Identification of the Chromosome Localization Domain of the *Drosophila* Nod Kinesin-like Protein

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Abstract. The nod kinesin-like protein is localized along the arms of meiotic chromosomes and is required to maintain the position of achiasmate chromosomes on the developing meiotic spindle. Here we show that the localization of ectopically expressed nod protein on mitotic chromosomes precisely parallels that observed for wild-type nod protein on meiotic chromosomes. Moreover, the carboxyl-terminal half of the nod protein also binds to chromosomes when overexpressed in mitotic cells, whereas the overexpressed amino-terminal motor domain binds only to microtubules. Chromosome localization of the carboxyl-terminal domain of nod depends upon an 82–amino acid region comprised of three copies of a sequence homologous to the DNA-binding domain of HMG 14/17 proteins. These data map the two primary functional domains of the nod protein in vivo and provide a molecular explanation for the directing of the nod protein to a specific subcellular component, the chromosome.

THE kinesins constitute a family of microtubule-based motors that use the energy of ATP hydrolysis to produce force and transport cargo along microtubules (Goldstein, 1993; Bloom and Endow, 1994). Kinesin holoenzymes are assembled from kinesin-like polypeptides that consist of a conserved motor domain linked to divergent tail domains. The motor domains bind to microtubules and generate force, whereas the tails are thought to function both as oligomerization and cargo-binding domains (Vale and Goldstein, 1990; Cole and Scholey, 1995).

Although different kinesins are known to transport different types of cargo (e.g., membrane-bounded vesicles or chromosomes), the mechanisms by which kinesins recognize and attach to their specific subcellular cargoes is not understood. For example, although the carboxyl-terminal tail domain of conventional kinesin has been shown to bind saturably to membranes in vitro (Skoufias et al., 1994), the molecular nature of the interaction between KHC and its membranous cargo in vivo has not been characterized. Clearly, if we want to understand mechanisms of intracellular transport, it will be important to identify the mechanisms that specify the targeting and binding of each kinesin and kinesin-like protein (KLP) to its cargo.

In this paper we provide a description of the mechanism by which one kinesin-like protein (nod) is localized to its proper cellular compartment, namely the chromosomes. The nod protein is required for correct segregation of the nonexchange chromosomes during *Drosophila* meiosis (Carpenter, 1973; Zhang and Hawley, 1990; Theurkauf and Hawley, 1992). Cytological studies have shown that the nod protein is required to control the proper movement of nonexchange chromosomes during spindle assembly at prometaphase and, in doing so, determines the position of these chromosomes on the metaphase spindles.

Sequence analysis revealed that the presumptive motor domain is localized at the amino-terminal end of the 666–residue nod polypeptide (Zhang et al., 1990). The carboxyl-terminal domain of nod shows no extensive homology to any other protein in the data base. However, it does contain three repeated sequences with homology to the 25–amino acid DNA-binding domain of the High Mobility Group (HMG)[1] proteins 14/17 (Afshar et al., 1995). HMG 14/17 proteins are members of a group of small nonhistone chromosomal proteins that, as predicted for the tail of nod, do not display obvious secondary structure but do bind DNA in a sequence-specific manner (Johns, 1982; Bustin et al., 1990). The significance of this homology is supported by the observation that the tail of the nod protein binds to AT-rich DNA efficiently in vitro (Afshar et al., 1995). Taken together these findings suggest that the tail of the nod protein binds to the DNA component of its chromosomal cargo.

Immunolocalization studies have demonstrated that the nod protein is localized along the entire length of meiotic chromosomes in stage 14 oocytes (Afshar et al., 1995). This discovery is consistent with genetic studies which identified multiple regions within a small region of X chro-

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1. *Abbreviations used in this paper*: HMG, high mobility group; nod, kinesin-like protein.

mosomal heterochromatin whose deletion increases the instability of a mini-chromosome in heterozygous *nod*/+ oocytes but not in otherwise wild-type oocytes (Murphy and Karpen, 1995). This result has been interpreted to mean that a minichromosome with fewer nod-binding sites competes less well for a reduced number of nod molecules and is thus functionally impaired (Murphy and Karpen, 1995). These data strongly suggest, as do the immunolocalization experiments cited above, that meiotic chromosomes interact with nod proteins at a very large number of sites along their length.

To improve our understanding of the mechanism of nod function in particular, and to gain insights into the mechanisms by which kinesins bind cargo in general, we have undertaken in vivo studies to map those regions within the carboxyl-terminal tail of the nod protein that direct its binding to chromosomes. We demonstrate that the nod protein, expressed under the control of the heat shock promoter, can associate with mitotic chromosomes in embryos, that the carboxy-half of the nod protein directs the nod protein to the chromosomes, and that a 82– residue segment of the tail (which contains three copies of the se-

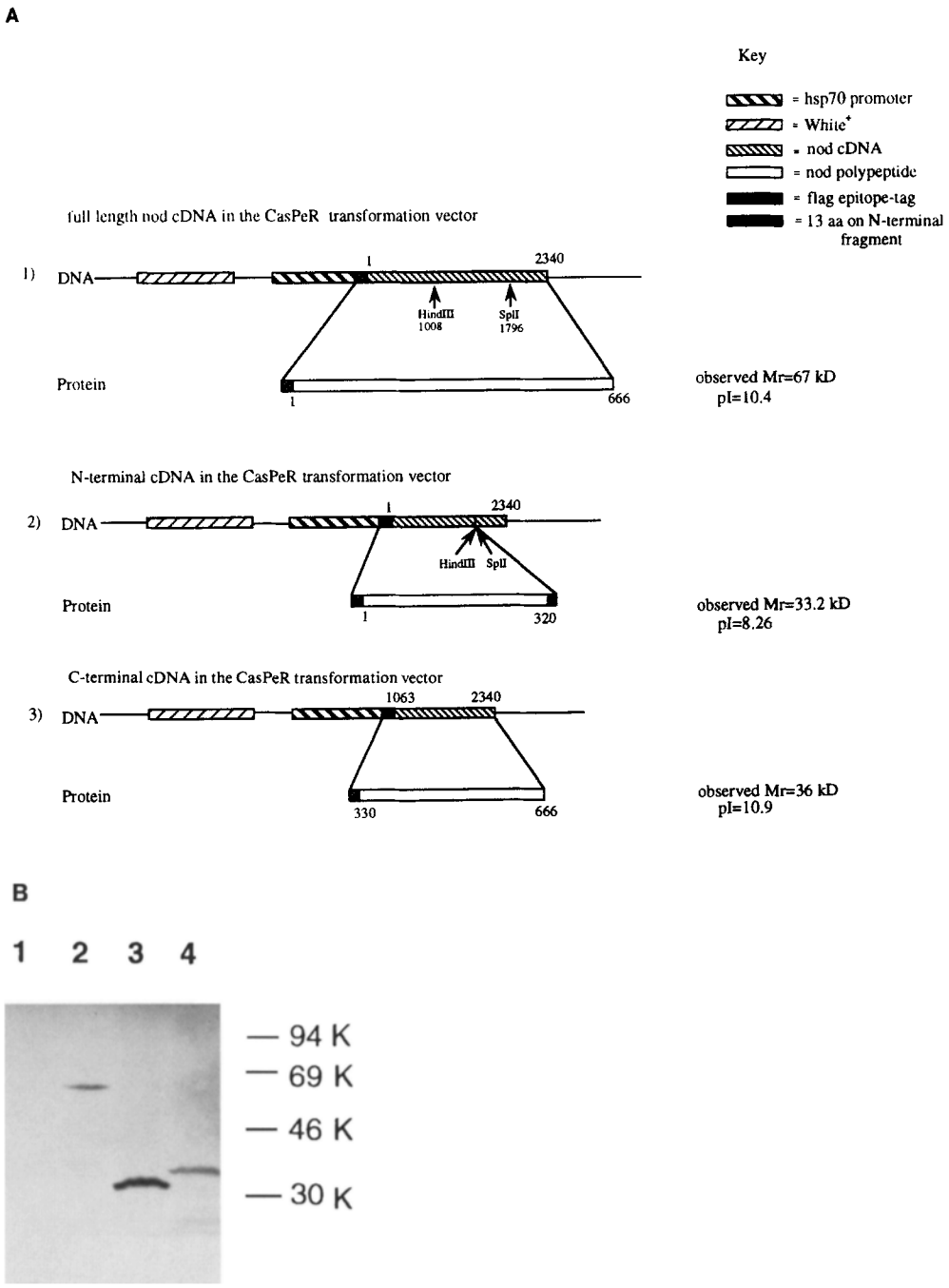


Figure 1. Expression of flag epitope-tagged full-length, amino-terminal or carboxyl-terminal domains of the nod protein in embryos. (*A*) Schematic representation of the transformation constructs carrying coding sequence for the full-length nod protein (*1*), the amino-terminal domain of nod (*2*) and the carboxyl-terminal domain of nod (*3*). The diagram of the respective proteins is shown below each construct. The transformation vector, phspCasPeR was used for expressing fusion proteins consisting of the expressed proteins fused to the flag epitope at their amino-terminal end. The proteins are expressed under the control of the heat shock 70 promoter. The *white*$^+$ gene served as a selection marker for transformation. The numbers refer to nucleotide residues in the nod cDNA sequence and amino acid residues in the nod protein sequence. (*B*) Immunoblot analysis of protein extracts from embryos expressing the full-length nod protein (lane *2*; M_r predicted from sequence 67.4 kD, migrates at 67 kD), the NH_2-terminal domain of the nod protein (lane *3*; predicted M_r=34.9 kD, migrates at 33.2 kD), and the COOH-terminal domain of the nod protein (lane *4*, predicted M_r=33.8, migrates at 36 kD). Fusion proteins were detected using the anti-flag antibody. Estimates of apparent M_r values were obtained from the blots using rainbow molecular weight markers as standards. The carboxyl-terminal domain of nod appears slightly larger than the size predicted from the sequence for unknown reasons. The same effect was seen with some of the truncated forms of the nod carboxyl-terminal domain (see Fig. 5 *B*). Extracts from nontransformed embryos were used as a control for the specificity of the anti-flag antibody (lane *1*).

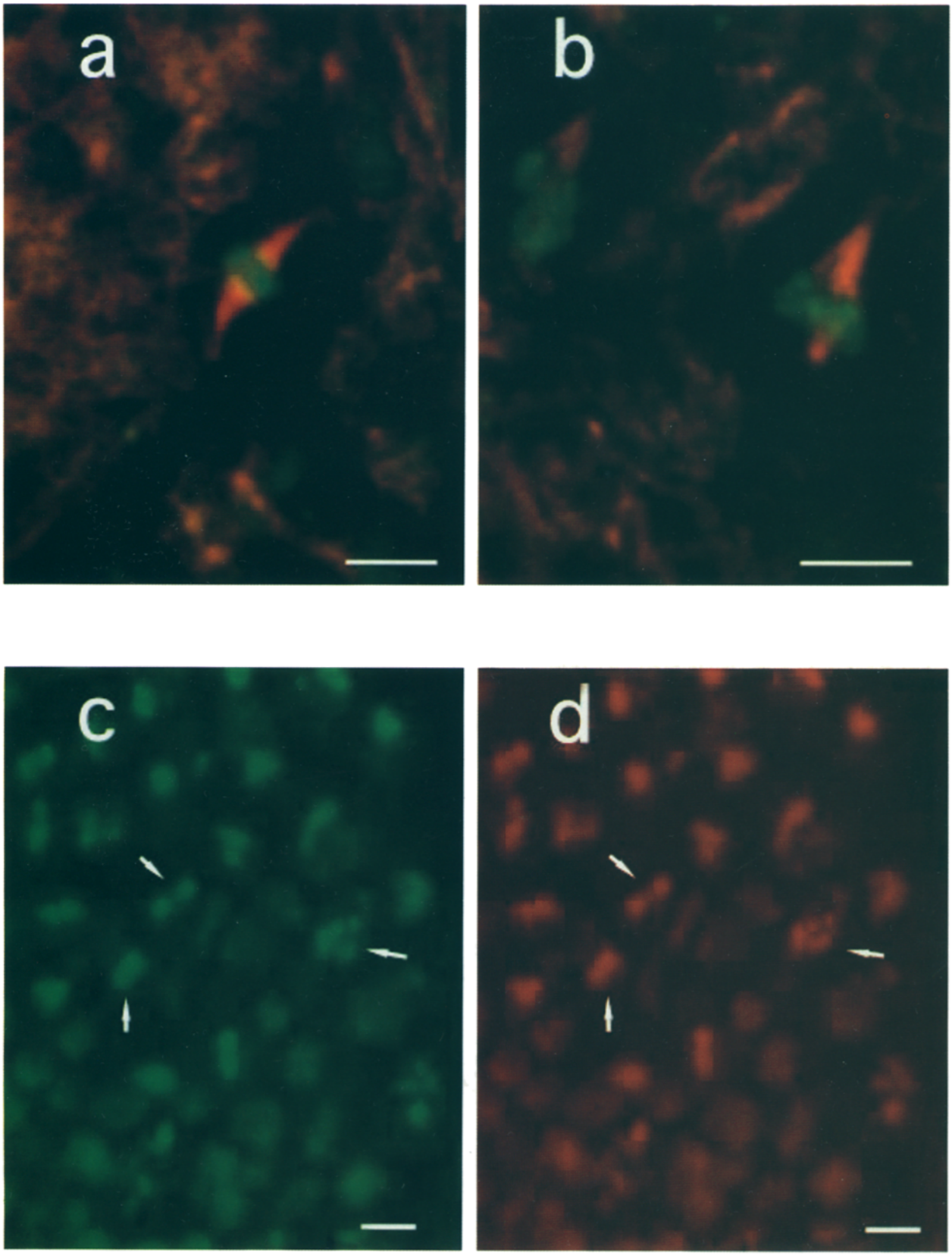


Figure 2. Localization of epitope-tagged nod protein in 2–6-h embryos. *a* and *b* represent confocal images of mitotic cells in an embryo double stained with anti-flag (*green*) antibody and anti-tubulin antibody (*red*). The relative position of anti-flag staining with respect to spindle microtubules indicates that the overexpressed nod protein is associated with chromosomes in mitotic cells. *c* and *d* present an embryo double stained with anti-flag antibody (*c*), and propidium iodide (*d*). Note that the staining shown in *c* and *d* overlaps almost exactly as indicated by arrows, confirming the localization of nod protein on mitotic chromosomes. Bar equals 5 μm.

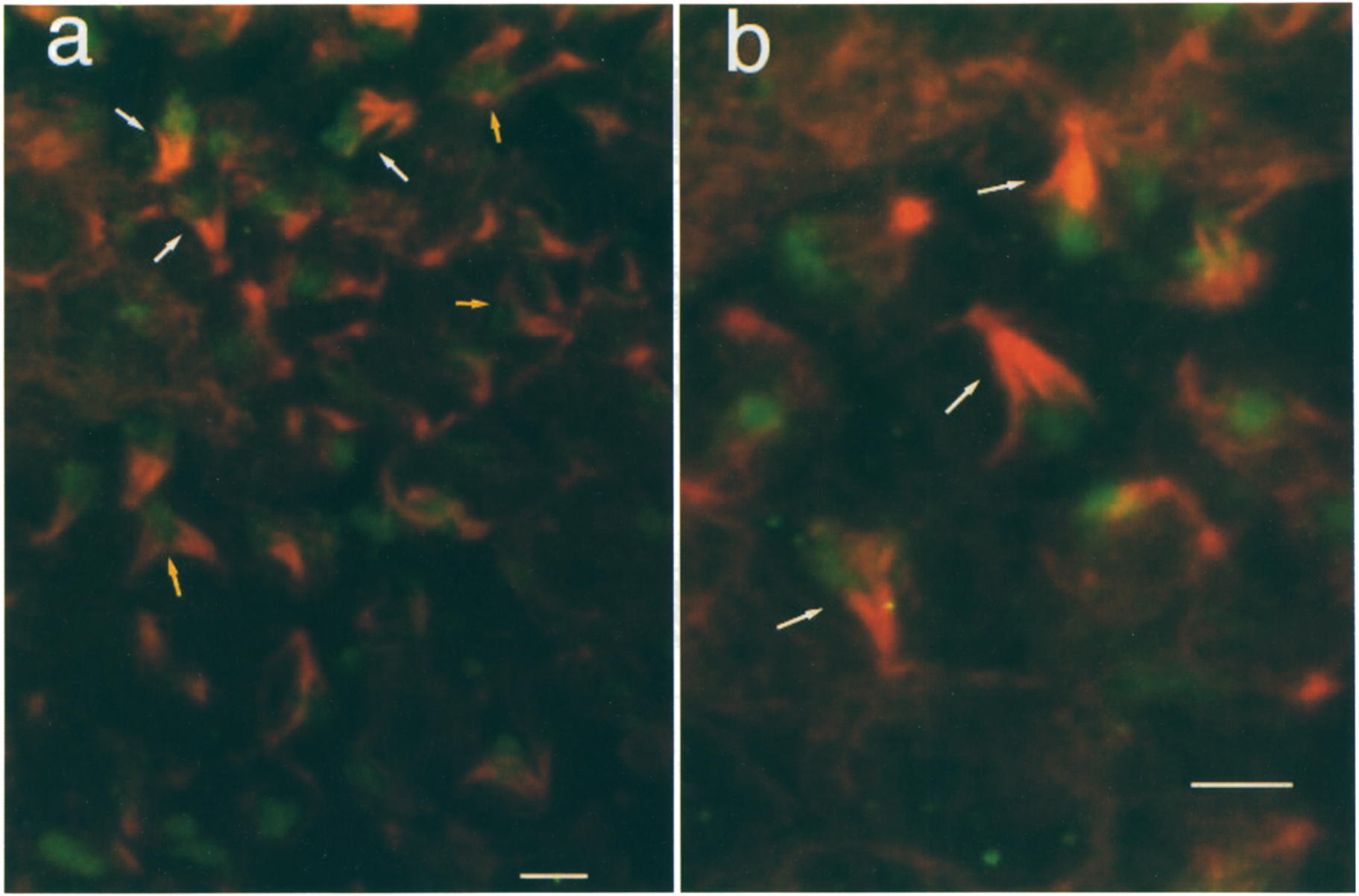


Figure 3. Overexpression of the nod protein causes spindle abnormalities. Images *a* and *b* show mitotic domains in the embryos in which nod is overexpressed. nod staining is shown in green and microtubule staining is in red. Note the presence of asymmetric spindles (*yellow arrows*) and monopolar spindles (*white arrows*) in these embryos. Such spindle abnormalities were absent in wild-type embryos that were heat shocked, fixed, and stained under identical conditions. Bar equals 5 μm.

quence homologous to the DNA binding domain of HMG 14/17 proteins) is necessary for the localization of nod on chromosomes.

Materials and Methods

Plasmid Construction

The construction of plasmid pflag-nodhsp70CasPeR expressing full-length nod has been reported before (Afshar et al., 1995). The construct p-flag-Nnodhsp70CasPeR, expressing the flag epitope tagged amino-terminal domain of nod was made by cutting pflag-nodhsp70CasPeR with SplI restriction enzyme and filling the recessed ends by Klenow enzyme, producing a fragment that was cut with BglII, and then ligated to a HindIII-blunt-ended BglII fragment of the nod cDNA. This created a stop codon 41 bp downstream of the SplI site. The construct pflag-Cnodhsp70CasPeR, expressing the COOH-terminal domain of nod was made as follows: a 745-bp sequence was amplified by PCR using primers flagC-nod and AK-12. Primer flagC-nod contains the sequence CGGGATCCCGCATGG-ATTACAAGGACGATGACGATAAGAAGCAATCGCTGGCC that has a BamHI restriction site, 4 bp from the nod 5′ untranslated sequence followed by 21 bp encoding the flag epitope-tag and 16 bp of nod sequence, from nucleotide 1063 to 1079, that encodes the start of the COOH-terminal domain. Primer AK-12, CACAACAACGGAGGTG-GCATGT, is derived from nucleotides 1743 to 1777 in the nod cDNA sequence. The 745-bp PCR fragment was cut with SphI and BamHI and was exchanged for a BglII–SphI fragment of pnodhsp70CasPeR. The construct expressing nod protein containing amino acids 460–666 (pCN3) was made similarly by using primer flag-nod1452, whose sequence is CGG-GATCCCGCATGGATTACAAGGACGATGACGATAAGGAGCC-CAAGGAATCG, i.e., the same design as primer flagC-nod but different in the last 16 bp in having sequence drived from bp 1452 to 1468, and primer AK-4, whose sequence is AGCTCTTAGAAAAAGTTCAGTT, containing nucleotides 1983 to 2006 of nod cDNA. These primers were used to amplify a 554 fragment that was eventually cut with BamHI and SplI and exchanged with a BglII–SplI fragment of the pnodhsp70CasPeR. The construct expressing nod protein from amino acids 513 to 666 (pCN4) was made exactly the same way, except for the use of primer flag513nod, whose sequence is CGGGATCCCGCATGGATTACAAGGACGAT-GACGATAAGCGTACGGTAGTGGCTTCGCCA, that contains the sequences from nucleotide 1611 to 1627 of the nod cDNA following the flag epitope encoding sequence. To construct the plasmid expressing regions of the nod protein from amino acid 330 to 522 (pCN1) primer pflagC-nod was used in combination with primer AK-08, GGAAT-TCAGCATGGCTGCTGTGGCGA, that contain EcoRI restriction site and residues 1620-1638 of the nod cDNA. Similarly, the plasmid expressing polypeptide 330 to 594 (pCN2) was made using primers pflagC-nod and primer Ak-09, GGAATTCACATCCAGGCCTTGGGCGC, containing EcoRI restriction site and the sequence 1836-1854 of the nod cDNA. The resulting fragments from PCR were treated with EcoRI restriction enzyme and then with Klenow enzyme, to produce blunt-ends, followed by treatment with BamHI restriction enzyme. Those fragments were then ligated individually to pflag-nodhsp70CasPeR that was cut with BglII and SplI restriction enzymes, where the recessed end from the SplI cut was filled. This created a stop codon 8 bp downstream of SplI site. All the constructs were sequenced to confirm the absence of mutations resulting from errors during PCR.

Transformation, Heat Shock Treatment, Embryo Collection, and Protein Analysis

Constructs were injected into white embryos according to established procedures (Rubin and Spradling, 1982). For each construct at least 15 independent lines were generated. To select for the best transformed line, between twenty and thirty embryos were heat shocked on a grape juice agar plate in a 37°C water bath for 1 h. Because the hsp 70 promoter cannot be induced in embryos at early preblastoderm stages (Zimmerman et al., 1983), we performed all of our experiments on 2–6-h embryos. The embryos were allowed to recover from the heat shock for 1 h at room temperature, and then homogenized in 50 ml of the SDS-PAGE sample buffer for further protein analysis by immunoblotting. Those lines that expressed the protein were used for immunostaining. The Western blotting was performed by the procedure described by Sambrook et al. (1989). The protein extracts containing the full-length nod, COOH-terminal and NH_2-terminal domains of nod were run on 8% SDS-PAGE, whereas 12% SDS-PAGE was used to analyze the protein fragments derived from the COOH-terminal domain of nod. Proteins were transferred to PVDF membrane for immunoblotting and detected using M5 anti-flag antibody and horse radish peroxidase–conjugated anti–mouse antibody. Antibody binding was visualized using the ECL system (Amersham Life Science, Arlington Heights, IL).

Embryo Preparation and Immunostaining

2–6-h embryos were collected and heat shocked as described previously. The embryos were dechorionated in 50% chlorox for 90 s, and washed several times with distilled water. They were then placed in 1:1 methanol-heptane solution with moderate shaking. After 5 min the heptane layer was replaced with methanol and fixation was continued for another 5 min. Embryos were rehydrated in 60% methanol in PBST (Phosphate buffered saline, PBS + 0.1% Triton X-100), 30% methanol-PBST, and then PBST alone. Nonspecific protein binding was blocked by placing embryos in PTB solution (3% BSA in PBST) for at least 1 h at room temperature. Embryos were incubated for 3 h at room temperature or overnight at 4°C with M5 anti-Flag Ab at 1:100 dilution in PTB, and then washed in PBST three times for 20 min and incubated with fluorescein-conjugated horse anti–mouse IgG (Vector Laboratories, Burlingame, CA) at 1:200 dilution in PTB. The secondary antibody was preincubated for at least 2 h with Oregon R embryos that were fixed in methanol and blocked for more than 30 min in PTB to block nonspecific reaction with endogenous fly proteins. After 3 h incubation, the embryos were washed three times in PBST, once in PBS, and mounted in 90% glycerol +10% of 10 mg/ml *p*-phenyldiamidine in PBS. For double-labeling experiments, rabbit anti-tubulin Ab was added, along with M5 anti-Flag Ab followed by Texas red–conjugated horse anti–rabbit IgG (Vector Laboratories) and horse anti–mouse Ab to the staining solutions. For double staining with propidium iodide, embryos were first incubated with 2 mg/ml RNAase A solution, 1 h, followed by incubation in 1 mg/ml of propidium iodide solution at 1:100 dilution for 5 min right before the last wash with PBS. Embryos were mounted as before and examined using an MRC-600 confocal microscope (BioRad Labs., Hercules, CA).

Results

Immunolocalization of the Nod Protein in Mitotic Cells

The nod protein can be immunolocalized to chromosome arms in meiotic cells (Afshar et al., 1995). Using the same anti-nod sera, we were not able to detect nod protein in wild-type embryos either on mitotic chromosomes by immunofluorescence (data not shown) or on immunoblots of the crude extract from embryos (Afshar et al., 1995). These observations suggest that either there is not enough nod protein to be detectable by our anti-nod sera in mitotic cells, or that meiotic, but not mitotic cells contain factors necessary for the binding of nod to chromosomes.

To test whether overexpressed nod protein can localize to mitotic chromosomes, we constructed transgenic lines that express Flag epitope-tagged nod protein under the control of the heat shock 70 promoter (Fig. 1 *A*). As shown in Fig. 1 *B*, the anti-flag antibody specifically recognizes a single band of 67 kD in protein extracts from induced transgenic embryos, but no signal is detected in extracts from control noninduced or nontransformed embryos. Two- to six-hour-old transgenic embryos were induced by heat shock and processed for double label immunofluorescence with anti-flag antibody and anti-tubulin antibody (Fig. 2 *a* and *b*). Nod staining was examined in mitotic domains (a cell or clusters of cells undergoing synchronous mitosis; Foe, 1989) containing a single cell (Fig. 2 *a*), two cells (Fig. 2 *b*) or clusters of cells (Figs. 2, *c* and *d*, 3, *a* and *b*). We observed an almost exact correspondence in the

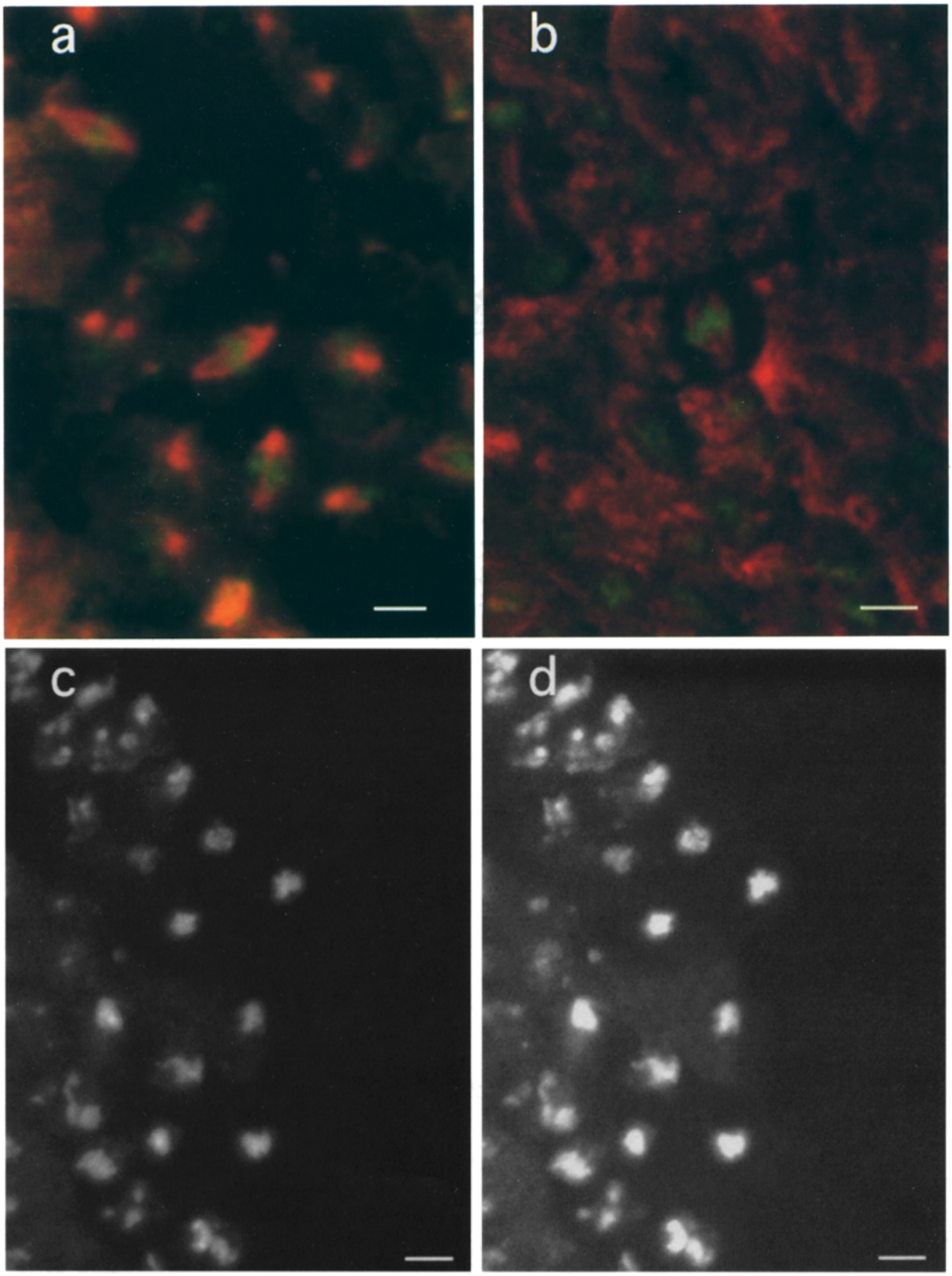
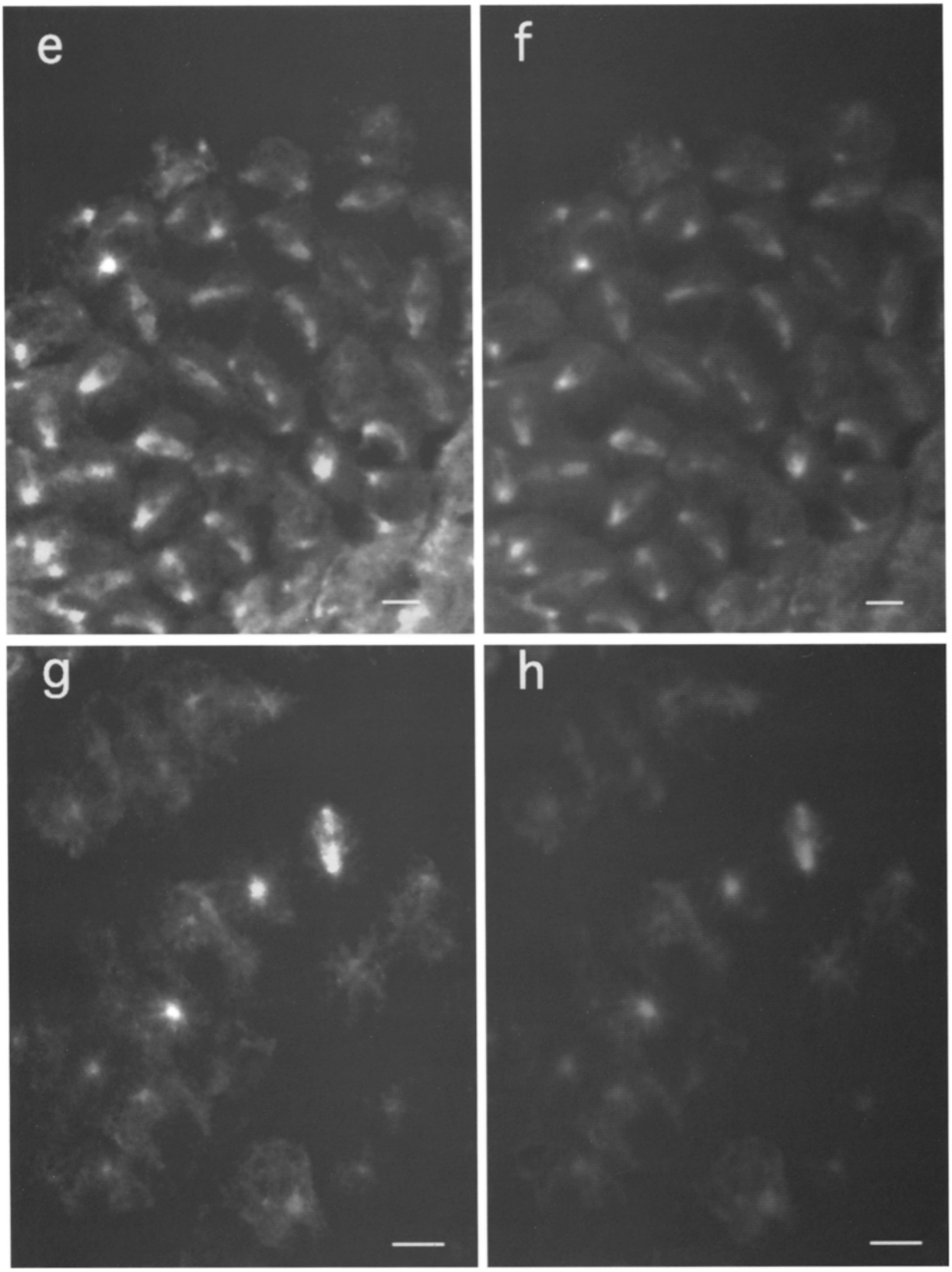


Figure 4. Localization of overexpressed carboxyl-terminal and amino-terminal domains of the nod protein in mitotic cells. *a* and *b* represent embryos expressing the flag epitope-tagged COOH-terminal domain of nod protein stained with anti-flag antibody to detect nod protein (*green*) and with antitubulin antibody to detect microtubles (*red*). This image shows that COOH-terminal domain of nod associates with chromosomes. This conclusion is supported by double staining of similar embryos with anti-flag antibody (*c*) and propidium io-



dide (*d*). *e–h* represent embryos expressing the flag epitope-tagged NH_2-terminal domain of nod that were double stained with anti-flag (*e* and *g*) and anti-tubulin (*f* and *h*). Note the association of the NH_2-terminal domain of nod with microtubules of the mitotic spindle (*e* and *f*) and interphase cytoplasm (*g* and *h*) in the embryos. Bar equals 5 μm.

staining pattern of expressed nod protein (detected using the anti-flag antibody) (Fig. 2 *c*), and the staining pattern of DNA (detected using propidium iodide) (Fig. 2 *d*), indicating that nod protein is localized all over the chromosome arms. Identical results were obtained using antisera made against *E. coli*–expressed nod protein and described in Afshar et al. (1995) (data not shown). This result demonstrates that nod protein binds to mitotic chromosomes in a similar (or identical) fashion to that exhibited by native nod protein in meiotic cells. Our data also show that nod-binding sites on the arms of mitotic chromosomes are unoccupied, and thus are available for binding of overexpressed nod protein. Therefore, no specific factor in meiotic cells appears to be required for nod to bind to chromosomes.

The overexpression of full-length nod protein driven by the hsp 70 promoter in embryonic cells results in a high frequency of spindle abnormalities. Approximately 70–80% of the spindles examined in 1,000 embryos overexpressing full-length nod construct were morphologically abnormal and no mitotic domain composed of clusters of cells with entirely normal bipolar spindles was observed. Most of the abnormal mitotic spindles are highly asymmetric, and in some extreme cases, apparently monopolar (Fig 3, *a* and *b*). Such abnormalities were rarely observed in either heat shock–treated wild-type embryos or in heat shock–induced embryos expressing either the COOH- or NH_2-terminal domains of nod (see below).

Ectopic Expression and Localization of the NH_2- and COOH-terminal Domains of Nod in Embryonic Cells

To test the hypothesis that the carboxyl-terminal domain of nod protein functions as a cargo binding domain, whereas the amino-terminal presumptive motor domain binds to microtubules, plasmids containing flag epitope-tagged NH_2-terminal or COOH-terminal domains of nod in the phsp70-CasPeR vector were constructed, and embryos transformed with these recombinant plasmids were induced to express the corresponding fusion proteins by heat shock (Fig. 1).

Double-label immunofluorescence of the transformed embryos revealed that the COOH-terminal domain of nod protein alone binds efficiently to chromosomes (Fig. 4, *a–d*), consistent with our previous in vitro DNA-binding studies (Afshar et al., 1995). The pattern of staining from the COOH-terminal domain of nod in embryos largely overlaps with the staining by propidium iodide (Fig. 4, *c* and *d*). Thus, like the full-length nod protein, the COOH-terminal domain of nod binds along the length of chromosomes rather than being restricted to a specific region. This suggests that all of the information necessary for chromosomal localization of nod is contained in the tail domain.

Double-label immunofluorescence of embryos expressing the NH_2-terminal domain of nod revealed that this protein fragment is bound to microtubules throughout the cell both in interphase and mitotic cells (Fig. 4, *e–h*), consistent with its proposed function as a motor domain (Zhang et al., 1990). As shown in Fig. 4, *e–h*, in the absence of the COOH-terminal domain, the NH_2-terminal domain of nod binds to astral microtubules and cytoplasmic microtubule networks, as well as the spindle microtubules.

These experiments argue that the nod protein can be broken down into two functional domains, an NH_2-terminal motor domain that binds to microtubules and a COOH-terminal chromosome binding domain that directs and links this putative motor to the chromosomes.

Mapping the Chromosome Localization Domain of Nod

To identify the regions of the COOH-terminal domain of nod that are important for chromosome localization, we tested the chromosome-binding ability of a series of four epitope-tagged truncated proteins (pCN1, pCN2, pCN3, and pCN4) derived from the COOH-terminal domain of nod (Fig. 5 *A*). The expression of the truncated proteins was tested by immunoblotting (Fig. 5 *B*).

As summarized in Fig. 5 *A*, the anti-flag staining observed in cells expressing constructs pCN3 and pCN4 was similar, if not identical, to the pattern of staining observed in cells expressing the entire COOH-terminal domain of nod. These results show that deletions encompassing the amino-terminal regions up to residue 513 have no effect on the chromosome-binding activity of nod. Similarly, fragment pCN2 associated with chromosomes, demonstrating that the last 70 amino acids of nod also are dispensable for binding of nod to chromosomes. In contrast, the pCN1 fragment which possesses residues 330-522, but lacks amino acids 522 to 666, did not display chromosome-binding activity.

Taken together, these results show that the minimal chromosome-binding region of nod is located between amino acid 513 to 594, since this portion of the protein is necessary for localization of nod to chromosomes. As shown in Fig. 5 *A*, this region exactly brackets the three HMG 14/17-like putative DNA-binding domains. This finding pinpoints the significance of the sequence homology between this region and the DNA-binding domain of HMG 14/17 proteins.

Discussion

Here we show both that the tail of nod protein binds to chromosomes in vivo, and that the tail is required to localize the full-length nod protein to chromosomes since the putative motor domain alone simply binds to microtubules throughout the cell. These studies confirm the importance of the COOH-terminal domain of nod for its chromosomal localization and are consistent with our previous finding that the nod COOH-terminal domain binds to AT-rich sequences of cloned satellite DNA in vitro, under conditions where the NH_2-terminal domain did not (Afshar et al., 1995). Moreover, we have also mapped the minimal DNA localization domain of nod (residues 522-594) to an 82–amino acid sequence that encompasses the three sequences homologous to the DNA-binding domain of the HMG 14/17 proteins.

We observed that a nod construct lacking this 82–amino acid sequence did not localize to chromosomes, whereas constructs containing this region but lacking any flanking residues on their carboxyl or amino-terminal ends (Fig. 5) were capable of localizing to chromosomes just as well as the full-length nod tail. This is consistent with data showing that the homologous region of HMG 14/17 proteins also physically interacts with DNA (Abercrombie et al., 1978; Cary et al., 1980; Crippa et al., 1992). The simplest

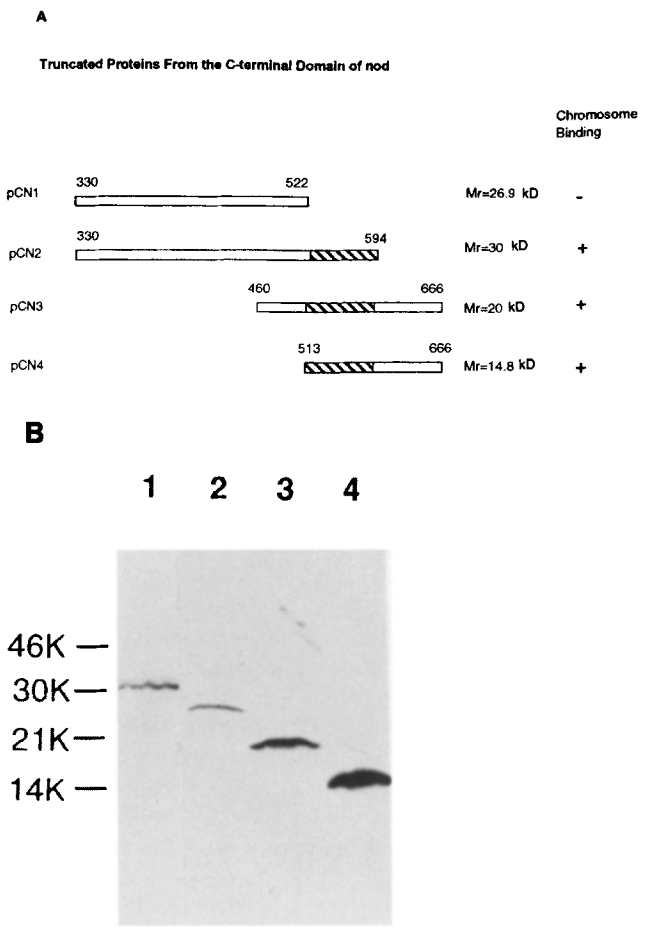


Figure 5. Expression of COOH-terminal fragments of nod and mapping of the chromosome localization domain of nod. (*A*) Diagram of protein fragments derived from the COOH-terminal domain of nod, with numbers indicating residue position in the nod protein. Amino acid 330 marks the motor domain-tail boundary within the 666–residue nod polypeptide. The hatched box depicts the region of the nod protein that contains three repeated sequences related to the DNA-binding domain of the HMG 14/17 proteins. All the protein fragments are tagged with the flag-epitope at their amino terminal end. Chromosome-binding activity of these fusion proteins was assayed by immunofluorescence and the results are summarized on the right. (*B*) Detection of COOH-terminal nod fragments in transformed embryos by immunoblotting. Lanes *1–4*, protein extracts from embryos expressing pCN2, pCN1, pCN3, and pCN4, respectively. Note that the pCN1 protein is highly expressed in embryos, but it cannot be detected on chromosomes by immunofluorescence (not shown).

interpretation of these results is that the region corresponding to residues 522-594 of the nod polypeptide is necessary for binding of nod to chromosomal DNA although we cannot rule out the possibility that flanking sequences might also play some accessory role in mediating interactions between the nod protein and the chromosomes.

Curiously each of the three chromosomal kinesins (nod, Chromokinesin, and Xklp1) possesses a different DNA-binding domain (Afshar et al., 1995; Vernos et al., 1995; Wang and Alder, 1995). As noted above, nod contains three copies homologous to the DNA-binding domain of the HMG 14/17 proteins. Chromokinesin contains a basic-leucine zipper DNA-binding domain that is required for efficient binding of chromokinesin to DNA in vitro. DNA binding of the Xklp1 protein is thought to be mediated by a cysteine-rich domain similar to that found in zinc-finger type transcription factors (although there is no direct evidence for interaction of this region with DNA). This observation suggests that there are multiple evolutionary paths by which motors may have become appended to DNA and that these differences may have functional significance in terms of when and where these motors become localized to their chromosomal cargo.

In the absence of such a chromosome localization domain, one might expect chromosomal motors to be localized only by their microtubule-binding domains, and thus promiscuously attach to microtubule-containing structures throughout the cell. Indeed, our results show that in the absence of the cargo-binding domain, the nod motor domain associates with microtubules throughout the cytoplasm.

The proper association of the nod protein with meiotic chromosomal DNA is crucial for the correct positioning and control of movement of the nonexchange chromosomes on meiotic spindles. In the absence of functional nod, achiasmate chromosomes are ejected from the mass of the karyosome usually resulting in chromosomal loss. Thus, nod protein associated with the chromosome arms may act as a component of the "polar ejection force" which opposes the poleward directed force on chromosomes. In the absence of nod function therefore achaismate chromosomes move precociously toward the spindle pole (for reviews see Hawley and Theurkauf, 1993; Fuller, 1995).

Functional Domains of Nod

Based both on sequence homologies (Zhang et al., 1990) and on the expression studies reported here, we conclude that residues 1-320 comprise the microtubule-binding domain of nod. This observation is also consistent with genetic studies of a dominant, potentially "rigor-binding," mutant of *nod* (nod^{DTW}) and three of its missense intragenic revertants (Rasooly et al., 1991, 1994). The nod^{DTW} mutation results in a single amino acid change in the putative ATP-binding domain. Two of the revertants, which behave as loss-of-function *nod* alleles, map within the putative microtubule-binding domain, while the third lies in the conserved region between the putative ATP- and microtubule-binding domains. The phenotypic analysis of these mutations strongly suggests that amino acid substitutions in the NH_2-terminal half of the nod protein alter the ability of nod to bind microtubules.

The function of the portion of the nod protein between residues 330 and 512 is unknown, but it may serve as a linker between the motor domain and the cargo-binding domain of nod, as a site of interaction with other molecules, or for proper folding or structural integrity of the protein. None of the existing point mutants in the *nod* gene map within this region, and thus there is no basis on which to assign it a function.

As noted above, the region between 512 and 594 is necessary for DNA binding and thus comprises the cargo (DNA)-binding domain of nod. We also note that the exons encoding the putative DNA-binding domain of nod are repeated three times within a 5-kb sequence at the 3′ end of the nod gene (Rasooly et al., 1994). Although only

the first of these repeats is used by the nod gene, preliminary studies suggest that the next two repeats are part of another gene with an exon located some 20 kb upstream of the beginning of the nod gene (i.e., *nod* lies within the intron of this gene) (Afshar, K., and R. S. Hawley, unpublished data). This observation suggests that there may be a second protein which also carries a DNA-binding domain virtually identical to that borne by nod.

The function of the last 70 amino acids of nod is also unknown. We show here that this region is not essential for the binding of nod to chromosomes. However, previous work has shown that two alleles of *nod*, nod^a and nod^{b1}, carry mutations that affect this portion of the protein and give rise to chromosome loss and nondisjunction (Zhang and Hawley, 1990; Rasooly et al., 1994). Therefore, this region does contain important information for the function of nod. Further structure-function analysis of the nod protein will be important for better defining the role of this region in nod function.

Is There a Mitotic Function for the Nod Protein?

In situ hybridization studies demonstrate that the *nod* transcript is present in most, if not all, populations of dividing mitotic cells (Zhang et al., 1990). The mitotic phenotype of the nod^{DTW} mutation also provides evidence for a role of the nod protein in mitosis as well as in meiosis. This mutation exhibits a cold-sensitive lethality due to frequent chromosome breakage and loss during mitosis (Hawley et al., 1993).

Hemizygous nod^{DTW}/Y males (or nod^{DTW}/nod^{DTW} females) die when raised at 16°C, regardless of whether they received the nod^{DTW} mutation from their father or their mother and thus the observed mitotic phenotypes are not simply due to perdurance of the maternal meiotic product (Rasooly et al., 1991). Given that the transcription pattern of nod^{DTW} females is identical to that of nod^+ females (Rasooly et al., 1991), these genetic studies strongly argue that the *nod* locus is expressed in mitotic cells.

Despite this evidence for mitotic expression of *nod*, we were unable to detect nod protein in embryonic mitotic cells from either wild-type or nod^{DTW} embryos, using the same antibody that readily detects nod protein on meiotic chromosomes (Afshar, K., and R. S. Hawley, unpublished data). One explanation for this conundrum is the possibility that the nod protein is present in mitotic cells in a only a very low concentration, perhaps because levels similar to those observed in meiotic cells might be harmful to the cell.

In support of this hypothesis, we note that the overexpression of the full-length nod protein causes high levels of spindle abnormalities, whereas spindle morphology in embryos containing overexpressed NH_2-terminal or COOH-terminal fragments of nod is normal. These results suggest that it may be necessary to maintain the concentration of nod at a low level. Perhaps the binding of large amounts of nod protein to mitotic chromosomes sterically hinders chromosome binding by other proteins that are important for mitotic spindle assembly. For example, inhibition of function of the chromosomal kinesin, Xklp1 is reported to interfere with spindle morphology in vitro (Vernos et al., 1995). Thus, one might imagine that the full-length nod protein could interfere with the chromosomal localization and function of an Xklp1 homologue in *Drosophila* mitotic cells, thereby causing the observed spindle abnormalities. Alternatively, the overexpressed full-length nod protein may provide an excess of plus end–directed force which interferes with the balance of motor forces that are necessary for spindle assembly. For example, we note that spindles with abnormal morphology can result from depletion of the normal activity of plus end–directed motors such as KLP61F or minus end–directed motors such as dynein (Heck et al., 1993; Vaisberg et al., 1993). In this context, it should be noted that determining the polarity of nod driven motility is an important problem that remains to be solved.

Common Problem for Kinesin Targeting

In addition to the nod kinesin-like protein which associates with chromosomes (Afshar et al., 1995), several members of the kinesin super-family have been localized to different components of meiotic or mitotic spindles, such as microtubules, spindle vesicles, chromosomes, and spindle poles (Sawin et al., 1992*a*,*b*, 1995; Houliston et al., 1994; Wright et al., 1991; Henson et al., 1995; Vernos et al., 1995; Wang and Alder, 1995; Hogan et al., 1993; Hagan and Yanagida, 1992; Yen et al., 1992; Nislow et al., 1992). How all these kinesins are targeted to different sites within the spindle is not understood. We do not know, for example, if cargo-localization is a property of the kinesin-like polypeptides themselves or whether accessory proteins are also required for cargo binding.

Our study suggests that cargo localization is a property of a discrete sub-region of the nonmotor region of the nod kinesin-like polypeptide itself. Similarly, a discrete region of the conventional kinesin has also been shown to play a crucial role in attachment to membranes at least in vitro (Skoufias et al., 1994). These results suggest that such distinct "cargo-localization" domains may be a general feature of the nonmotor "tail" regions of those kinesins that function like nod, by cross-linking microtubules to a nonmicrotubular cargo.

However, it may not be possible to generalize our result to all kinesins. In a recent study, Sawin and Mitchison (1995) observed that the COOH-terminal tail of the Eg5 kinesin-like polypeptide was needed for spindle localization but did not localize to spindles when ectopically expressed in cultured cells without an intact NH_2 terminus, leading to the conclusion that the COOH-terminal tail of Eg5 does not have any distinct "spindle localization" signal. These results might reflect the fact that Eg5 and its close relatives must oligomerize via head-tail interactions into higher order structures, in order to associate with spindle microtubules and cross-link them (Cole et al., 1994; Sawin and Mitchison, 1995).

In summary our data allow us to describe the nod protein simply as a motor-like domain appended to a DNA-binding domain. While this picture improves our understanding about the linkage between the track (microtubules) and the cargo (chromosomes), it does not explain the manner in which nod-dependent chromosome movement is regulated. We imagine that a number of other proteins serve to regulate the activity of the nod motor-like domain and to control the expression of the *nod* gene. The identifi-

cation of these proteins will require biochemical assays for proteins that physically interact with nod, and genetic screens for mutations that enhance or suppress existing nod alleles. Such efforts are currently underway in our laboratories.

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