Direct visualization of the *elt-2* gut-specific GATA factor binding to a target promoter inside the living *Caenorhabditis elegans* embryo

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Communicated by Gary Felsenfeld, National Institutes of Health, Bethesda, MD, August 17, 1999 (received for review May 8, 1999)

In analyzing the transcriptional networks that regulate development, one ideally would like to determine whether a particular transcription factor binds directly to a candidate target promoter inside the living embryo. Properties of the *Caenorhabditis elegans elt-2* gene, which encodes a gut-specific GATA factor, have allowed us to develop such a method. We previously have shown, by means of ectopic expression studies, that *elt-2* regulates its own promoter. To test whether this autoregulation is direct, we fused green fluorescent protein (GFP) close to the C terminus of *elt-2* in a construct that contains the full *elt-2* promoter and the full *elt-2* zinc finger DNA binding domain; the construct is expressed correctly (i.e., only in the gut lineage) and is able to rescue the lethality of an *elt-2* null mutant. Multicopy transgenic arrays of this rescuing *elt-2*::GFP construct were integrated into the genome and transgenic embryos were examined when the developing gut has 4–8 cells; the majority of these embryonic gut nuclei show two discrete intense foci of fluorescence. We interpret these fluorescent foci as the result of ELT-2::GFP binding directly to its own promoter within nuclei of the developing gut lineage. Numerous control experiments, both genetic and biochemical, all support this conclusion and support the specificity of the binding. The approach should be applicable to studying other transcription factors binding target promoters, all within the living *C. elegans* embryo.

A major focus of current developmental biology is to determine the downstream genes that are controlled by a particular transcription factor. Because developmental gene regulation invariably occurs as part of a complex redundant network, the usual experimental approaches can have serious limitations, both logical and practical. For example, biochemical experiments demonstrating that a factor can bind directly to a candidate regulatory site *in vitro* are no guarantee that the factor binds the same site *in vivo*. On the other hand, *in vivo* approaches that monitor expression of candidate target genes in response to ablation or ectopic expression of a factor usually are unable to determine whether regulation is direct or indirect.

Ideally, one would like to image the interaction between an active transcription factor and a specific target promoter inside the living unperturbed embryo. Several features of embryonic development in the nematode *Caenorhabditis elegans* suggest that this goal might be attainable. The *C. elegans* embryo is transparent, and the cell lineage is known completely (1); in particular, the cells (and nuclei) of the intestine lineage are relatively large and easily identified in the early embryo. In *C. elegans*, transgenes exist as extrachromosomal concatenated arrays (2) that can be integrated into the genome at a single locus by means of γ-irradiation (see, for example, refs. 3–5). With few exceptions, genes in these arrays appear to be correctly regulated. The copy number of a typical integrated *C. elegans* transgene can range from 10 to several hundred (3, 6). Thus, direct microscopic detection of a factor binding to a transgenic promoter might be possible where it would not be possible with the promoter of a single copy gene. Indeed, Carmi *et al.* (7) and Dawes *et al.* (8) recently have visualized *C. elegans* transgenic arrays in fixed permeabilized embryos by using an antibody to candidate DNA binding factors; their experiments provided an elegant demonstration that these factors were directly involved in controlling genes in the *C. elegans* sex determination pathway. However, to study transcription factor-promoter interactions inside living embryos, we wanted to avoid the use of exogenous antibodies and embryo fixation. Thus, we have investigated the approach of Belmont and coworkers (9–11), who have showed that green fluorescent protein (GFP) fused to *lac* repressor can be used within living cells to detect arrays containing several hundred copies of the *lac* operator. In the present paper, we establish that a similar GFP-based approach can indeed be used to detect a lineage-specific transcription factor binding to a target promoter inside the living *C. elegans* embryo. Specifically, we have been able to observe the gut-specific GATA factor *elt-2* (12, 13) binding to its own promoter inside nuclei of the *C. elegans* embryonic intestine.

Materials and Methods

Plasmid Constructs. The genomic sequence of the *C. elegans elt-2* gene can be obtained from GenBank (accession no. U25175) or in cosmid C33D3 of the *C. elegans* genomic sequence. The 7.4-kb *Ppu*MI–*Pst*I fragment (see Fig. 1*a*) was end-filled and cloned into the *Sfr*I site of pCR-Script SK+ (Stratagene) to produce construct pJM85; we previously have shown that pJM85 rescues the *elt-2* null mutant (13). The 6.3-kb *Not*I–*Nru*I fragment (the *Not*I site is in the vector multiple cloning site) was excised from pJM85, end-filled, and inserted into the *Sma*I site of the GFP expression vector pPD95.77 (kindly provided by A. Fire, J. Ahnn, G. Seydoux, and S. Xu, Carnegie Institute of Washington, Baltimore) to produce JM86 (Fig. 1*b*). The plasmid pSV2-dhfr-8.32 containing 256 *lac* operator binding sites was kindly provided by A. Belmont (University of Illinois, Urbana-Champaign).

***C. elegans* Strains.** Transgenic arrays were produced and integrated as described (2, 4, 5); phenotypic marker plasmids used were either pRF4 for injection into wild-type worms (2) or plasmid pDP#MM016B (kindly provided by M. Maduro and D. Pilgrim, University of Alberta) for injection into *unc-119* (*ed4*) worms (14). Gene copy numbers in integrated transgenic arrays were determined by quantitative Southern blotting, using several different restriction enzyme digests and a Molecular Dynamics PhosphorImager.

Abbreviations: GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole.

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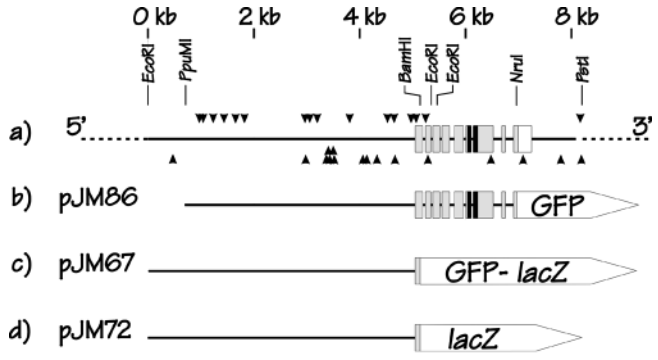

Fig. 1. Schematic representation of the *C. elegans elt-2* gene and *elt-2*::reporter constructs used in the present study. (*a*) The chromosomal region surrounding the *C. elegans elt-2* gene near the middle of the X chromosome. Gray boxes represent exons; the black region represents the *elt-2* DNA binding domain, i.e., the GATA-type zinc finger + 25 aa downstream; unfilled box represents the 3′ untranslated region. Coordinates are marked at the top as kbp downstream from an *Eco*RI site (≈5,100 bp upstream of the *elt-2* ATG codon). Selected restriction enzyme cleavage sites used in the various constructs are shown. Arrowheads represent positions of potential *elt-2* binding sites, i.e., WGATAR or YTATCW for down and up facing arrowheads, respectively. (*b*) Schematic representation of the plasmid pJM86, the main construct used in the present paper. pJM86 contains the coding sequences of GFP fused at the *Nru*I site such that only the nine C-terminal amino acids of ELT-2 are removed. The full *elt-2* DNA binding domain is included. The plasmid vector is pPD95.77 (*c* and *d*) Schematic representation of two control constructs, pJM67 and pJM72, in which either GFP/lacZ or lacZ reporters, respectively are fused to the *elt-2* gene at the *Bam*HI site, 32 aa downstream of the *elt-2* ATG codon. The ELT-2 DNA binding domain is not included in either construct.

***lac* Repressor and Antibody Staining.** For the control experiment in which *lac* operator binding sites and bound *elt-2*::GFP molecules were detected simultaneously, embryos were fixed and permeabilized as described (13), then incubated with pure *lac* repressor protein as described (9), followed by simultaneous incubation with a rabbit anti-LacI polyclonal serum (Stratagene no. 217449) and a chicken polyclonal antiserum against GFP (Chemicon AB16901). A variety of fluorescently labeled secondary antibodies were used with equivalent results. Highly purified *lac* repressor protein was kindly provided by T. Record and M. Capp (University of Wisconsin, Madison).

Microscopy and Image Processing. Embryos were visualized by using a 1.32 numerical aperture 63× Plan Apo lens with either a Leica DM-R or Zeiss AxioPlan II upright fluorescence microscope. Images were collected by using either a Princeton Instruments 14-bit cooled charge-coupled device (CCD) with a pixel size corresponding to 140 nm or a Cooke Corporation Sensicam 12-bit cooled CCD with a pixel size corresponding to 100 nm. Embryos were sectioned in the *z*-axis at 400-nm intervals. The *z*-axis stacks then were corrected for GFP fading through the *z*-axis by equalizing the intensity of light in each image plane by using Universal Imaging (Media, PA) METAMORPH software. After the normalization of signal intensity, the *z*-axis stack was projected onto a single image plane. To reduce out-of-focus light, digital deconvolution was performed by using the nearest neighbor algorithm of AUTOQUANT AUTODEBLUR version 5.1 for Windows. Deconvolution was performed on intensity-corrected images, and the deconvolved image stack was projected onto a single image plane. After initial processing, the images were further processed by subtracting the dark current and the autofluorescent signal and then rescaling the resulting image so that the minimum and maximum signal intensities corresponded to gray values of 0 and 255, respectively.

Results

Background Information on *elt-2*. The *C. elegans elt-2* gene encodes a GATA factor with a single zinc finger DNA binding domain that shows 72–84% amino acid identity to the corresponding domains of gut-associated GATA factors such as *serpent* of *Drosophila* and GATA-4,5,6 of vertebrates (12). Expression of *elt-2* is completely gut specific, beginning when the embryonic gut has only two cells (the 2E cell stage) and continuing throughout the life of the worm (13). Null mutations in *elt-2* are lethal, and afflicted animals die as early larvae with malformed intestines (13). The observation relevant to the current study is that ectopic expression of *elt-2* drives expression throughout the embryo of a reporter gene fused to the *elt-2* promoter, i.e., *elt-2* can positively autoregulate (13). The present paper provides strong evidence that this autoregulation is associated with direct binding of ELT-2 to its own promoter.

Fig. 1*a* summarizes the structure of the *C. elegans elt-2* gene and indicates the position of potential *elt-2* binding sites (WGATAR sequences). Fig. 1*b* shows the principal construct (pJM86) used: 4.3 kb of the *elt-2* promoter and all but the C-terminal 9 aa of the *elt-2* coding region are fused to coding sequences for GFP. Because the fusion to GFP is C terminal to the *elt-2* zinc finger domain, the construct is expected to have normal DNA binding properties and indeed is capable of rescuing the lethality of the *elt-2* null mutant (data not shown); pJM86 is referred to below as the full rescuing *elt-2*::GFP construct. Fig. 1 *c* and *d* show two control constructs in which 5.1 kb of *elt-2* promoter and the N-terminal 32 aa of *elt-2* are fused either to *lacZ*/GFP (pJM67) or to *lacZ* (pJM72); neither construct contains the *elt-2* DNA binding domain but both constructs are strongly expressed in nuclei of the developing gut (ref. 13 and see below). Several transgenic lines were produced for each construct, and selected transgenic arrays were integrated into the genome. One particular strain (JM73) of transgenic worms was the starting point for the majority of the experiments reported below; in this strain, the transgenic array contained 90 (+/− SD = 20) copies of construct pJM86 (along with marker gene *unc-119*) integrated into the genome on chromosome III.

Transgenic *elt-2*::GFP Binding to Its Own Promoter Leads to Discrete Fluorescent Foci in Embryonic Gut Nuclei. Fig. 2*A* shows three examples of young *C. elegans* embryos (8E cell stage) from strain JM73, i.e., transformed with the full rescuing *elt-2*::GFP construct pJM86. Images were prepared by collecting serial optical sections at 400-nm intervals and then projecting these serial sections onto a single plane. Of the several hundred total nuclei in the embryo at this stage, only the large gut nuclei fluoresce green. The important observation is that, in these projections, the majority of gut nuclei clearly show that the GFP fluorescence is concentrated into foci. Many of the gut nuclei show two striking and intense foci of fluorescence (small arrows). In nuclei where apparently only a single focus is observed, a second focus often can be revealed by rotating the stacked images through 90 degrees. In those nuclei where the foci appear less discrete and possibly fibrillar, we suggest that this may represent a bona fide structural variant of the transgenic array associated, for example, with a particular stage of the cell cycle (see below).

Foci are seen in the raw data without deconvolution (Fig. 2*A Upper*) but become much more distinct after deconvolution (Fig. 2*A Lower*). The foci are easily detectable whether the subtracted background is taken as the average intensity of all pixels within the nucleus (i.e., assuming the localization phenomenon is nonspecific; see ref. 15) or taken as the average pixel intensity of an area clearly outside of the fluorescent foci (i.e., assuming the foci do indeed represent specific structures within the nucleus). Line scans through foci show intensity ratios (i.e., peak/nuclear average) ranging from 1.5 to 4 (data not shown). The foci do not

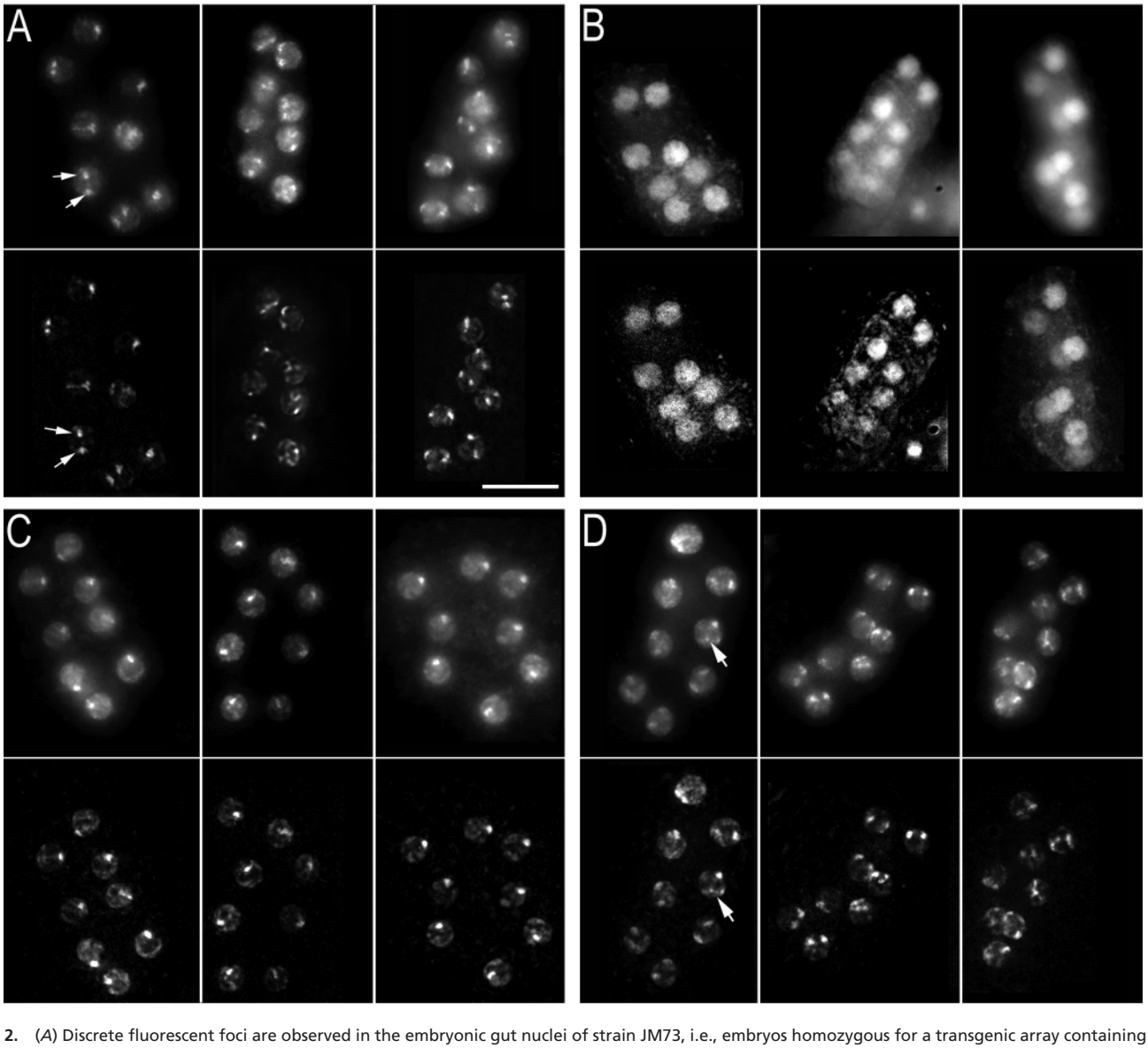

Fig. 2. (*A*) Discrete fluorescent foci are observed in the embryonic gut nuclei of strain JM73, i.e., embryos homozygous for a transgenic array containing the full rescuing *elt-2*::GFP construct pJM86. Three representative embryos (left to right) at the 8E cell stage are shown. (*Upper*) The stack of serially collected 400-nm optical sections was projected without further manipulation onto a single plane. (*Lower*) The same images were first subjected to digital deconvolution and then projected onto a single plane. The arrows point to an example of two distinct fluorescent foci within a single gut nucleus. The scale bar represents 10 μ. (*B*) Discrete fluorescent foci are not observed in gut nuclei of embryos transgenic for the construct pJM67, i.e., a GFP fusion to *elt-2* that removes the DNA binding domain (see Fig. 1*c*). Three typical 8E cell stage embryos are shown; all nuclei of the eight gut cells are intensely fluorescent but no discrete foci are observed. Shown are image stacks before (*Upper*) and after (*Lower*) digital deconvolution. (*C*) Single fluorescent foci are observed in gut nuclei of embryos heterozygous for the transgenic array containing the full rescuing *elt-2*::GFP construct pJM86. As described in the text, male worms of strain JM73 were crossed with wild-type hermaphrodites; fluorescent embryos in the next generation therefore must be heterozygous cross progeny and were imaged as usual at the 8E cell stage. Projected images of three typical embryos are shown, either before (*Upper*) or after (*Lower*) digital deconvolution. (*D*) An extra fluorescent focus appears in the gut cells of embryos carrying one copy of a transgenic array containing pJM86 (i.e., the full rescuing *elt-2*::GFP construct) and a second independent transgenic array containing pJM72 (i.e., the *elt-2* promoter fused to a *lacZ* reporter gene; see Fig. 1*d*). The images represent the projected image stack without (*Upper*) or with (*Lower*) digital deconvolution. The arrow points to a gut nucleus in which one fluorescent focus is condensed and the second is extended or fibrillar. For each of the above experimental situations, full data sets were collected for at least 25 embryos.

result from a chance superposition of high-intensity pixels on a grainy background: when the image stack is rotated around some arbitrary axis, the foci also are seen to rotate as cohesive spots in space (data not shown).

In a healthy well-fed population of JM73 worms, the majority of embryos exhibit ≈2 easily detectable fluorescent foci in their gut nuclei. The foci look similar whether the embryos are left unperturbed inside the maternal uterus or are isolated by dissection. Foci can be detected at the 4E and 16E cell stages (data not shown), but images taken at the 8E cell stage provide the highest clarity and signal-to-noise ratio, presumably because this stage represents the optimal compromise between decreasing nuclear size and increasing levels of *elt-2* expression. In a second independently constructed strain, the copy number of pJM86 in the (integrated) transgenic array was measured as 40 (+/− SD = 10), i.e., roughly half of that in strain JM73; fluorescent foci in gut nuclei were clearly detectable in this second strain but were less intense, as would be expected (data not shown).

In other experimental systems, usually in mammalian cells that

have been fixed and permeabilized, fluorescent antibody staining or *in situ* hybridization have been used to visualize a variety of subnuclear structures (reviewed in ref. 16). However, the interpretation of some of these structures has been questioned (see, for example, ref. 15), and there is the omnipresent concern that such structures might be produced during the process of fixation and staining. Thus, most of the remainder of this paper describes control experiments that support our interpretation that the discrete fluorescent foci observed inside embryonic gut nuclei do indeed represent the ELT-2::GFP molecule binding to its own promoter, present in multiple copies in the transgenic array.

An *elt-2*::GFP Fusion Construct Lacking the ELT-2 DNA Binding Domain Does Not Produce the Fluorescent Gut Foci. Fig. 2*B* shows three examples of embryos (8E cell stage) from a strain transformed with pJM67, the control construct in which GFP is fused close to the N terminus of *elt-2*, thereby deleting the ELT-2 DNA binding domain (see Fig. 1*c*). These embryos show intense but rather uniform fluorescence in their gut nuclei; any graininess observed in the images (especially after deconvolution) is quite unlike the discrete intense foci observed with the full rescuing *elt-2*::GFP construct pJM86.

This particular control strain expresses GFP at a much higher level than do any of our transgenic strains containing pJM86. To rule out the possibility that (two) fluorescent foci are actually present in embryonic gut nuclei of this control strain but are masked by the high expression levels, we inspected embryos at an early stage when transgene expression is low; no fluorescent foci were observed (data not shown). Likewise, no believable foci could be produced by determined computer manipulation of the digital images.

Single Fluorescent Foci Are Seen in Gut Nuclei from Strains Heterozygous for the Rescuing *elt-2*::GFP Construct. If the foci do indeed represent integrated *elt-2*::GFP arrays and not some unspecified bipartite nuclear structure, then the number of such foci should be halved in heterozygous embryos. Males homozygous for the integrated rescuing *elt-2*::GFP array were mated to wild-type (nontransgenic) hermaphrodites; in this way, only cross-fertilized embryos should be fluorescent. Fig. 2*C* shows three examples of heterozygous embryos at the 8E cell stage; single intense foci are evident in the majority of the gut nuclei. We find that the single foci observed in such heterozygous embryos are considerably more discrete (and convincing) than are foci observed in embryos homozygous for the transforming array (for example, Fig. 2*A*). As noted above, we suggest that the occasional focus that appears extended may reflect a real structural variant of the transgenic array.

An Additional Copy of the *elt-2* Promoter Leads to an Additional Fluorescent Focus in Embryonic Gut Nuclei. Male worms transgenic for the full rescuing *elt-2*::GFP construct were crossed to hermaphrodites of a strain homozygous for an integrated transgenic array containing the *elt-2* promoter fused to the *lacZ* reporter gene (construct pJM72; see Fig. 1*d*). Fig. 2*D* shows three cross-progeny embryos at the 8E cell stage. As predicted, many of the gut nuclei clearly show two discrete fluorescent foci. The arrow in Fig. 2*D* indicates a nucleus in which one fluorescent focus appears compact and condensed but the second focus appears extended.

***elt-2*::GFP and *lac* Repressor Molecules Colocalize to the Same Nuclear Foci If the Transforming *elt-2*::GFP Array Also Includes Copies of the *lac* Operator.** Transgenic *C. elegans* strains were produced by injecting pJM86 (i.e., the full rescuing *elt-2*::GFP fusion gene), together with plasmid pSV-dhfr-8.32 (which contains 256 copies of the *lac* operator; ref. 9) and the *unc-119* containing plasmid as the transformation marker; a selected transgenic array then was integrated into the genome. Embryos from this strain were fixed, permeabilized, exposed to purified *lac* repressor protein, and incubated with antibodies to GFP and *lac* repressor. Bound primary antibodies then were detected with fluorescently labeled secondary antibodies. This particular control experiment is basically a variation of the approach used by Carmi *et al.* (7) and Dawes *et al.* (8).

Fig. 3*A* shows a 400-nm optical section, generated by digital deconvolution, of an embryo costained for *elt-2*::GFP (*Upper Left*), *lac* repressor (*Upper Right*), and DNA (i.e., 4′,6-diamidino-2-phenylindole, DAPI; *Lower Left*). Because these are individual optical sections, not all nuclei show clear examples of foci. However, it is clear that the *elt-2*::GFP foci are detected only in nuclei of gut cells whereas the *lac* repressor foci are found in nuclei throughout the embryo. Fig. 3*A* (*Lower Right*) shows a three-color merged image in which *elt-2*::GFP is colored green, *lac* repressor is colored red, and DNA is colored blue. The small arrows in the upper panels indicate several foci that are obviously similar in both the *elt-2*::GFP and the *lac* repressor images; in the merged image (*Lower Right*), the two signals overlap almost completely (yellow).

The two large arrows in the merged color image indicate the positions of *lac* repressor foci colocalizing with metaphase chromosomes aligned on the metaphase plate, providing further evidence that these signals are DNA dependent. *elt-2*::GFP foci also can be detected on condensed metaphase chromosomes (data not shown). The fact that the *lac*-repressor detected foci can be observed in nuclei throughout the embryo suggests that GFP-tagged transcription factor-promoter interactions also can be investigated in cells other than the large gut cells (see also refs. 7 and 8).

The Fluorescent Gut Foci Require Specific Binding. An important control is to show that the fluorescent foci do not derive from nonspecific binding of the *elt-2*::GFP fusion protein to any DNA that contains WGATAR sequences. A strain of worms was produced carrying an integrated transgenic array containing the *lac* operator plasmid pSV-dhfr-8.32, the transformation marker plasmid (*unc-119*), and plasmid pPD95.77, the empty vector used to construct the rescuing *elt-2*::GFP fusion. Hermaphrodites from this strain of worms were crossed to males from the strain JM73 containing the full rescuing *elt-2*::GFP fusion and an F2 strain isolated that was homozygous for both of the independent arrays. Embryos from this doubly homozygous strain were treated with *lac* repressor and anti-GFP antibodies as described in the previous section. Fig. 3*B* represents a 400-nm optical section through such an embryo showing staining of *elt-2*::GFP (*Upper Left*), *lac* repressor (*Upper Right*), and DNA (*Lower Left*). Once again, because a single optical section is shown, foci are not observed in all nuclei. The merged image (*Lower Right*) clearly demonstrates that the *elt-2*::GFP and the *lac* repressor foci are now independent of each other, i.e., in the gut cells of the merged image, *elt-2*::GFP foci appear green, rather than yellow. We used semiquantitative PCR to show that the copy number of the empty vector in this control array is comparable to (and if anything higher than) the copy number of the full rescuing *elt-2*::GFP gene in strain JM73 (data not shown); thus it is unlikely that the lack of detectable *elt-2*::GFP binding to this array is the result of lack of sensitivity.

Discussion

In the present paper, we have shown that a GFP-labeled transcription factor can be observed binding to a target promoter in the developing gut cells of the living *C. elegans* embryo. In this particular case, the transcription factor is the *C. elegans* gut-specific GATA factor *elt-2*, binding to its own (auto-regulated) promoter. This binding is observed as distinct fluorescent foci,

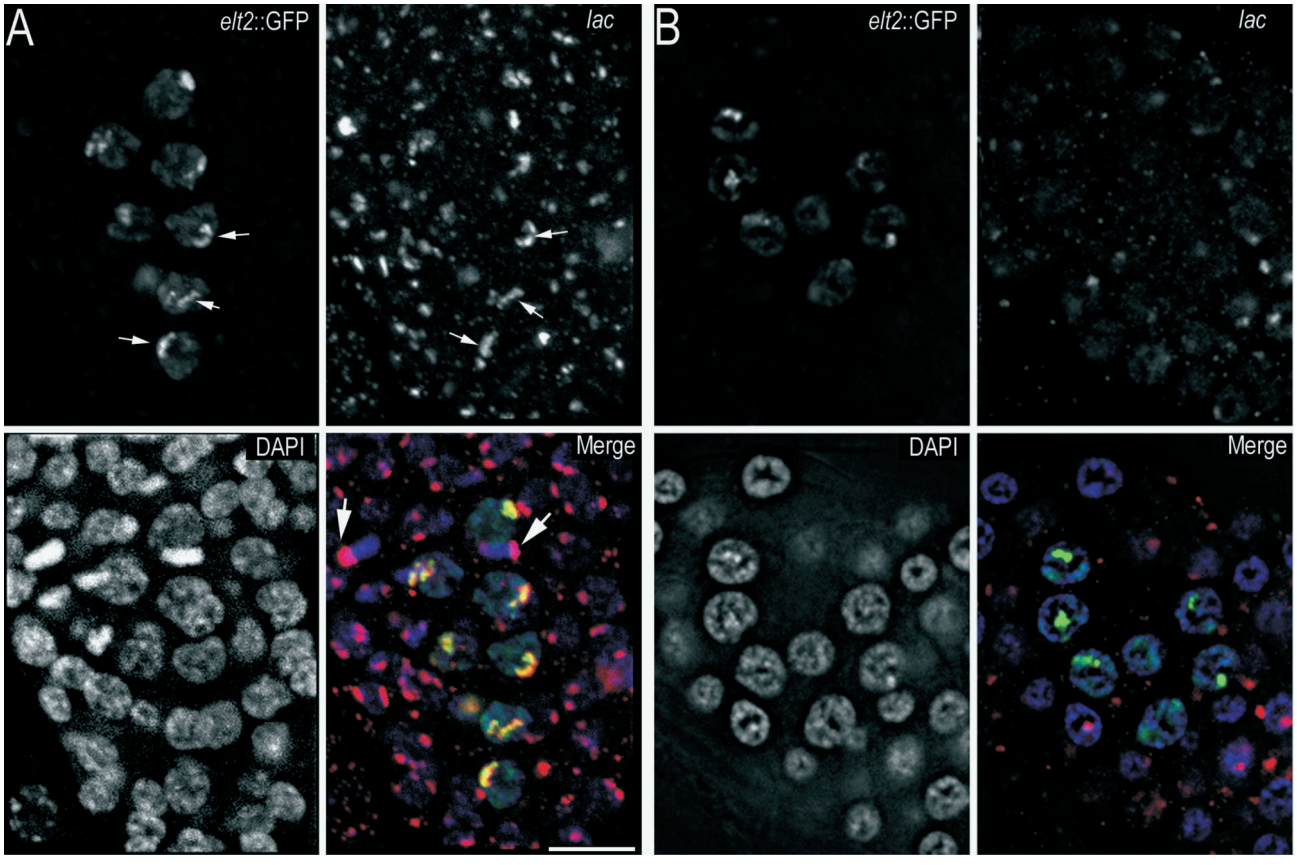

Fig. 3. (*A*) A mixed transgenic array containing multiple copies of the *lac* operator as well as the full rescuing *elt-2*::GFP construct pJM86 produce colocalizing fluorescent foci in embryonic gut nuclei, as detected by (*Upper Left*) antibody against GFP (followed by fluorescently labeled secondary antibodies) and by (*Upper Right*) exogenously added *lac* repressor (followed by anti-*lac* repressor antibody and fluorescently labeled secondary antibodies). Arrows point to several fluorescent foci detected by GFP antibodies that overlap with foci detected by *lac* repressor binding. The DAPI-stained image of the same field is shown (*Lower Left*). Each of these three panels represents a 400-nm optical section through a transgenic embryo (8E cell stage). (*Lower Right*) A three-color merged image in which *elt-2*::GFP is colored green, *lac* repressor is colored red, and DNA is colored blue. The yellow area reveals the extensive overlap between nuclear foci detected by *elt-2*::GFP and *lac* repressor. Thirty-two such embryos were examined in detail, all of which showed colocalized foci. Parallel treatment of wild-type embryos showed no significant staining ($n = 20$). The two arrows in the merged image indicate *lac* repressor binding to condensed metaphase chromosomes. The scale bar represents 10 μm. (*B*). Binding of *elt-2*::GFP is specific for its own promoter. Embryos homozygous for two independent transgenic arrays, one containing the full rescuing *elt-2*::GFP construct pJM86 and the second containing a mixture of *lac* operator sites and empty plasmid vector, were stained and processed exactly as for *A*. A 400-m optical section of a typical embryo is shown, revealing the disposition of (*Upper Left*) *elt-2*::GFP, (*Upper Right*) *lac* repressor, and (*Lower Left*) DNA (DAPI). (*Lower Right*) The three-colored merged image is shown. Foci detected by *elt-2*::GFP (green) do not overlap with foci detected by *lac* repressor (red); 33 embryos were examined.

easily detectable by confocal microscopy. In genetic crosses, these fluorescent foci behave as expected for simple genetic loci. Foci are not seen if the transgenic *elt-2*::GFP construct lacks the *elt-2* DNA binding domain. When the transgenic arrays also contain *lac* operator sequences, the GFP-detected foci colocalize with exogenously added *lac* repressor. All of these observations rule out the possibility that the fluorescent foci derive from intranuclear aggregation of GFP or aggregation of the *elt-2* GATA factor itself, as has been reported for vertebrate GATA factors (17).

Fluorescent nuclear foci are not produced by a transgenic array that contains empty vectors lacking the *elt-2* promoter. In other words, the foci reflect specific interaction at the *elt-2* promoter; apparently, nonspecific binding to chance WGATAR sites in the transforming DNA either does not occur or does not produce a detectable fluorescent focus. An important point for the future will be to explore the nature and extent of this binding specificity, for example by incorporating into the transgenic arrays known numbers and precise arrangements of either wild-type or mutated WGATAR binding sites. In particular, we should be able to approach questions of *in vivo* specificity of GATA factors. If hypodermal-specific GATA factors such as *elt-1* and *elt-3* (18, 19) are expressed in the developing gut, will they also bind to the *elt-2* promoter? Does the molecular basis of GATA factor specificity lie with the specificity of promoter binding or with the specificity of subsequent gene activation (see, for example, ref. 20)?

Belmont and coworkers (9–11) estimated that several hundred *lac* repressor::GFP molecules can be detected in a discrete fluorescent structure inside a cell nucleus. At the moment, we can give only the roughest (but nonetheless comparable) estimate for the number of bound ELT-2::GFP molecules that can be detected inside a *C. elegans* embryonic gut nucleus. We have observed discrete fluorescent foci in the gut cells of embryos from a strain in which the transgenic array has ≈40 copies of the *elt-2*::GFP gene. The results of the control experiment discussed in the previous paragraph indicate that none of the intensity in a fluorescent focus is contributed by ELT-2::GFP binding nonspecifically, i.e., to the vector or to the plasmid used as a transformation marker. Thus, if all of the 20 or so WGATAR sites found in the *elt-2* promoter (see Fig. 1*a*) are involved in ELT-2 binding, then the fluorescent focus would correspond to

≈800 bound ELT-2::GFP molecules. If, as seems more likely, only a fraction of these sites are involved in interaction with ELT-2, our estimate of the minimal detectable number of DNA-bound factors would be correspondingly lower. Other sources of uncertainty can be imagined, but overall it seems clear that the present approach depends heavily on the fact that *C. elegans* transgenes are present in multiple copies.

Does the ability to visualize direct factor-promoter binding reveal anything about the chromatin structure of an active gene? At the moment, we feel that the only safe conclusion is that the gene array must be highly condensed, with a compaction ratio in the range of hundreds. That is, the transgenic array may contain several million base pairs of DNA but the dimensions of the fluorescent foci are only fractions of microns. The compaction ratio would be correspondingly lower if only a portion of the array contributes to the fluorescent dot. Also, we can presently say little about how the foci behave during gut cell duplication, for example, whether the occasional extended fibrillar structures observed are associated with a particular stage of the cell cycle or stage of development. These questions should be approached in the future by using multiphoton microscopy to avoid possible photo-toxicity or light perturbed development (see, for example, ref. 21).

In summary, we anticipate that the present GFP-based approach can be used to demonstrate that a particular factor can bind to a particular promoter inside a particular cell in a nonpermeabilized nonfixed embryo. Unlike other *in vivo* techniques, such as ectopic expression or gene knockouts, the present method can provide strong evidence that a particular regulatory relation is direct rather than indirect. There remains, of course, the question whether the binding interaction that leads to the fluorescent focus is the same interaction that leads to gene regulation; we hope that this question can be approached at the ultrastructural level in the future. A future extension of the present confocal approach should be to investigate the simultaneous binding of multiple factors to the same promoter, using techniques such as fluorescence resonance energy transfer between spectral variants of GFP.

We thank Dr. A. Belmont (University of Illinois) for providing the *lac* operator containing plasmid, Dr. A. Fire (Carnegie Institute) for providing convenient reporter plasmids, and Drs. T. Record and M. Capp (University of Wisconsin) for providing purified *lac* repressor protein. We especially thank Dr. J. White (Integrated Microscopy Resource, University of Wisconsin) for providing a preliminary image of an *elt-2*::GFP transgenic embryo that convinced us that the current approach was feasible. This work was supported by operating grants from the Medical Research Council of Canada (to D.P.B.-J. and J.D.M.) and the Cancer Research Society, Inc. (to D.P.B.-J.). The University of Calgary Microscopy and Imaging Facility is supported by a maintenance grant from the Medical Research Council of Canada. T.F. is a postdoctoral fellow, and J.D.M. is a Medical Scientist of the Alberta Heritage Foundation for Medical Research.

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