sites are fewer in number, are located more peripherally and are generally larger. In addition, the

Fig. 8. Hatching rates of embryos after control procedure (no injection, ○) and injection of Bj6 antibody (■), control antibody (△) and physiological buffer (□). For each curve >100 embryos have been used.
number and magnitude of the intensely labeled loci appear to be higher in vivo, although a complete quantitative assessment has not been made. These observations are best explained by assuming that active loci in vivo are more tightly clustered and that fixation disrupts or rearranges these chromatin-protein complexes. The functional significance of the in vivo clustering of active sites will have to be explored further. We note, however, that clustering of active sites has been observed in intact polytene nuclei (Gruenbaum et al., 1984). Our data are a cogent reminder that extrapolation of information obtained from fixed cells to the living organism can be dangerous.

NONA is co-localized with RNA polymerase II, as determined by indirect immunofluorescence on polytene chromosomes, and fewer than 10% of the puffs stained by anti-RNA polymerase II antibodies lack NONA (H.S., unpublished observations). We believe that the NONA labeling we have observed in vivo largely reflects the distribution of active transcriptional sites in interphase chromatin (Fig. 7). The preferential location of these sites in the nuclear periphery is interesting and is consistent with the data of Hutchison and Weintraub (1985) obtained in cells from mouse and chicken by completely different methods. Following the NONA distribution in single amnioserosa nuclei for longer times we noted a change in the pattern which is best explained by invoking restricted movement of active loci. Our data do not suggest that changes in the pattern in these nuclei originate from silencing or activation of additional genes during the observation period. Rather, the data are more consistent with limited local movement of parts of the chromatin within the nuclei. However, a more sophisticated comparison of the loci by image processing techniques of temporal sequences is required to shed further light on this issue. In other nuclei (such as the ectodermal neurons shown in Fig. 7E-F) we detected very rapid, dramatic and complex changes in the NONA distribution in vivo which could be explained either by rapid chromatin movements or by newly appearing and disappearing sites. We are presently pursuing studies to distinguish between these two possibilities. Our data clearly indicate that in vivo studies will be essential for unraveling complex questions about transcriptional activity and chromatin structure, and to ask, in addition, to what extent the spatial arrangement of active chromatin loci are conserved in cell lineages during development.

Drosophila embryos prior to blastoderm formation are advantageous in that many nuclei are present in a large syncytial cell with a peripheral cytoplasm. Injected probes can reach all of these nuclei. In addition, cells in a similar state of determination can be compared side by side and cells belonging to different lineages can be inspected in the same animal, in terms of their behavior and that of the probe, in an in vivo situation. This technique has obvious advantages over the use of cell cultures for similar types of studies.

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