

# Targeting of Cardiac Muscle Titin Fragments to the Z-bands and Dense Bodies of Living Muscle and Non-Muscle Cells

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A 6.5-kb N-terminal region of embryonic chick cardiac titin, including the region previously reported as part of the protein zeugmatin, has been sequenced, further demonstrating that zeugmatin is part of the N-terminal region of titin, and not a separate Z-band protein. This Z-band region of cardiac titin, from both 7- and 19-day embryos as well as from adult animals, was found to contain six different small motifs, termed z-repeats [Gautel et al., 1996: *J. Cell Sci.* 109:2747–2754], of approximately 45 amino acids each sandwiched between flanking regions containing Ig domains. Fragments of Z-band titin, linked to GFP, were expressed in cultured cardiomyocytes to determine which regions were responsible for Z-band targeting. Transfections of primary cultures of embryonic chick cardiomyocytes demonstrated that the z-repeats play the major role in targeting titin fragments to the Z-band. Similar transfections of skeletal myotubes and non-muscle cells lead to the localization of these cardiac z-repeats in the Z-bands of the myofibrils and the dense bodies of the stress fibers. Over-expression of these z-repeat constructs in either muscle or non-muscle cells lead to the loss of the myofibrils or stress fibers, respectively. The transfection experiments also indicated that small domains of a protein, 40 to 50 amino acids, can be studied for their localization properties in living cells if a suitable linker is placed between these small domains and the much larger 28 kDa GFP protein. *Cell Motil. Cytoskeleton* 45:67–82, 2000. © 2000 Wiley-Liss, Inc.

**Key words:** titin; z-repeats; Green Fluorescent Protein; Z-bands; alpha-actinin; myofibrils; myofibrillogenesis; cardiomyocytes; myotubes; dense bodies; stress fibers

## INTRODUCTION

Titin, also known as connectin, is an exceptionally large protein, 3.0–3.7 MDa in molecular weight, found predominantly in cross-striated muscles [for reviews see Maruyama, 1994; Trinick, 1994]. A single titin polypeptide spans the entire half sarcomere, a distance up to 1.2  $\mu\text{m}$  in rest length muscle or 1.8  $\mu\text{m}$  in fully stretched muscle. Titin is embedded at its amino terminus in the Z-band [Gregorio et al., 1998; Young et al., 1998] and extends longitudinally across the I-band and then binds along half of the length of the myosin filaments in the

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A-band [Trinick, 1994]. Titin filaments extending from the opposing Z-bands of a sarcomere, thus, anchor the myosin filaments, keeping them centered despite changes in sarcomere length that occur during contraction. In addition, titin is also thought to be responsible for the passive resistance of sarcomeres to stretching [Maruyama et al., 1977; Wang, 1994; Horowitz and Podolsky, 1987].

The full-length 81-kb titin cDNA, derived from human cardiac muscle, encodes a 3.0 MDa polypeptide of 26,926 amino acid arranged predominantly in 132 fibronectin type III domains (FN3) and 112 immunoglobulin-like domains (Ig) [Labeit and Kolmerer, 1995; Labeit et al., 1997]. The Z-band region of titin consists of approximately 1,000 amino acids arranged in 4 Ig domains that are interspersed with non-domain residues and a tandem array of novel motifs composed of 40 to 50 amino acids termed z-repeat motifs or z-repeats [Gautel et al., 1996]. Titin isoforms in various mammalian muscles have variable copy numbers of 4, 5, 6, or 7 z-repeats that may account for variations in Z-band size or strength [Gautel et al., 1996; Sorimachi et al., 1997].

Several lines of evidence indicate that the N-termini of titin in adjacent sarcomeres overlap in an antiparallel manner in the Z-band with the N-termini at each edge of the Z-band [Gregorio et al., 1998; Young et al., 1998]. With yeast two-hybrid assays and *in vitro* binding assays, this Z-band region of titin has been shown to interact with at least two Z-band proteins: alpha-actinin [Ohtsuka et al., 1997b; Sorimachi et al., 1997; Turnacioglu et al., 1997a; Young et al., 1998] and telethonin/T-cap [Mues et al., 1998; Gregorio et al., 1998]. Alpha-actinin binding domains of titin reside in the z-repeats [Ohtsuka et al., 1997a; Sorimachi et al., 1997; Young et al., 1998] and in a non-modular region adjacent to the C-terminus of the z-repeat motif [Young et al., 1998]. Telethonin binds to the region that includes the first two Ig domains [Mues et al., 1998; Gregorio et al., 1998].

We report here the sequence of the first 6.5 kb of the N-terminus of chick cardiac titin. This includes a previously sequenced region of chick cardiac titin [Turnacioglu et al., 1996], that reacts with antibody to zeugmatin [Maher et al., 1985]. These data add further evidence that zeugmatin is part of the N-terminal region of titin, and not a separate Z-band protein. Comparison of chicken cardiac titin with chicken skeletal titin shows that the titins from the two muscles differ in the number of z-repeats, as do mammalian titins from different muscles.

To determine which sub-regions of titin can target to the Z-bands in a live cell assay, we linked GFP to different parts of the Z-band region of titin and transfected chick cardiomyocytes in culture. The results indicate that a single z-repeat composed of 40 to 50 amino acids can target titin fragments to the Z-band. The

extent of incorporation into Z-bands, however, varied between the repeats. Furthermore, the specificity of the targeting of individual z-repeats was enhanced when a 335 amino-acid segment of the I-band region of titin, that does not target to Z-bands, was inserted between the C-terminus of the z-repeat and the GFP tag. Similar transfections of quail skeletal muscle cells and non-muscle PtK2 cell line lead to localization of the cardiac muscle z-repeat peptides in the Z-bands of the myotubes and in the alpha-actinin-rich dense bodies of stress fibers, the non-muscle homologues of Z-bands and myofibrils in non-muscle cells [Mittal et al., 1987]. Over-expression of the z-repeat constructs leads to the disassembly of myofibrils in both types of striated muscle cells and to the loss of the stress fibers in PtK2 cells. This work was presented in a preliminary form at the American Society for cell Biology meeting [Turnacioglu et al., 1997c].

## MATERIALS AND METHODS

### Cardiac Muscle RNA Extraction, RT-PCR, and Construct Assembly

Hearts of 7-day-old (Hamburger-Hamilton [HH] Stage 31) and 19-day-old (HH Stage 45) Leghorn chick embryos and adult chickens were dissected, flash frozen in liquid nitrogen, and pulverized with a mortar and pestle that was bathed in liquid nitrogen. Additional adult cardiac tissue was dissected, cut into strips, and stored at 4°C in RNAlater, a tissue and RNA storage solution (Ambion, Austin, TX). Total RNA was then extracted with a guanidinium thiocyanate-phenol-chloroform extraction procedure [Chomczynski and Sacchi, 1987] or by using the QuickPrep Total RNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ). Reverse transcription and the PCR reaction (RT-PCR) were done in a single tube as described in Turnacioglu et al. [1997a] using primers derived from a 11.5 kb chicken skeletal titin cDNA [Yajima et al., 1996]. The entire Z-band portion and part of the I-band portion of cardiac titin was derived from RT-PCR reactions using the following five primer pairs as follows: for TN1 (bp 1–1,334): agggatccatgacaacgaaagcacca & gcctgcagtcattgctgtccttttagcagct; for TN2 (bp 1,334–2,439): gactgcagtcattgctgtccttttagcagc & cagaagcttctccaattgttaccgaatgacaa; for TN3 (bp 2,439–4,010): cgagaagcttctgcaacgggtctcct & ccgtaagctttgctgcctattctgagcaa; for TN4 (bp 4,010–5,075): ccgtaagcttctgtatcagtgactgtctg & cgggatccatcataacggagcttaaac; and for TN 5 (bp 5,075–6,169): cgggatccacttacctggatattgtgac & ccgtaagctttgagtagttttacagattcatc. Base pair numbering is from Yajima et al. [1996]. Primers flanking the z-repeat region were also used in RT-PCR reactions: (caagttaccattagtggtgctgct & ttgggctcattggtcttttacagc).

The RT-PCR products were subcloned into the multiple cloning site of pBluescript SK+ (Invitrogen, San Diego, CA) and sequenced from both strands by an automated sequencer (Applied Biosystems, Foster City, CA) to obtain a contiguous open reading frame of 6.5 kb. Some RT-PCR products were cloned using the Topo TA cloning kit (Invitrogen) and were also sequenced. The cDNAs corresponding to TN 1–5 were then subcloned into the green fluorescent protein plasmid, pEGFP-N1 (Clontech, Palo Alto, CA) to produce the TN1/EGFP, TN2/EGFP, TN3/EGFP, TN4/EGFP, and TN5/EGFP constructs with the GFP on the C-terminus of the titin fragments. A 2,912 bp fragment, beginning at the N-terminus, was also derived by RT-PCR. A further series of constructs were produced from this Z-band fragment to express various combinations of the z-repeat motifs (z-repeats) using primers based on the sequence of Gautel et al. [1997].

The fragments were subcloned into pEGFP-N1 or -C1 to generate the probes in Figure 3. Several of the probes had a 355 amino acid I-band section of titin, i.e., TN4, between the z-repeats and the GFP (see Fig. 3, constructs 7–9). Other probes were designed with the z-repeat separated from GFP by 17 amino acids in the multiple cloning site (Fig. 3, constructs 11–14). In one case, a 264-bp fragment (base pairs 1980–2244) encoding 88 amino acids of collagen XV [Myers et al., 1992], was placed between the sequence for the first z-repeat and the GFP in pEGFP-N1. A control construct was also assembled that encoded just the collagen fragment linked to GFP. All plasmid DNAs were purified with a Qiagen column (Qiagen, Chatsworth, CA) and all constructs were confirmed by sequencing.

### Cell Culturing, Transfections, and Staining of Cells

Cardiac myocytes were isolated from 7-day embryonic chicks [Sanger et al., 1984; Dabiri et al., 1999a] and grown on glass bottom dishes (MatTek, Ashland, MA) or regular coverslips. Skeletal myoblasts were isolated from 10-day-old quail embryos and cultured on collagen coated dishes [Dabiri et al., 1999b]. PtK2 cells were cultured as previously reported [Sanger et al., 1998, 2000]. The cardiac muscle cultures were transfected with the various GFP constructs after 1 or 2 days of culture using Lipofectamine (Gibco BRL, Grand Island, NY) [Turnacioglu et al., 1997a; Dabiri et al., 1999] or FuGene6 (Boehringer Mannheim, Mannheim, Germany). No difference in transfection efficiency was noted between these two transfection reagents or the length of days in culture (i.e., transfection after 1 or 2 days of cell culture). However, since FuGene6 can be used in the presence of serum we are now using this reagent on cardiomyocytes. Skeletal muscle cells were transfected using a Cal-Phos Maximizer kit (Clontech Inc., Palo Alto, CA). PtK2 cells were transfected using Lipofectamine

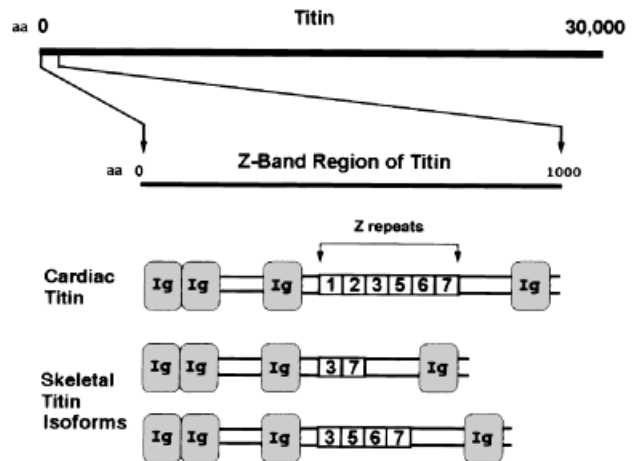


Fig. 1. Cardiac muscle titin is composed of about 30,000 amino acids of which approximately 1,000, beginning at the N-terminus, are in the Z-band. A diagram comparing the arrangement of the N-terminal region of the chicken cardiac titin, reported in this paper, with the chicken skeletal muscle titin [Yajima et al., 1996]. The rounded boxes represent Ig-like domains and the numbered boxes represent the z-repeat motifs. Note that the N-terminal region of cardiac titin has 6 z-repeats, whereas the skeletal has only 2 or 4. The 6 cardiac z-repeats have been numbered 1–3 and 5–7 to correspond to the numbering of the homologous sequences of mammalian cardiac titin [Sorimachi et al., 1997]. The chicken skeletal z-repeats have been numbered 3, 5, 6, and 7 by homology with the chicken cardiac titin sequence. The skeletal repeats were originally designated as 2, X, Y, and 5 [Ohtsuka et al., 1997a].

[Ayoob et al., 2000]. At 12–96 h after transfection with the titin-EGFP constructs, the GFP fluorescence was recorded in the live cells. In some experiments, cells were fixed for 15 min at room temperature in 3.0% paraformaldehyde in sodium phosphate buffer, rinsed several times with standard salt (0.1 M KCl, 0.01 M  $K_2PO_4$ , 1 mM  $MgCl_2$ , pH 7.0), and permeabilized with 0.1% Nonidet P-40, and then stained with antibodies to muscle-specific alpha-actinin (Sigma, St. Louis, MO) or muscle myosin II (gift of Dr. F. A. Pepe, University of Pennsylvania, described in Sanger et al. [1986]) as previously described [Rhee et al., 1994; LoRusso et al., 1997]. Some of the transfected cultures were stained with rhodamine labeled phalloidin (Fluka, Ronkokomo, NY) as previously reported [Rhee et al., 1994]. Live cells were maintained on the microscopic stage by supplying heat and 5%  $CO_2$  as described [Dabiri et al., 1999]. The transfected cells were viewed with a Nikon Diaphot 200 microscope with either a phase-contrast 100 $\times$ , or a phase-contrast 63 $\times$  objective. Images were acquired with a liquid-cooled CCD (CH 220 with Kodak KAF 1400 CCD, Photometrics, Tuscon, AZ) or (C 4742–95, Hamamatsu, Bridgewater, NJ) and were processed with Metamorph image processing system (Universal Imaging Inc., West Chester, PA) or Image Pro Plus (Media Cybernetics, Silver Spring, MD) and Adobe Photoshop (Adobe, Mountain View, CA).



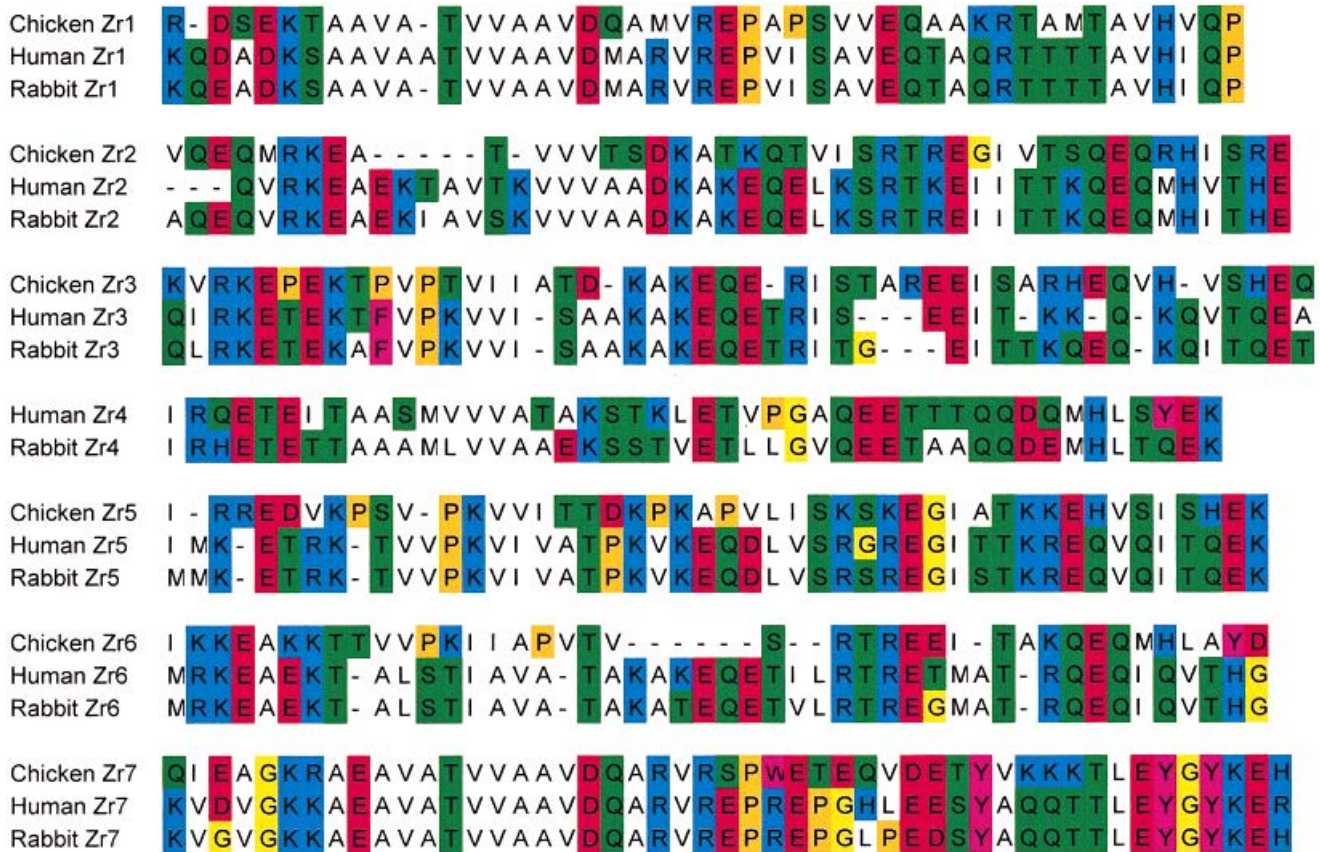


Fig. 2. This chart compares the sequences of the six z-repeats of chicken cardiac titin with those of human and rabbit cardiac titin, which have seven repeats. Z-repeats were aligned according to similarity of amino acids as defined by the Colour Interactive Editor for Multiple Alignments (CINEMA)v2.1. The color coding indicates: blue, polar

positive (H K R); red, polar negative (D E); green, polar neutral (S T N Q); white, non-polar aliphatic (A V L I M); purple, non-polar aromatic (F Y W). Prolines (P), glycines (G), and cysteines (C), orange, gold, and yellow, respectively, are given separate colors to highlight their unique structural roles in proteins.

## RESULTS

### Sequence of the N-Terminus of Chicken Cardiac Titin

The deduced amino acid sequence from the N-terminus of chicken cardiac titin to the beginning of the z-repeat motifs is 99.5% identical to the N-terminus region of chicken skeletal connectin (or titin) [Yajima et al., 1996]. In this first 403 amino-acid segment there are three Ig domains and 5 SPXR consensus sites for ERK and cdc kinases, as was noted in this region of human cardiac titin [Gautel et al., 1996]. The first 6.5 kb of embryonic (19 days old) chick cardiac titin, encoding the full length of the Z-band region and part of the I-band region of titin is available from GenBank (accession number AF159173). This sequence includes part of a region previously thought to represent zeugmatin [Turnacioglu et al., 1996], adding further evidence that zeugmatin is a proteolytic fragment of titin [Turnacioglu et al., 1996, 1997a,b; Sanger et al., 2000].

Six repeating motifs, homologous to the z-repeats of human cardiac titin [Gautel et al., 1996], are present in the chicken cardiac titin and 4 of these are 97% identical to the 4 chicken skeletal z-repeats identified by Ohtsuka

et al. [1997b] (Fig. 1). The color chart in Figure 2 compares the amino acid sequences of the z-repeats of chicken cardiac titin with those of human and rabbit cardiac z-repeats [Young et al., 1998; Sorimachi et al., 1997]. In contrast to the adult mammalian hearts, which have seven z-repeats, the chicken heart possesses only six z-repeats, lacking a repeat homologous to the fourth z-repeat present in both mammalian titins. If the chicken cardiac z-repeats are numbered consecutively 1–3 and 5–7 beginning with the most N-terminal, the cardiac z-repeats that match the chicken skeletal z-repeats (previously termed 2, X, Y, 5 by Ohtsuka et al. [1997b]) are numbers 3, 5, 6, and 7 (Fig. 1).

### No Changes in the Number of Z-Repeats During Development of the Chicken Heart

To determine if there is a difference in the number of z-repeats at an earlier or later stage of cardiac development, RNA was isolated from 7-day-old embryonic chick hearts and adult chicken hearts and used as a template to isolate cDNAs encoding the entire z-repeat region of this

titin. Our gel electrophoresis and sequencing data revealed that there was only one band and it contained the same six z-repeats discovered in 19-day-old embryonic cardiac titins, i.e., z-repeats 1–3 and 5–7 (data not shown).

### Localization of Fragments of the Z-band Region of Titin in Transfected Cardiomyocytes

Transfection of cardiomyocytes with a probe expressing a 42 kDa fragment of titin, coupled to Green Fluorescent Protein (GFP), had demonstrated that the fragment could target to the Z-bands of living chick cardiac muscle cell provided that the GFP probe was coupled to the C-terminus of the fragment (Fig. 3, construct no. 4 vs. non-targeting construct no. 5) [Turnacioglu et al., 1997a,b]. To determine which sub-regions of the Z-band sequence of titin were capable of targeting to the Z-bands in living cardiomyocytes, a series of GFP-linked probes were constructed. The chart in Figure 3 summarizes all of the constructs and their ability to target. Not surprisingly, the complete sequence of the Z-band region of titin, linked at its C-terminus to GFP, readily incorporated in the Z-bands of living cardiac muscle cells as judged by position of the GFP fluorescence with respect to the phase-contrast image of the Z-bands in live cells and to muscle-specific antibodies that were used to counterstain the same cell (Fig. 3, construct no. 1; Fig. 4A,B). We found that the Z-band incorporation of this probe had no short-term effect on the spontaneous contractions of the cells.

Expression of the fragment encoding just the 6 z-repeats coupled at its C-terminus to GFP also leads to incorporation of the GFP probe into the Z-bands of cardiac muscle cells (Fig. 3, construct no. 3; Fig. 4C,D). If the 6 z-repeat region was excluded from the probe encoding the full Z-band region of titin, no localization was detected in Z-bands (Fig. 3, construct no. 2; Fig. 5A,B). The fluorescent product of this z-repeat-free construct was distributed diffusely throughout the cytoplasm as well as in nuclei. Occasionally (less than 5% of the transfected cells), there was a low level of the labeled probe along some myofibrils and bright fluorescence inside the nucleus, in addition to the diffuse cytoplasmic fluorescence (Fig. 5C,D). The cells, transfected with full Z-band, six z-repeats, and the deletion construct, exhibited spontaneous contractions, and, when fixed, permeabilized and stained with muscle specific antibodies, normal myofibrils were detected (Figs. 4 and 5).

If the cDNAs for single z-repeats were coupled to GFP with a linker of eight amino acids, they did not localize to Z-bands but were diffusely distributed in the cytoplasm. To determine if the 28 kDa GFP protein might be interfering with the ability of the much smaller z-repeats (only 40 to 50 amino acids in length) to target to the Z-band, we inserted different linkers between the individual z-repeats and the GFP. A linker of 17 amino acids was created by recloning the probes into the pEGFP-N1 vector further upstream in the multiple clon-

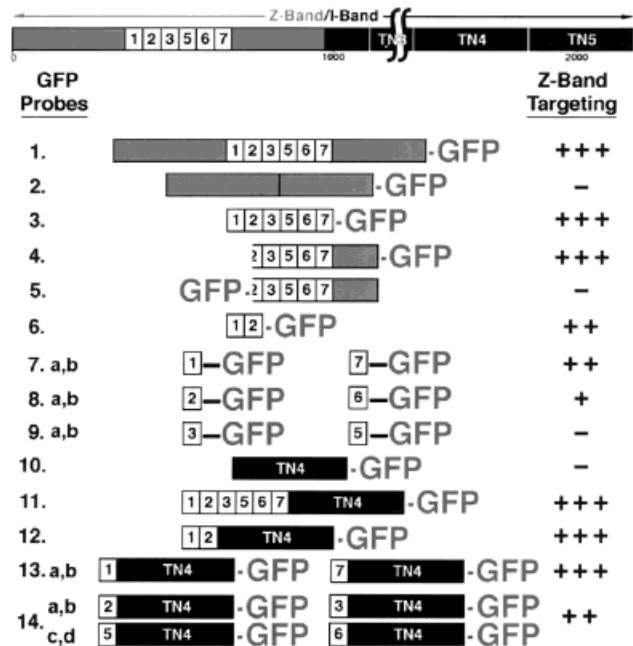


Fig. 3. Chart of the titin fragment-GFP constructs that were introduced into embryonic chick cardiac cells via transfection. The probes that were constructed for this experiment included fragments encoding: (1) the full-length Z-band sequence of titin; (2) the full-length of the Z-band with the 6 z-repeats excluded; (3) the 6 z-repeats alone; (4,5) subfragments of the Z-band piece; (6) the first 2 z-repeats alone; (7–9) single z-repeats separated by 17 amino acids from the linkage to GFP; (10) an I-band fragment that does not localize to the Z-band; and (11–14) multiple or single z-repeats fused to TN4. A summary of the Z-band targeting of these constructs is indicated on the right side. +++: strong localization with bright fluorescent Z-bands and a low level of diffuse fluorescence. ++: Z-band localization accompanied by diffuse cytoplasmic fluorescence; +: weak and rare localization in the Z bands and a high level of diffuse cytoplasmic fluorescence; -: predominately diffuse fluorescence with some cells showing faintly fluorescent myofibrils.

ing site. With this strategy, the first and last z-repeats localized to Z-bands in the live cells, but there was diffuse cytoplasmic fluorescence and bright nuclear fluorescence as well (Fig. 3, construct no. 7; Fig. 6). A similar result was seen with the first two z-repeats linked in the same way to GFP (Fig. 3, construct no. 6). A single z-repeat from the middle of the group rarely (z-repeats 2 and 6) or never (z-repeats 3 and 5) localized in Z-bands (Fig. 3, construct nos. 8 and 9).

A piece of titin 355 amino acids long from the I-band, about 800 amino acids away from the Z-band (TN4 in Fig. 3), was also used as a linker to separate GFP from the z-repeats (Fig. 3). In nearly all transfected cells, the TN4-GFP did not localize to any substructures of the cells (Fig. 3, construct no. 10; Fig. 7A), but in a few cells, expression of this construct led to some association of the fluorescent peptide along myofibrils (Fig. 7B). When the TN4 was linked to the 6 z-repeat construct, the TN4 linker did not interfere with the ability of the z-repeats to target strongly to the Z-band (Fig. 3, construct no. 11).



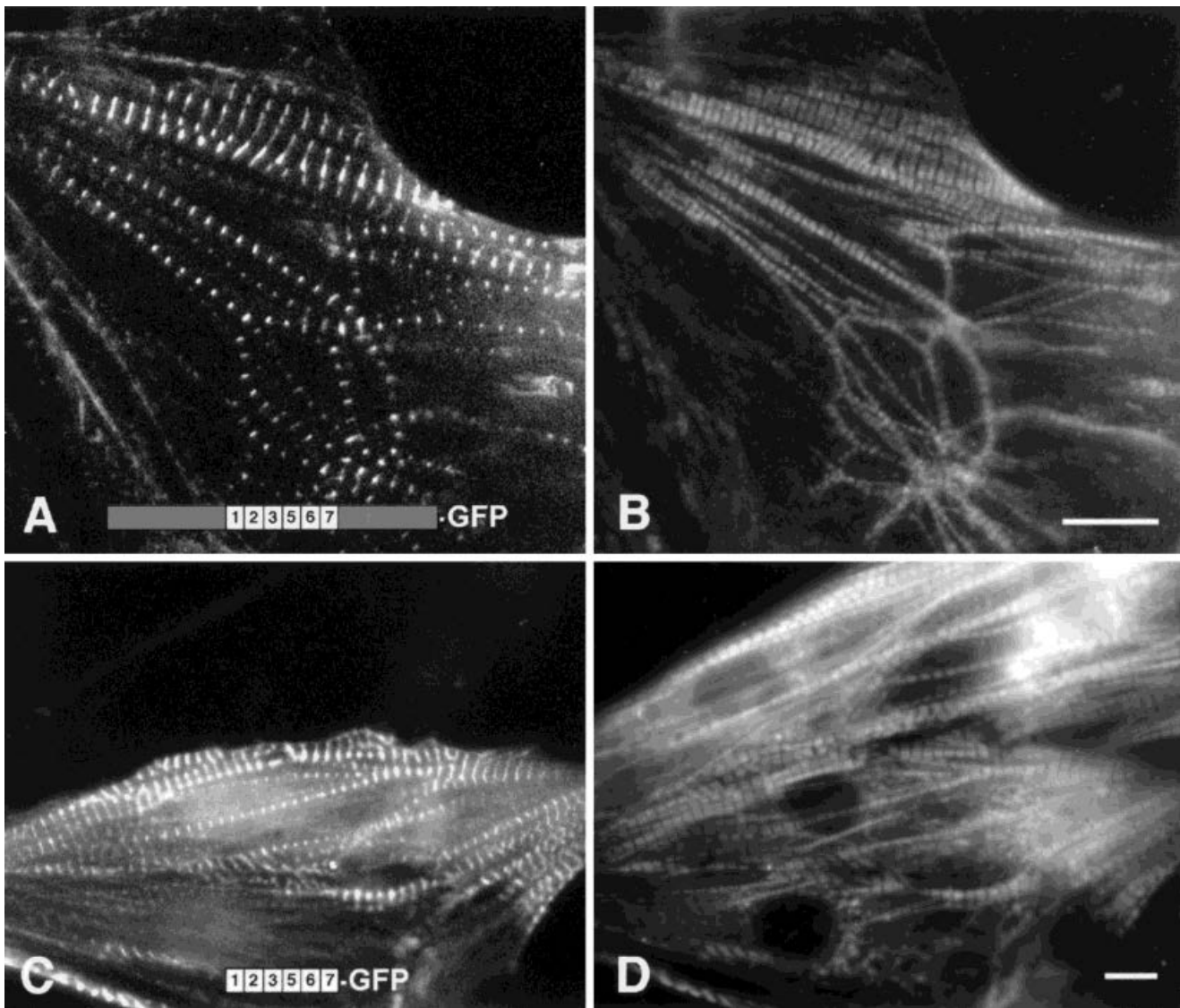


Fig. 4. Chick embryonic cardiomyocytes transfected after 1 day in culture with the full length Z-band titin-GFP probe (A) or the six z-repeat fragment (C). Both constructs target to the Z-bands. **B,D**: A-bands in the same cells counterstained with a muscle specific myosin IIB antibody. Scale bar = 10  $\mu$ m.

Linking the first two z-repeats or a single z-repeat to GFP via TN4 resulted in improved targeting of each fragment to Z-bands (Fig. 3, construct nos. 12, 13, 14; Figs. 8 and 9). These constructs, like the other constructs expressing the z-repeats, targeted to both the Z-bodies of the premyofibrils [Rhee et al., 1994] and the Z-bands of the mature myofibrils (Fig. 8). The strongest targeting was seen with the first and last z-repeats (Fig. 9A and F) and was comparable to that detected when all 6 z-repeats were expressed (Fig. 4). If the first z-repeat was separated from GFP by a fragment of collagen XV, the fusion protein also targeted to the Z-bands of the cardiac myofibrils (Fig. 10A). A control plasmid encoding just the collagen fragment linked to GFP produced a fusion protein that was not incorporated into any fibrils in transfected cardiomyocytes (data not shown).

#### Over-Expression of Z-Repeats Leads to Loss of Myofibrils

Over-expression of single z-repeat fusion proteins lead to myofibril disassembly (Figs. 9C,D; 10B). This was particularly noticeable in constructs containing z-repeats 2, 3, 5, or 6 (Fig. 9C,D). Over-expression was accompanied by concentrations of the fluorescent probe in the nuclei (Figs. 9C,D; 10B). Initially, in the early stages of transfections, the z-repeats localized in closely spaced bands in premyofibrils (Fig. 8) as previously reported by Turnacioglu et al. [1997a] for construct no. 4 (Fig. 3). In the over-expressing cells, no signs of any fibrils could be detected. New myofibrils were not detected to form in these over-expressing living cells. In cells overexpressing the z-repeat1-collagen fragment-GFP, myofibrils were disassembled (Fig. 10 B).

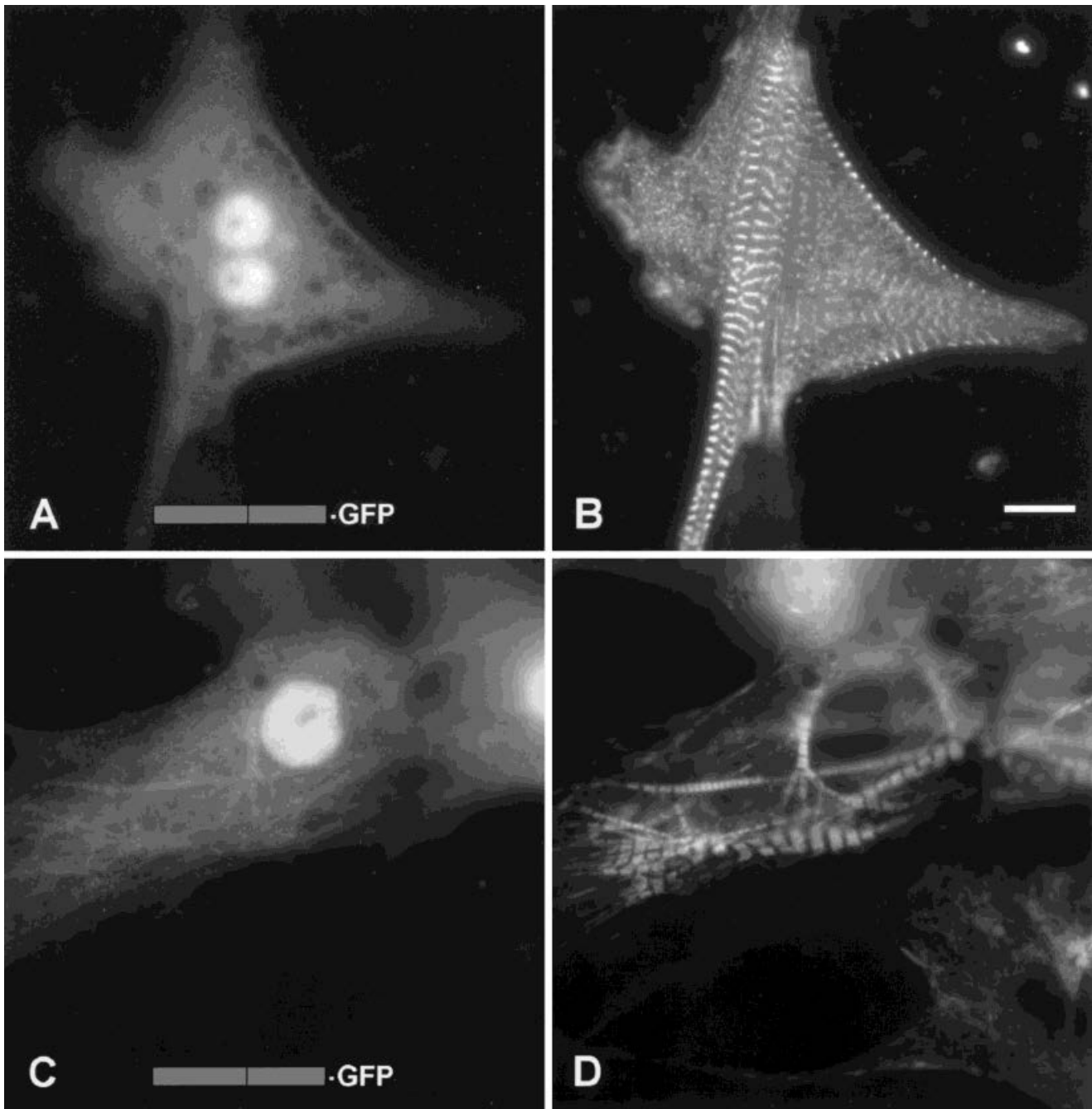


Fig. 5. Cardiomyocytes, transfected with the region of Z-band titin from which the z-repeats were deleted, show diffuse cytoplasmic fluorescence that is often very intense in nuclei (A, C) and occasionally shows some un-banded association with a few of the myofibrils (C). These cells were fixed, permeabilized and stained with a sarcomeric alpha-actinin antibody (B) or a muscle myosin II specific antibody (D). Deletion of the z-repeats from the Z-band region of titin abolishes the ability of this region to target to Z-bands. Scale bar = 10  $\mu$ m.

#### Expression and Over-expression of the Constructs in Skeletal Muscle Cells

In quail myotubes transfected with a plasmid encoding the cardiac muscle z-repeat 1-TN4-GFP (Fig. 3, construct 13a), the fusion protein incorporated into the Z-bands of the myofibrils (Fig. 11). Over-expression of z-repeat 1-GFP (Fig. 3, construct 7a) in myotubes led to the loss of myofibrils with the myosin filaments scattered in the cell (Fig. 12).

#### Expression and Over-expression of the Constructs in Non-Muscle Cells

In the cultures of cardiomyocytes, fibroblasts that were transfected often showed stress fiber fluorescence in a beaded pattern with spacings about 1  $\mu$ m apart (data not shown). To examine the targeting of z-repeats to stress fibers, we transfected PtK2 cells with single z-repeats coupled to TN4-GFP. PtK2 cells were selected

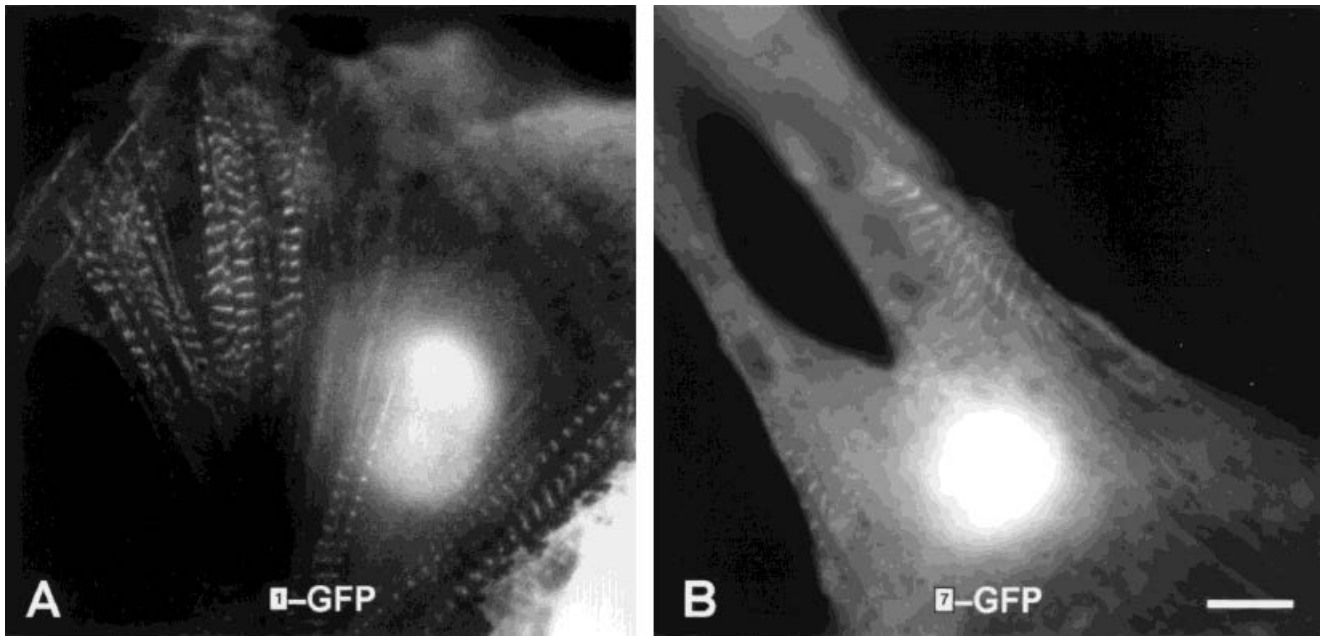


Fig. 6. These cardiomyocytes were transfected with a construct in which the first z-repeat (A) or the last z-repeat (B) was separated by 17 amino acids from the GFP. These 17 amino acids are part of the multicloning site in the GFP plasmid. The fusion protein does target to the Z-bands, but there is also some diffuse cytoplasmic and nuclear fluorescence in the cardiomyocytes. Scale bar = 10  $\mu$ m.

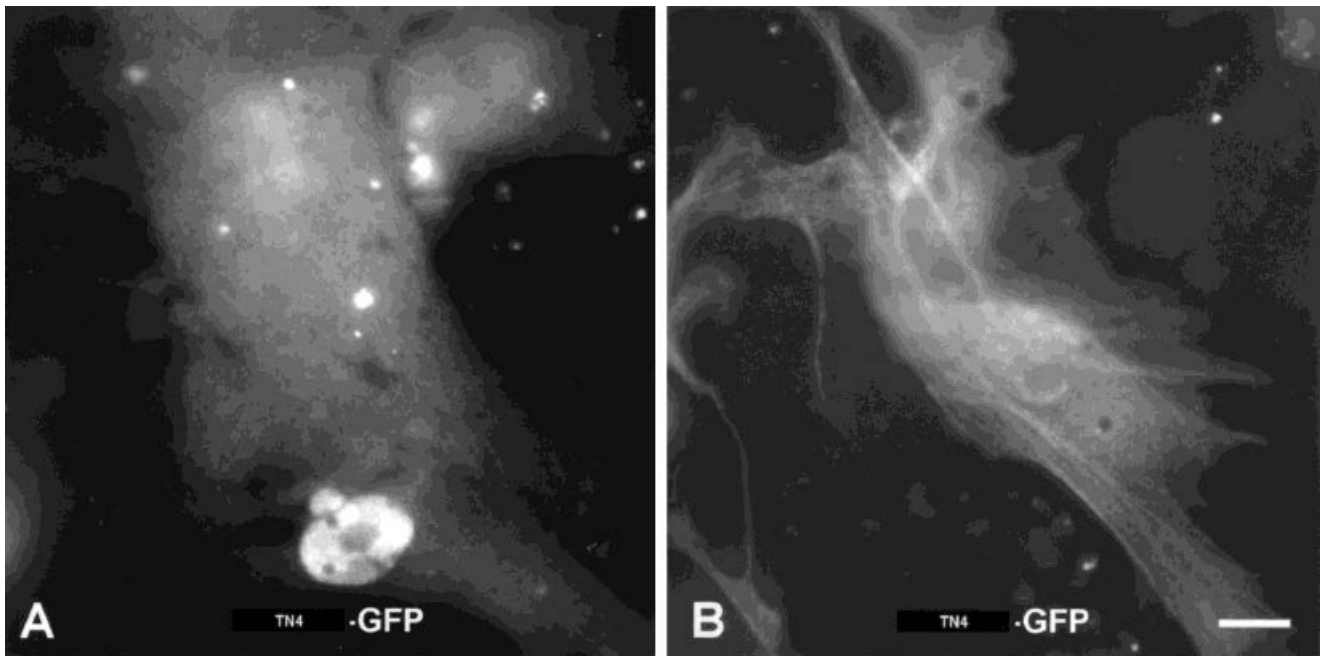


Fig. 7. These cardiomyocytes were transfected with the TN4-GFP construct; the TN4 encodes part of the I-band region of titin. The fusion protein shows two types of localization patterns. In most cases (A), the fluorescence is diffuse. In a few cells (B), TN4-GFP is weakly distributed along the fibers in addition to the diffuse cytoplasmic fluorescence. Scale bar = 10  $\mu$ m.

because the sarcomeric structure of their stress fibers has been carefully documented and it is known that alpha-actinin is concentrated in their dense bodies [Sanger et al., 1983; Mittal et al., 1987; Turnacioglu et al., 1998]. Expression of single z-repeats linked to TN4-GFP lead to localization of

fluorescence in a punctate pattern along the stress fibers (Fig. 13A–F). As in the muscle cells, z-repeats 2, 3, 5, and 6 showed weaker targeting to the stress fibers (Fig. 13B–E). These fluorescent densities correspond to the known positions of the dense bodies of the stress fibers in



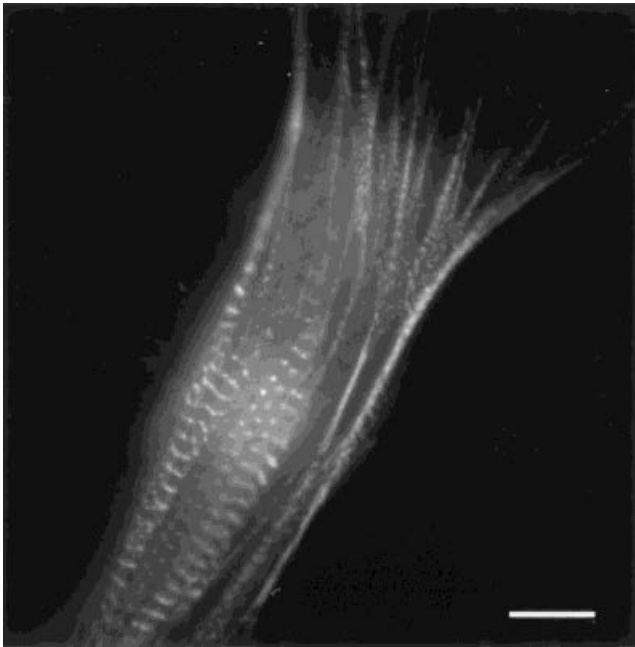


Fig. 8. This cardiomyocyte was transfected with the z-repeat 1-TN4-GFP construct. The fluorescent fusion protein localized to the Z-bodies of the premyofibrils in the top of the cell and to the Z-bands of the mature myofibrils in the middle and lower half of the beating cell. Scale bar = 10  $\mu$ m.

PtK2 [Turnacioglu et al., 1998]. As a control we transfected PtK2 cells with a plasmid encoding just GFP. These experiments led to a diffuse distribution of the fluorescent protein with no localization in stress fibers. In some cells, concentrations of the GFP were detected in the nuclei. These control GFP-transfected cells were fixed and stained with rhodamine phalloidin. The expression of just GFP had no effect on the presence of stress fibers in the PtK2 cells. (data not shown).

Over-expression of z-repeat-GFP constructs lead to the loss of the stress fibers and concentrations of the fluorescent probes inside the nuclei (Fig. 14). As the level of the probe increased in the nuclei, there was a decrease in the number of stress fibers in the transfected cells. Control PtK2 cells, transfected with plasmids encoding just GFP, revealed no signs of stress fiber disruption in the presence of high levels of the fluorescent protein (data not shown).

## DISCUSSION

### N-terminal Sequence Data Confirms That Zeugmatin Is Part of Titin

Zeugmatin was identified originally as a novel Z-band protein on the basis of immunofluorescence and biochemical evidence [Maher et al., 1985]. Sequencing of a 1.1 kb cDNA from clones generated by screening a chicken cardiac muscle lambda g11 expression library with the anti-zeugmatin monoclonal antibody, indicated that zeugmatin was part of the Z-band region of titin [Turnacioglu et al., 1996, 1997a,b]. In the present study,

RT-PCR of 19-day embryonic chick heart RNA using primers from the sequence of the N-terminus of chicken skeletal titin cDNA [Yajima et al., 1996] yielded a contiguous 6.5-kb sequence starting from the N-terminus and including the previously reported partial sequence of cardiac zeugmatin. This supports our suggestion that zeugmatin is not a separate Z-band protein but rather part of the N-terminus of titin [Turnacioglu et al., 1996]. This region of the chicken cardiac titin before the start of the z-repeats is 99.5% identical to the corresponding region of chicken skeletal titin recently sequenced by Ohtsuka et al. [1997b]. The differences between the Z-band regions of titin in chicken cardiac and skeletal muscle are found in the z-repeat region. The cardiac muscle has 6 z-repeats, which can be numbered 1, 2, 3, 5, 6, 7 (Fig. 2) by comparison with mammalian titin and adopting the numbering suggested by Sorimachi et al. [1997]. In the two chicken skeletal muscle isoforms, the z-repeats [Ohtsuka et al., 1997b] are virtually identical to z-repeats 3, 5, 6, and 7 of the chicken cardiac isoform (Fig. 1). These results support the hypothesis advanced by Gautel et al. [1996] and Sorimachi et al. [1997] that the coding sequence for the Z-band region of titin is alternatively spliced in different cross-striated muscle cells. The number of z-repeats in chicken hearts from embryonic (7 and 19 days) and adult hearts did not vary, however, as it does in fetal and adult mammalian cardiac titins [Sorimachi et al., 1997]. It is noteworthy that a GFP construct containing the first z-repeat of cardiac muscle (Fig. 3, construct 13a) can target to the Z-bands of skeletal muscle cells even though this repeat is not present in chicken skeletal muscle.

### Single Z-Repeat-GFP Can Target to Z-bands In Vivo

Although the Z-band region of cardiac titin is composed of about 1,000 amino acids [Gautel et al., 1996; Yajima et al., 1996], a small region of about 300 amino acids, containing novel motifs called z-repeats, is chiefly responsible for the targeting of titin to the Z-band. One z-repeat alone, linked to GFP, usually targeted to the Z-band, provided the GFP was linked to the C-terminus and not to the N-terminus of the z-repeats and a suitable linker was placed between it and the GFP probe. The relatively large size of the GFP (about 28 kDa) may inhibit the binding properties of the single z-repeat if it is in close proximity. When a long I-band region of titin, i.e., TN4 (Fig. 3) was used as a linker, targeting of the first and last z-repeats was improved (Fig. 9A and F vs. Fig. 6), and was as strong as that of the construct containing all 6 z-repeats (Fig. 9A and F vs. Fig. 4). The use of the TN4 linker also allowed for the improved targeting of the four inner z-repeats, two of which could not target with the smaller linker of 17 amino acids. Whereas the region

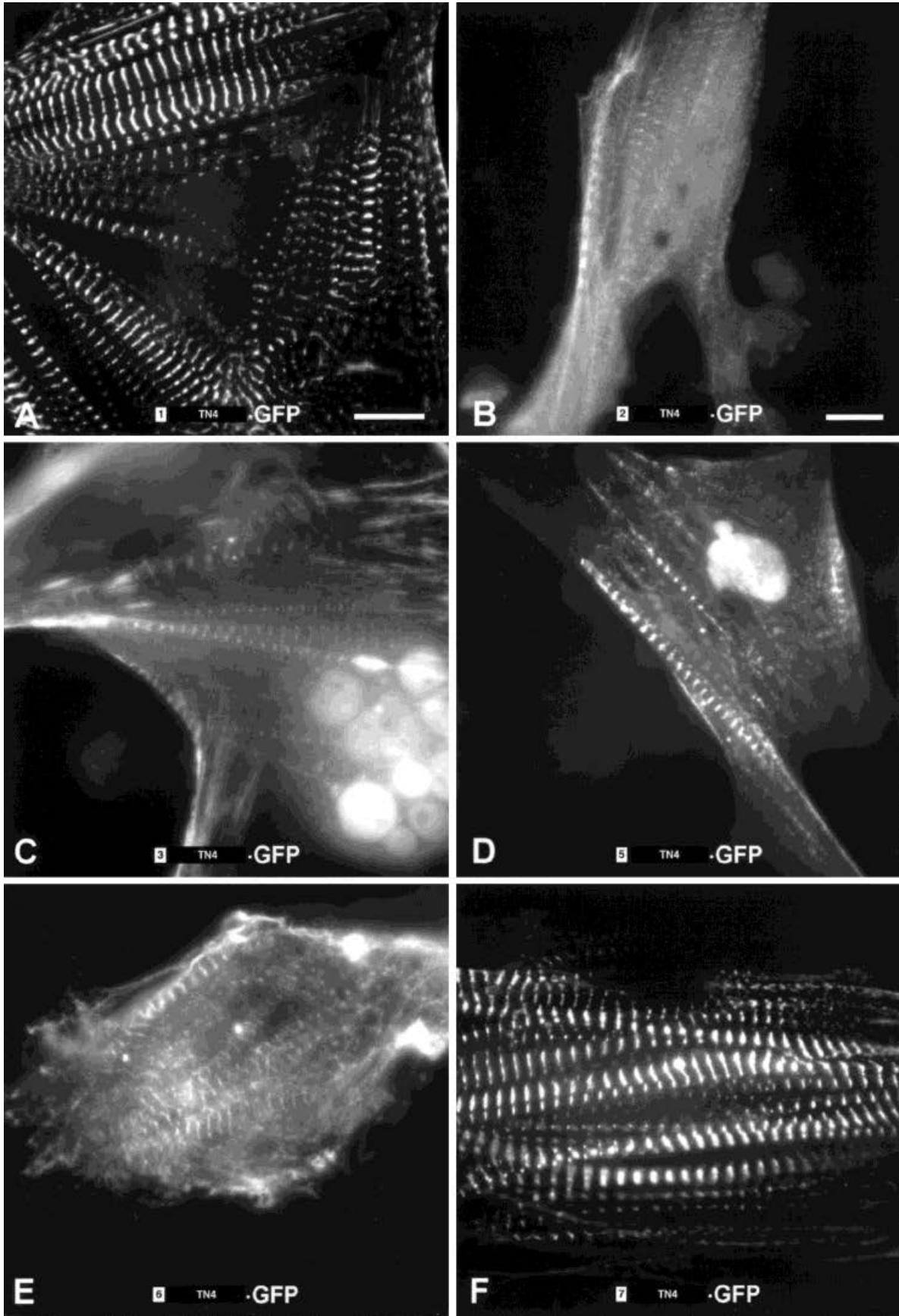


Fig. 9. Single z-repeats, linked with TN4 to GFP, target strongly (A,F) or less so (B–E) to Z-bands in cardiomyocytes. Z-repeats 1, 2, 3, 5, 6, and 7 are shown in order in A to F. Note that nuclei of cells transfected with the two middle z-repeats often exhibit nuclear concentrations of the GFP-construct (C, D). Scale bar = 10  $\mu$ m (A, C–F are the same magnifications).

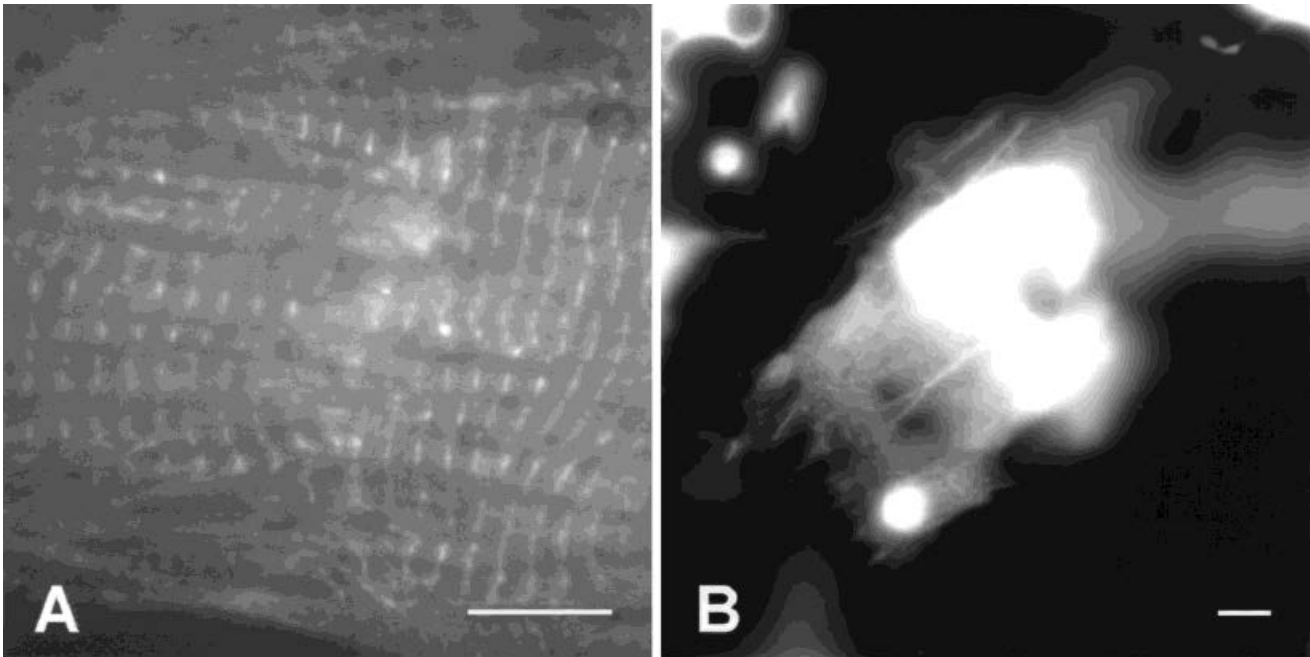


Fig. 10. Cardiomyocytes transfected with a plasmid expressing z-repeat 1 linked to GFP via a collagen XV fragment. **A:** The fluorescent fusion protein localized to the Z-bands. **B:** Overexpression of this fusion protein led to the loss of the myofibrils. Scale bar = 10  $\mu\text{m}$ .

of titin in the I-band, adjacent to the Z-band, is believed to be able to bind to actin filaments, there is no evidence that the more C-terminal I-band region of titin (TN4) binds actin [Linke et al., 1997]. Only rarely did we detect TN4-GFP associating with actin filaments in the myofibrils. Nevertheless, it is possible that the interactions of the z-repeats with the Z-bands of the myofibrils and the dense bodies of the stress fibers are enhanced by the presence of the flanking regions and TN4. However, the transfections of cardiomyocytes using the 17 amino acids of the polylinker cloning site or the collagen fragment to separate a single z-repeat from GFP also permitted Z-band targeting (Fig. 10).

There are two regions of titin, in addition to z-repeat fragments, that bind to Z-band proteins: a non-modular region of titin, adjacent to the C-terminus of the z-repeat motif, that binds alpha-actinin [Young et al., 1998]; and the first two Ig domains of titin that bind the Z-band protein telethonin in yeast two-hybrid assays [Mues et al., 1998; Gregorio et al., 1998]. A GFP-tagged fragment including these regions, but lacking z-repeats, did not target to the Z-band in transfected cardiomyocytes (Fig. 5). It may be that these residues are not accessible in the GFP-tagged fragment. The *in vitro* binding of telethonin to the two Ig domains of titin, in fact, appears to be dependent on the conformation of the titin fragment [Mues et al., 1998].

We previously reported that a 42 kDa fragment of the N-terminal region of titin bound alpha-actinin [Turna-

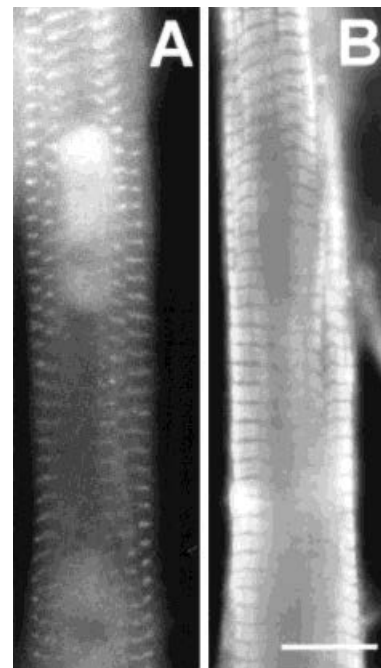


Fig. 11. **A:** A quail myotube that had been transfected with the z-repeat 1-TN4-GFP construct. Note the localization of the fusion protein in the Z-bands of the myofibrils. Although this first z-repeat is a cardiac muscle-specific z-repeat, it is able to target to the Z-bands of skeletal muscle cells. **B:** The myotube was fixed, permeabilized, and stained with a muscle-specific myosin II antibody, revealing A-bands with normal appearance. Scale bar = 10  $\mu\text{m}$ .



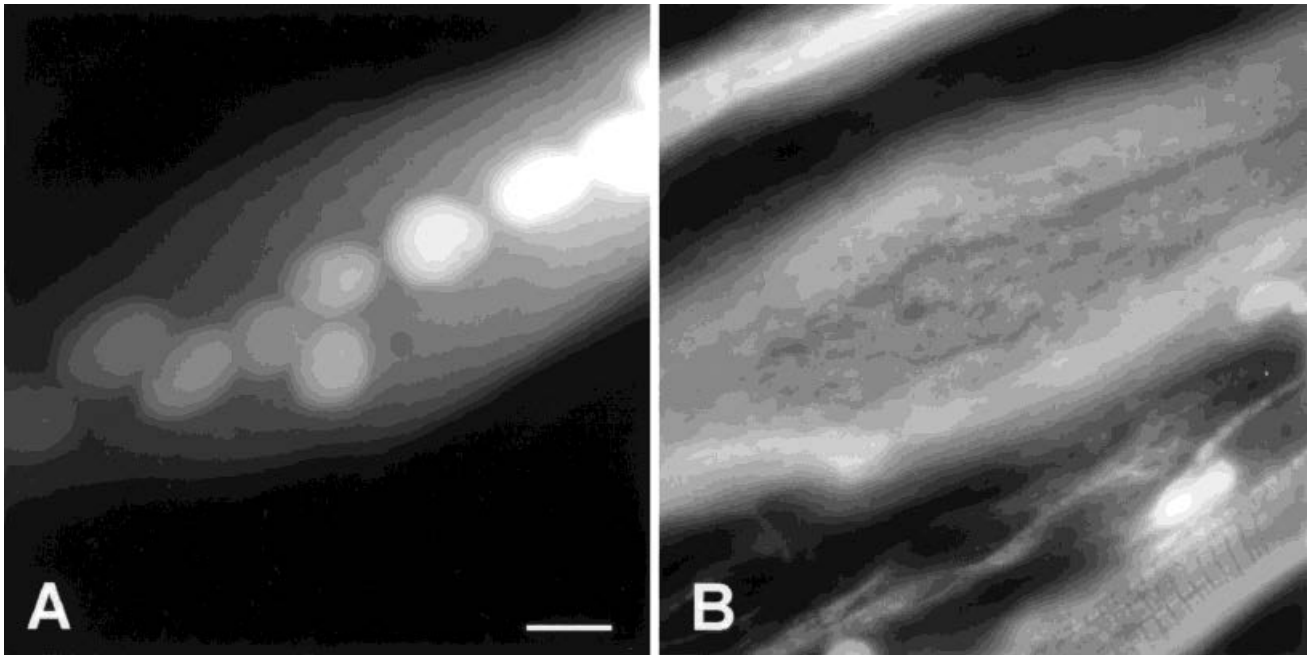


Fig. 12. Quail myotubes transfected with a plasmid expressing z-repeat 1 fused to GFP via a 17 amino acid linker. **A:** Overexpression of the construct resulted in myotubes with brightly fluorescent nuclei and no evidence of Z-bands. **B:** No A-bands were seen when the cells were fixed, permeabilized, and stained with a muscle-specific myosin

antibody, indicating that the myofibrils were completely disrupted in the transfected myotube. The myosin II filaments have been scattered along the length of the transfected myotube. An adjacent untransfected myotube in the lower right has a normal distribution of myosin II in A-bands. Scale bar = 10  $\mu$ m.

cioglu et al., 1996]. We suggested that a KIKK motif in the sequence might be involved in this binding since this sequence in ICAM was found to bind alpha-actinin [Carpen et al., 1992]. However, the segment of a 63 kDa fragment of chicken skeletal titin that binds alpha-actinin [Ohtsuka et al., 1997a,b], lacks the z-repeat in which this motif is found. The same is true of the z-repeats of rabbit skeletal titin that bind alpha-actinin [Sorimachi et al., 1997]. Furthermore, the observation in this report that each z-repeat is able to target titin fragments to the Z-band, suggests that motifs other than KIKK must also mediate the binding of titin to alpha-actinin or perhaps other Z-band proteins. It is of interest that the differential ability of the z-repeats to target to the Z-bands is reflected in their differential ability to bind alpha-actinin. Thus, the highly homologous z-repeats 1 and 7 that target the best to the Z-band also exhibit the strongest binding to alpha-actinin [Young et al., 1998].

Recent evidence suggests that an isoform of titin is localized in the nucleus [Machado et al., 1998]. Overexpression of the z-repeat-GFP probes leads to a concentration of these probes in the nuclei. This could occur if the titin fragments were binding to some of the nuclear sites that normally bind nuclear titin. On the other hand, we have noticed that over-expression of GFP alone often leads to its concentration inside nuclei. Thus, our observations of occasional titin fragments localizing to nuclei

cannot be used to support the existence of a nuclear or chromosomal titin.

### Insights for Myofibrillogenesis

We have proposed that the formation of myofibrils in cultured cardiac cells proceeds via a three-stage process in which premyofibrils convert to nascent myofibrils that, in turn, convert to mature myofibrils [Rhee et al., 1994; Turnacioglu et al., 1997a; LoRusso et al., 1997; Sanger et al., 2000]. Premyofibrils are characterized by closely spaced densities (Z-bodies) of alpha-actinin between which are filaments of non-muscle myosin IIB, and sarcomeric isoforms of actin, alpha-actinin, tropomyosin, and troponin-forming "minisarcomeres" [Dabiri et al., 1997; Sanger et al., 2000]. Anti-zeugmatin and anti-titin (T-11) antibodies as well as muscle myosin II specific antibodies do not stain premyofibrils in spreading chick cardiomyocytes [Rhee et al., 1994]. In the transition to mature myofibrils, nascent myofibrils form with muscle-specific myosin II colocalized with non-muscle myosin IIB, and Z-bodies that stain positively with anti-titin and anti-zeugmatin antibodies. In mature myofibrils, Z-bodies are replaced by Z-bands, bands of non-muscle myosin II B are lost, and A-bands with C-protein are present in full-length sarcomeres [Rhee et al., 1994]. Time-lapse observations of sarcomeric alpha-actinin coupled to green fluorescent protein (GFP) in living

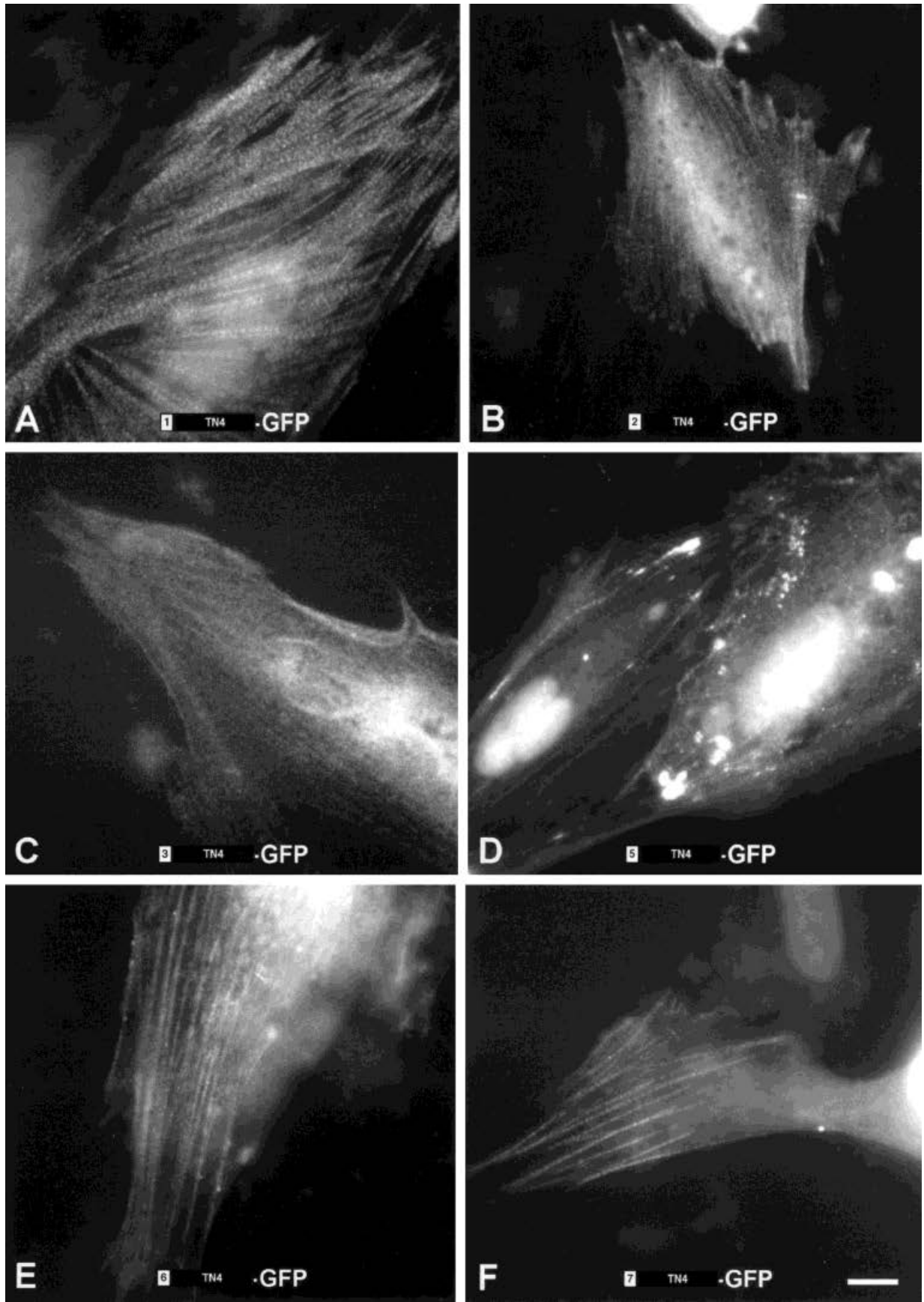


Fig. 13. A–F: Each of these PtK2 cells, a non-muscle kidney cell line, were transfected with a different z-repeat-TN4 construct. There is some localization of each construct in the dense bodies of the stress fibers. As in muscle, the first (A) and last z-repeat (F) constructs yield the best localizations. Disassembly of stress fibers and limited localization in dense bodies is characteristic of the two middle z-repeats (C,D). (B) z-repeat 2; (C) z-repeat 3; (D) z-repeat 5; (E) z-repeat 6. Scale bar = 10  $\mu$ m.

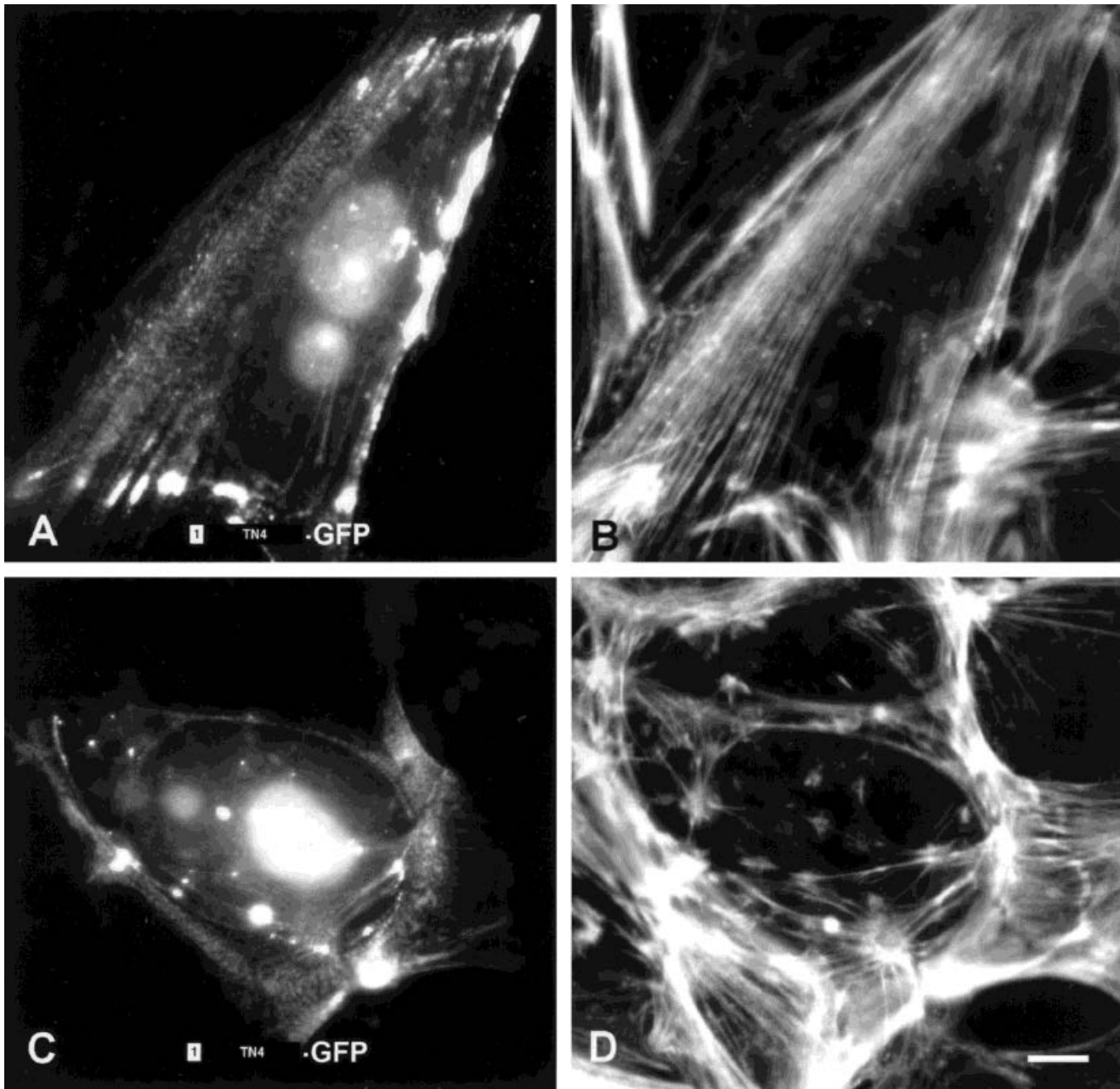


Fig. 14. Over-expression of the first z-repeat linked by TN4 to GFP causes stress fiber disassembly in PtK2 cells. The extent of stress fiber disassembly increases with increasing levels of expression as judged by intense GFP fluorescence in cytoplasmic aggregates and nuclei. (A,C) GFP and (B,D) phalloidin counter-stain to reveal the stress fibers. Scale bar = 10  $\mu$ m.

cardiac muscle cells undergoing myofibrillogenesis support several aspects of the premyofibril model [Dabiri et al., 1997].

Titin's role in myofibril formation could derive from its Z-band binding properties, its myosin filament binding properties, or both. It has been postulated to align the thick filaments of myosin into A-bands and link them

to the Z-bands in a sarcomere [Hill et al., 1986; Fulton and L'Ecuyer, 1993]. We suggested that zeugmatin, now known to be part of the N-terminal region of titin, might bind together adjacent Z-bodies of the nascent myofibrils to form the Z-bands of the mature myofibrils [Rhee et al., 1994]. The ability of the N-terminal region of titin to bind alpha-actinin and to target to Z-bands in transfected cells



could facilitate this [Turnacioglu et al., 1996, 1997a,b; Sorimachi et al., 1997; Ohtsuka et al., 1997a,b; Young et al., 1998]. Support for the role of the N-terminus of titin in myofibrillogenesis is suggested by experiments showing the disassembly of existing myofibrils and inhibition of new myofibril formation by over-expression of titin fragments containing single or multiple z-repeats. Once the N-terminal region of titin is bound to the alpha-actinin in the Z-bodies, the C-terminus of the titin molecule could then capture and induce the alignment of the thick filaments into an A-band. On the other hand, the C-terminal side of titin could be involved in the assembly of thick filaments while the N-terminal end of titin is captured by the Z-bodies to form nascent myofibrils.

It is of interest that titin fragments containing z-repeats can target to the dense bodies of stress fibers in non-muscle cells and Z-bodies of premyofibrils in cardiomyocytes, even though muscle titin antibodies do not stain these structures [Rhee et al., 1994]. The targeting of these constructs may be simply be a reflection of the ability of the z-repeats to bind alpha-actinin molecules, which are concentrated in dense bodies of stress fibers [Sanger et al., 1983] and Z-bodies of premyofibrils [Rhee et al., 1994]. On the other hand, there is some biochemical evidence that there is a non-muscle isoform of titin, i.e., cellular titin, that binds to alpha-actinin and non-muscle myosin II filaments in non-muscle cells [Eilertsen et al., 1994, 1997]. Although there is no sequence data for this cellular titin, it may prove to have similar z-repeats detected in the N-terminus of muscle titins. The transfection of PtK2 cells with GFP-constructs encoding z-repeats, may lead to competition between the cellular titins and the z-repeat-GFP probes. The over-expression of these muscle titin z-repeat-GFP probes would lead to the inability of the cellular titins to connect their myosin filaments to the dense bodies leading to the loss of the integrity of the stress fibers.

## Summary

In this article, we have presented additional evidence to support our previous suggestion that the Z-band protein, zeugmatin, is actually part of the Z-band targeting region of the N-terminus of titin. GFP was used as a tool for determining the Z-band targeting domains of titin fragments in living cardiac muscle cells. The position of the GFP probe with respect to the domains is important for binding. This GFP-assay indicated that the six z-repeats provide the Z-band targeting site in chicken cardiac titin. Each z-repeat, separated by an appropriate linker from GFP, can target to Z-bands, although the first and last z-repeats target most strongly.

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