positive clones were excised in vivo from the λZAP II vector into pBluescript II by coinfection with helper phage R408 [J. M. Short, J. M. Fernandez, J. A. Sorge, W. D. Huse, *Nucleic Acids Res.* **16**, 7583 (1988)]. From this screening we obtained cTY12-5A, which overlaps with cTYp1-2 and extends toward the 5′ untranslated end of the 49-kD protein mRNA.
9. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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13. In accordance with this finding, we also found that a polyclonal antibody raised against purified bovine arrestin stained the 49-kD spots of both *Drosophila* and *Musca* on two-dimensional gel blots (N. Komori and H. Matsumoto, unpublished data).
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15. We quantified the phosphorylation by scanning the autoradiograms with a Molecular Dynamics Computing Densitometer Model 300A and found that Ca^{2+} increased the 49-kD protein phosphorylation by 95 ± 53% (n = 5, with 99% confidence limit). We suspected that the *Drosophila* 49-kD protein may be homologous to phosphoproteins found in photoreceptors of other invertebrate species. There are at least two phosphoproteins that are phosphorylated in vitro in response to Ca^{2+}: a 46-kD protein of *Limulus* ventral and lateral eyes [E. M. Wiebe, A. C. Wishart, S. C. Edwards, B.-A. Battelle, *Vis. Neurosci.* **3**, 107 (1989)] and a 47- to 49-kD protein of the crab *Leptograpsus variegatus*, which exhibits 4-nitrophenylphosphatase activity [S. C. Trowell and M. Carter, in *Molecular Physiology of Retinal Proteins*, T. Hara, Ed. (Yamada Science Foundation, Osaka, Japan, 1988), pp. 415–416]. However, the polyclonal antibody to the 49-kD protein (*30*) failed to stain any protein bands in these molecular mass regions on immunoblots of *Limulus* ventral or lateral photoreceptors or of *Leptograpsus* photoreceptors (N. Komori and H. Matsumoto, unpublished data).
16. The decrease in phosphorylation of the 49-kD protein in lanes 3 and 4 of Fig. 4C by the addition of phosphatidylserine may be due to the decrease in the effective Ca^{2+} concentration by chelation. The reason for the lower background phosphorylation in lanes 3 and 4 is unknown.
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22. Potential phosphorylation sites of the 49-kD protein by protein kinase C or Ca^{2+}/calmodulin–dependent protein kinase II are shown in Fig. 3A.
23. Because we suspected that vertebrate arrestin might be a phosphoprotein, we attempted to phosphorylate it. Under illuminated or nonilluminated conditions under which several unidentified proteins became phosphorylated in bovine whole retinal homogenates, we failed to observe any phosphorylation of arrestin (N. Komori and H. Matsumoto, unpublished data).
24. In general, *p*I is one of the important parameters that determines the way in which a protein interacts with other macromolecular structures.
25. The rhodopsin binding site may differ between arrestin and the 49-kD protein because the COOH-terminal structures of the corresponding rhodopsins differ substantially [W. L. Pak, *Photobiochem. Photobiophys.* **13**, 229 (1986)].
26. Pig brain calmodulin (Sigma) also failed to activate the phosphorylation of the 49-kD protein in vitro (H. Matsumoto, unpublished data). Therefore, the class of protein kinase that phosphorylates the 49-kD protein is unknown.
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A Bacterial Enhancer Functions to Tether a Transcriptional Activator Near a Promoter

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The nitrogen regulatory protein NtrC of enteric bacteria activates transcription of the *glnA* gene by catalyzing isomerization of closed complexes between RNA polymerase and the *glnA* promoter to open complexes. NtrC binds to sites upstream of *glnA* that have properties of eukaryotic transcriptional enhancers. NtrC-binding sites were found to facilitate open complex formation when these sites and the *glnA* promoter were located on different rings of a singly linked catenane, but not when the two rings were decatenated. The results provide evidence that NtrC contacts RNA polymerase–promoter complexes in a process mediated by formation of a DNA loop. NtrC-binding sites serve to tether NtrC near the *glnA* promoter, thereby increasing the frequency of collisions between NtrC and polymerase-promoter complexes.

EUKARYOTIC TRANSCRIPTIONAL ENhancers are DNA sequences that serve as binding sites for proteins that increase (or in some cases decrease) the rate of transcription of nearby genes (*1*). Defining characteristics of enhancers include their ability to function efficiently over large distances, at least in vivo (*2*), and to function downstream as well as upstream of transcriptional start sites.

Sequences analogous to transcriptional enhancers have been identified in prokaryotes (*3*). One of the best studied of these is the enhancer upstream of the *glnA* gene of enteric bacteria (*4*), which encodes glutamine synthetase. This enhancer, which is composed of multiple binding sites for the nitrogen regulatory protein NtrC (*5*), is amenable to detailed analysis because *glnA* transcription can be studied in a purified in vitro system with well-defined DNA templates. Moreover, the functions of the enhancer-binding protein NtrC and its target protein σ^{54}-holoenzyme, an alternative holoenzyme form of RNA polymerase, are comparatively well understood. NtrC catalyzes isomerization of closed recognition complexes between σ^{54}-holoenzyme and the *glnA* promoter to open complexes in which DNA around the transcription start site is

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locally denatured (*6, 7*); formation of open complexes requires adenosine triphosphate (ATP) (*6*).

Models for the mechanism of enhancer action can be grouped into two classes according to the function ascribed to the DNA that connects an enhancer to its conjugate promoter (*8*). In models of one class, communication between the enhancer and the promoter is specifically mediated by the DNA between them, whereas in models of the second class, this intervening DNA functions only to tether the enhancer and promoter together. Examples in the first class are the tracking and topoisomerase models. In terms of NtrC function, the tracking model would postulate that NtrC first binds to its specific enhancer-like sites and then tracks (slides) along the DNA to contact σ^{54}-holoenzyme bound in a closed complex at the *glnA* promoter. The topoisomerase model would postulate that NtrC binds to its sites and then alters the superhelical tension of the DNA to facilitate open complex formation. The only example in the second class that pertains for our in vitro studies is the looping model, which would postulate that NtrC remains bound to its specific sites and contacts the RNA polymerase-promoter complex directly when the DNA between sites and promoter forms a loop.

To distinguish between models of the two classes, we used a strategy originally used to study the interaction of distant binding sites for the resolvase from transposon Tn3 (*9*) and to study the interaction of such sites for the transposase from phage Mu (*10, 11*). We placed NtrC-binding sites and the *glnA* promoter on separate rings of a singly linked catenane, thereby interrupting the pathway between them but keeping them tethered. We then determined whether the NtrC sites could facilitate the formation of open complexes by σ^{54}-holoenzyme in vitro.

In order to assess the function of NtrC-binding sites, we quantitated formation of open complexes by means of a single-cycle transcription assay (*6, 12*). Briefly, appropriate supercoiled DNA templates (see below), all of which contained the *glnA* promoter upstream of a strong transcriptional terminator (*13*), were combined with σ^{54}-holoenzyme and NtrC. Formation of open complexes was initiated by addition of 2′,3′-dideoxy ATP and was terminated by addition of heparin, which sequesters free RNA polymerase and thereby prevents multiple rounds of transcription on a single template. Open complexes, which are stable to heparin, were then detected by their ability to give rise to radiolabeled transcripts on addition of ribonucleoside triphosphates.

To obtain templates for comparing the function of NtrC-binding sites in cis to the *glnA* promoter and in trans on a catenane, we inserted the promoter and different numbers of NtrC-binding sites into plasmid pAB7.0 (*14*), which can be converted to a singly linked catenane by the resolvase from transposon Tn3 (*9, 15*). Plasmid pJES304 carries only the *glnA* promoter and lacks specific binding sites for NtrC; plasmid pJES305 contains a low-affinity NtrC-binding region located 3 kb downstream (4 kb upstream) of the *glnA* promoter, and plasmid pJES350 contains a high-affinity NtrC-binding region 3 kb upstream of the promoter (Fig. 1) (*5, 16*). The singly linked catenanes derived from these plasmids by resolution contained one ring bearing the *glnA* promoter, identical in all cases, and a second (companion) ring either lacking (304cat) or containing (305cat and 350cat) specific binding sites for NtrC (Figs. 1 and 2) (*17*). After gel purification, catenane preparations contained <7% of the residual parent plasmid (Fig. 2) and, like the parent plasmids, contained <5% nicked molecules at the time transcription assays were performed (*18*).

The presence of specific NtrC-binding sites on the catenanes or their corresponding parent plasmids stimulated the formation of open complexes similarly (Fig. 3) (*19, 20*) (compare 305cat or 350cat, which have specific sites, with 304cat, which lacks them, and compare pJES305 or pJES350 with pJES304). On both the catenanes and the parent plasmids, the presence of specific sites reduced the concentration of NtrC required for half-maximal formation of open complexes by three- and sevenfold for the low- and high-affinity NtrC-binding regions, respectively (*21*). Maximum utilization of each template was ~40%. Because the assay does not allow any template to be used more than once, the stimulatory effect of sites in the catenated configuration cannot be accounted for by use of residual parent plasmid (<3%) (Fig. 2) in the catenane preparations.

To determine if NtrC-binding sites had to be catenated to the *glnA* promoter in order to function in trans, we performed the following control experiment. We purified individual catenane rings (Fig. 1) and compared the activity of the promoter-bearing ring alone with that of the promoter-bearing

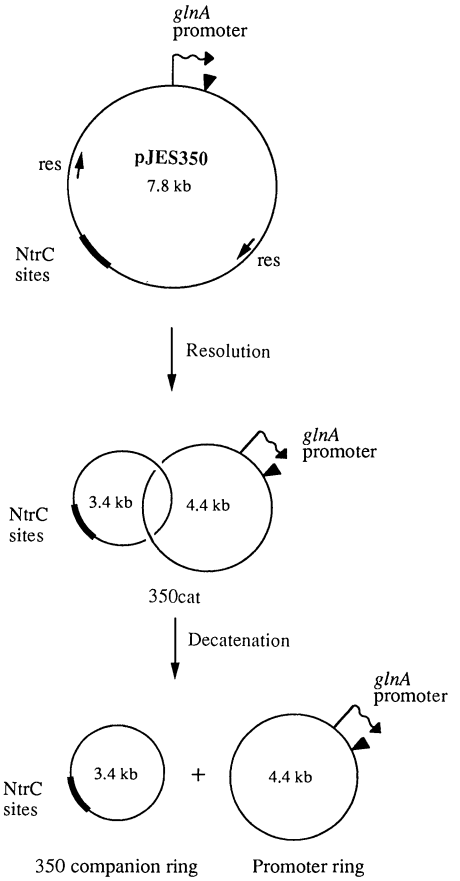

Fig. 1. Templates for testing the ability of NtrC-binding sites to function as transcriptional enhancers. Plasmid pJES350 was constructed by inserting NtrC-binding sites 1 to 4 (nucleotide positions −259 to −60 relative to the start site of transcription in the wild-type *glnA* promoter region) into pJES304 (see below) at a location 3.1 kb upstream of the *glnA* promoter (*16*). We refer to the NtrC-binding region in pJES350 as a "high-affinity" region (*5*). Plasmid pJES304 (7.6 kb; not shown) contains the *glnA* promoter (positions −32 to +17 relative to the start site of transcription) upstream of a strong transcriptional terminator from phage T7 (*13*). Plasmid pJES305 (7.6 kb; not shown) was constructed by inserting NtrC-binding sites 1 and 2 (nucleotides −200 to −101 relative to the start site of transcription in the wild-type *glnA* promoter region) into pJES304 at a location 3.0 kb downstream (equivalent to 4.0 kb upstream) of the *glnA* promoter (*16*). We refer to the NtrC-binding region in pJES305 as a "low-affinity" region because site 2 contains a point mutation (*5*), and hence is probably not a high-affinity binding site. Each of the above plasmids contains two directly repeated binding sites (res sites) for the resolvase from transposon Tn3, and this site-specific recombinase was used to convert them (*9, 15, 17*) to the singly linked catenanes 350cat, 304cat, and 305cat, respectively. These catenanes contain an identical 4.4-kb ring bearing the *glnA* promoter but differ in the number of NtrC-binding sites on the companion ring. Although indicated here as open circles, both parent plasmids and catenanes were supercoiled. Decatenation was accomplished by linearizing one ring of a catenane with an appropriate restriction enzyme and isolating the remaining ring. The ring bearing the *glnA* promoter (promoter ring) was isolated from 304cat after digesting with Ava I, which linearizes the companion ring. Companion rings lacking NtrC sites (304 companion) or having such sites (350 companion and 305 companion) were isolated from the corresponding catenanes after digesting with Bgl II, which linearizes the promoter ring. The intact supercoiled circles were separated from linear molecules by gel electrophoresis and recovered as for catenanes (*17*).

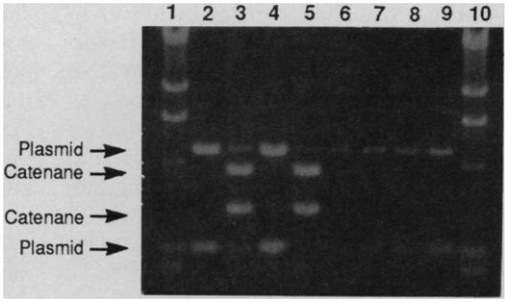

Fig. 2. Purity of catenane preparations. Catenanes (lanes 3 and 5) and parent plasmids (lanes 2 and 4) yield different restriction fragments when digested with Nco I. (Parent plasmids contain two sites for this enzyme, which segregate to different circles of the catenanes.) The amounts of contaminating parent plasmid in catenane preparations were assessed by comparing the amounts (intensities of ethidium bromide–stained bands) of parental Nco I fragments in digests of catenane preparations (lanes 3 and 5) to those in digests of parental standards (lanes 6 to 9). All DNA concentrations were determined with a Hoefer TKO 100 DNA fluorimeter. Lanes 2 to 5 contained 300 ng of Nco I–digested pJES304, 304cat, pJES305, and 305cat, respectively. Lanes 6 to 9 contained 10, 20, 30, and 60 ng of Nco I–digested pJES304, respectively. Parental bands in lane 5 were less intense than those in lane 6, indicating that 305cat was <3% contaminated by residual parent plasmid. Similar comparisons indicated that 304cat and 350cat were <7% and <3% contaminated, respectively. Lanes 1 and 10 contained λ DNA digested with Hind III as size standards.

ring mixed in equimolar concentration with companion rings having or lacking NtrC-binding sites. As expected, high concentrations of NtrC were required for the formation of open complexes on the isolated promoter-bearing ring (Fig. 4); these concentrations were comparable to those needed with other templates that lacked specific NtrC-binding sites (for example, pJES304 in Fig. 3, A and B). The presence of NtrC sites on an unlinked companion ring did not decrease the concentrations of NtrC required for open complex formation (*19*), indicating that the simple presence of NtrC-binding sites in trans is not sufficient to stimulate open complex formation at low NtrC concentrations.

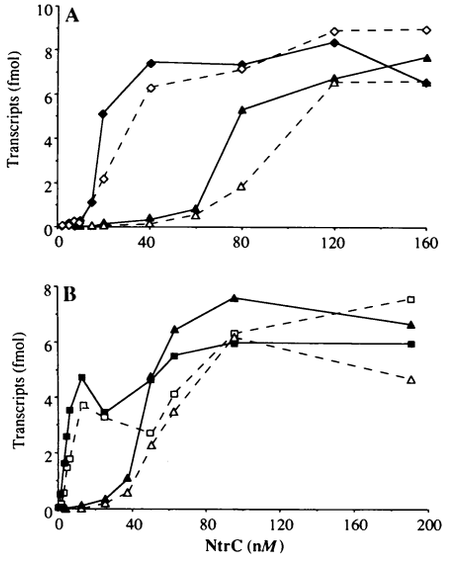

Fig. 3. The effect of catenation on the ability of NtrC-binding sites to function as transcriptional enhancers. Formation of open complexes on various supercoiled DNA templates (19 fmol) was measured as a function of NtrC concentration [mutant form NtrC (constitutive) = NtrC610 (*6*)]. Open complexes, which were formed in the presence of dideoxy ATP, were detected by their ability to yield transcripts in a single-round transcription assay (*6, 12*). (**A**) The templates were pJES305 (◆), 305cat (◇), pJES304 (▲), and 304cat (△). (**B**) Templates were pJES350 (■), 350cat (□) (*22*), pJES304 (▲), and 304cat (△).

Finally, we demonstrated that NtrC-binding sites at large distances from the *glnA* promoter (for example, in both parent plasmids and singly linked catenanes) stimulate open complex formation almost as well as they do in the wild-type *glnA* promoter-regulatory region (Fig. 5). NtrC titrations on plasmid pJES350, which carries the high-affinity NtrC-binding region at a distance of 3 kb from the *glnA* promoter, were strikingly similar to those on plasmid pJES131, which carries these sites in their normal location within 150 bp of the promoter (*22*). Successively higher concentrations of NtrC were required for the formation of open complexes on plasmid pJES305, which contains a low-affinity binding region for NtrC at a distance of 3 kb from the *glnA* promoter, and on plasmid pJES304, which lacks specific NtrC-binding sites. The maximum template utilization was the same in all cases, provided the NtrC concentration was sufficiently high.

Taken together, our results show that NtrC-binding sites function almost as well from a singly linked catenane as in their wild-type location in cis to the *glnA* promoter. This provides evidence that NtrC activates transcription by contacting RNA polymerase–promoter complexes directly, with such contact being mediated by a DNA loop (*23*). Evidence obtained in vivo led to the same conclusion (*24, 25*). Moreover, the DNA loop predicted to exist when NtrC interacts with the polymerase-promoter complex has been visualized directly by electron microscopy (*26*).

The finding that NtrC sites are stimulatory on a singly linked catenane contrasts with the fact that the ends of transposon Mu and binding sites for Tn3 resolvase can function in trans only on multiply linked catenanes (*9–11*). In the latter two cases,

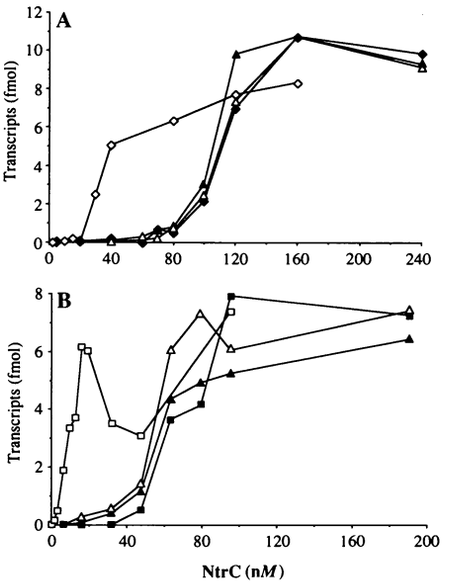

Fig. 4. The effect of decatenation on the ability of NtrC-binding sites to function as transcriptional enhancers. Formation of open complexes on various DNA templates was assessed as a function of NtrC concentration as described in the legend to Fig. 3. (**A**) Templates were 305cat (◇), promoter ring alone (▲), and promoter ring plus equimolar 304 companion ring (△) or 305 companion ring (◆). (**B**) Templates were 350cat (□) (*22*), promoter ring alone (▲), and promoter ring plus equimolar 304 companion ring (△) or 350 companion ring (■). Template concentrations were 0.8 n*M* (19 fmol) in all cases except for the promoter and companion rings in (A), for which the concentrations were 1 n*M* (24 fmol).

supercoiling—which is mimicked by the interlinks of a multiply linked catenane—is presumably required to direct the formation of functional synaptic complexes; supercoiling is apparently not required to direct interaction between NtrC-binding sites and the *glnA* promoter.

Because NtrC-binding sites do not function in trans unless they are catenated to the *glnA* promoter, the DNA between sites and promoter apparently serves as a tether to increase the frequency of collisions between NtrC and polymerase-promoter complexes. Tethering of regulatory proteins that form DNA loops is important in controlling the rate of transcription of a number of prokaryotic genes (*27*). Whereas our results indicate that tethering is important in NtrC function, they do not rule out additional roles for DNA binding in transcriptional activation. For example, DNA may be an allosteric activator of NtrC, or multiple NtrC-binding sites may direct assembly of several NtrC dimers into a complex that is required for efficient activation of transcription (*26, 28*).

Techniques similar to those we used to study the function of NtrC-binding sites have been applied to study the function of

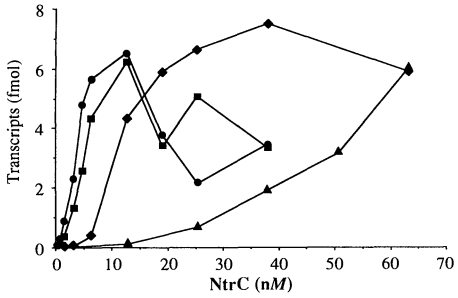

Fig. 5. The effect of number and position of NtrC-binding sites on their ability to function as transcriptional enhancers. Formation of open complexes on various DNA templates was assessed as a function of NtrC concentration as described in the legend to Fig. 3. Templates were: pJES131 (●), pJES350 (■) (*22*), pJES305 (◆), and pJES304 (▲). Plasmid pJES131 carries the wild-type *glnA* promoter-regulatory region (positions −1570 to +30). The most upstream NtrC site in this template (site 1) is centered at position −140 with respect to the startpoint of transcription.

two eukaryotic transcriptional enhancers. The simian virus 40 (SV40) enhancer was shown to function in trans to the β-globin promoter in nuclear extracts when the two were linked together by a protein bridge (*29*), and the ribosomal RNA enhancer of *Xenopus laevis* was shown to function in trans to its cognate promoter in oocytes when the two were carried on different rings of a multiply linked catenane (*30*). These findings and others (*1, 31*) are consistent with the view that at least some eukaryotic enhancers reach their targets (as yet undefined) by means of DNA loops. Because there is no a priori reason to think that all transcriptional enhancers act in the same manner, it will be of interest to learn how widely the looping mechanism pertains.

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5. NtrC has a bihelical DNA-binding motif [A. Contreras, M. Drummond, *Nucleic Acids Res.* **16**, 4025 (1987)]. On the basis of in vitro deoxyribonuclease I (DNase I) and methylation protection assays, NtrC binds to five sites of ~15 bp each in the *glnA* promoter-regulatory region (J. Keener, P. Wong, S. Kustu, unpublished data). These are centered at positions −140, −108, −88, −67, and −45 with respect to the transcriptional start site at +1. By themselves, sites 1 and 2, which are high-affinity sites with clear twofold rotational symmetry, have properties of eukaryotic transcriptional enhancers (*4*). Sites 4 and 5, which are low-affinity sites, do not appear to be important for activation of transcription (*4, 7, 25, 26*) (Fig. 5). It is not known whether site 3 is important.
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11. With an in vitro transposition assay, Craigie and Mizuuchi (*10*) demonstrated that the normal requirement for both ends of the transposon Mu to be present in cis on a supercoiled molecule could be circumvented if these ends were present on different circles of a multiply linked catenane. They thus provided evidence that molecules of transposase contacted one another by means of a DNA loop rather than by tracking.
12. Final concentrations of components present for the formation of open complexes were: 30 n*M* core RNA polymerase from *Escherichia coli*, 50 n*M* σ^{54} from *Salmonella typhimurium*, 0.8 n*M* template, and 0.4 to 320 n*M* NtrC protein [mutant form NtrC610 = NtrC (constitutive) (*6*)]. Components were mixed at 0°C in the order indicated above except that buffer [50 m*M* tris-acetate (*p*H 8.0), 100 m*M* potassium acetate, 8 m*M* magnesium acetate, 27 m*M* ammonium acetate, 1 m*M* dithiothreitol, and 3.5% (w/v) polyethylene glycol (6000 to 8000; Sigma)] was added after σ^{54}. Mixtures were then warmed to 37°C for 10 min, and reactions were initiated by adding dideoxy ATP to a final concentration of 2.2 m*M* in a total volume of 24 μl. Dideoxy ATP allows NtrC to catalyze formation of open complexes at the *glnA* promoter and also suppresses nonspecific initiation of transcription by core RNA polymerase. After 10 min, formation of open complexes was terminated by addition of heparin to a final concentration of 0.1 mg/ml. After an additional 10 min, nucleotides were added to allow synthesis of transcripts [ATP was added to 10 m*M* final concentration, guanosine triphosphate (GTP) and uridine triphosphate (UTP) to 0.4 m*M*, and cytidine triphosphate (CTP) to 0.1 m*M*; 5 μCi of [α-^{32}P]CTP was also added (10 mCi/mmol, Amersham)]. After 10 min, transcripts were precipitated, isolated by gel electrophoresis, and quantified as described (*6*). Radioactivity in transcript bands (counts per minute corrected for background) was converted to femtomole of transcript; the specific activity of transcripts was determined by multiplying the specific activity of CTP by 87, the number of cytidine residues per transcript. For reproducible results, it was necessary to carry out all mixing steps by tapping the reaction tubes gently.
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16. Plasmid pJES304 was constructed by replacing a 1.9-kb Pvu I fragment in pAB7.0 (*14*) with a 2.3-kb Pvu I fragment from pJES214 (*6*). Plasmid pJES305 was constructed by replacing a Pvu I–Eco RI fragment of ~100 bp in pJES304 with a 100-bp Pvu I–Eco RI fragment carrying NtrC-binding sites 1 and 2 from pJES138 (S. Porter and S. Kustu, unpublished data); pJES138 carries NtrC-binding sites 1 and 2 on a Hinf I–Hph I fragment that was derived from the wild-type *glnA* promoter region. Both pJES305 and pJES138 carry a point mutation in site 2 at position −103 with respect to the normal start site of transcription at *glnA* that converts a consensus half site GGTGC to GGGGC (S. Porter and S. Kustu, unpublished data). Plasmid pJES350 was constructed by replacing a Nde I–Ava I fragment of ~340 bp in pJES304 with a 520-bp Nde I–Bst EII fragment containing NtrC-binding sites 1 to 4 from plasmid pJES351 (*26*).
17. Plasmid template was combined on ice with Tn3 resolvase to a molar ratio of 2.8 resolvase dimers per dimer binding site, in a buffer containing 160 m*M* potassium glutamate, 50 m*M* tris-acetate (*p*H 7.8), and 10 m*M* magnesium acetate. Reaction mixtures were left on ice for 15 min, incubated for 1 hour at 37°C, and extracted with phenol-chloroform; the DNA was precipitated with ethanol. Catenanes were separated by gel electrophoresis [40 m*M* tris-acetate (*p*H 7.0), 1 m*M* EDTA; and 0.8% agarose (*32*)] from nicked parental DNA, which is not a substrate for resolution. The gel slice containing the catenane band, which comigrates with residual supercoiled parent plasmid, was excised, and DNA was recovered by a freeze-thaw procedure (*32*) with the following modifications: Gel slices were pulverized by forcing them through a 20-gauge needle into Eppendorf tubes (standard rather than low-melting point agarose was used) and were frozen slowly at −20°C. After they were thawed, ~1.5 volumes of phenol was added, and the mixture was spun in a vortex mixer for 5 min, frozen, thawed, and spun again in a vortex mixer. It was then centrifuged for 15 min in a microcentrifuge to recover the aqueous layer. The volume of this layer was reduced by repeated extraction with *n*-butanol (*32*), after which the DNA was precipitated with ethanol and suspended in water for use in transcription assays. To minimize nicking, templates were not exposed to ethidium bromide but rather were localized on gels by staining flanking marker lanes. In addition, gel-purified templates were stored in small aliquots at −70°C.
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19. We performed the experiment shown in Fig. 3A five times and that shown in Fig. 4A three times. For all experiments sites were clearly stimulatory in cis to the promoter and in trans on a catenane but not in trans after decatenation. The NtrC concentration required for 50% of maximum template utilization decreased by a factor of 3.6 ± 0.6 (mean ± standard deviation) when the binding sites were in cis to the promoter and by 2.6 ± 0.4 when they were in trans on a catenane. The experiments in Figs. 3A and 4A were done with an NtrC fraction that was less active than the one used for the experiments in Figs. 3B, 4B, and 5.
20. It is not clear whether NtrC must bind to templates that lack specific sites for it in order to stimulate open complex formation or whether it can do so from solution; we favor the former alternative (*6*).
21. Open complex formation reproducibly required somewhat more NtrC on catenanes than on their corresponding parent plasmids, whether or not these templates contained specific NtrC-binding sites. The difference may be due to decreases in superhelicity that are known to accompany catenane formation (*9*).
22. All templates carrying high-affinity NtrC-binding regions showed a decrease in open complex formation at 20 to 40 n*M* NtrC. High NtrC concentrations also decrease transcription from the wild-type *glnA* promoter in vivo (L. Reitzer, personal communication). The mechanism of this inhibition is not known.
23. To reconcile our results with the tracking or topoisomerase models would require that NtrC have a second DNA-binding site, a hypothesis for which we have no evidence.
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28. Because DNA is relatively rigid over the distance encompassed by the wild-type *glnA* promoter-regulatory region, details of local structure such as directed bends, intrinsic or protein-induced, might affect the efficiency of transcriptional activation. However, if such effects pertain, our in vitro assay does not detect them (Fig. 5).
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Primary Structure of the γ Subunit of the DHP-Sensitive Calcium Channel from Skeletal Muscle

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Affinity-purified, polyclonal antibodies to the γ subunit of the dihydropyridine (DHP)-sensitive, voltage-dependent calcium channel have been used to isolate complementary DNAs to the rabbit skeletal muscle protein from an expression library. The deduced primary structure indicates that the γ subunit is a 25,058-dalton protein that contains four transmembrane domains and two N-linked glycosylation sites, consistent with biochemical analyses showing that the γ subunit is a glycosylated hydrophobic protein. Nucleic acid hybridization studies indicate that there is a 1200-nucleotide transcript in skeletal muscle but not in brain or heart. The γ subunit may play a role in assembly, modulation, or the structure of the skeletal muscle calcium channel.

THE DHP-SENSITIVE Ca^{2+} CHANNEL from skeletal muscle consists of four subunits: α_1 (170 kD), α_2 (175 kD, nonreduced; 150 kD, reduced), β (52 kD), and γ (32 kD) (*1*). The α_1 subunit contains the binding sites for the three classes of Ca^{2+}-channel blockers (DHPs, phenylalkylamines, and benzothiazepines) (*2*) and is a substrate for protein kinases, as is the β subunit. The α_2 and γ subunits are glycoproteins and bind wheat germ agglutinin. Upon disulfide bond reduction, the α_2 subunit undergoes a characteristic mobility shift by SDS–polyacrylamide gel electrophoresis (PAGE) analysis with the concurrent appearance of the δ peptides (19 to 30 kD). The cDNAs for the α_1, α_2, and β subunits have been isolated and characterized (*3–5*). The α_1 subunit, with 24 putative membrane-spanning segments, is the principal transmembrane subunit of the complex and has significant sequence homology with several other members of the voltage-dependent ion channel family, including the α subunit of the Na^+ channels and the *Drosophila* K^+ channel (*3, 4, 6*). The transmembrane properties of the α_2 and β subunits are quite different from the α_1 subunit, with the α_2 subunit predicted to have as many as three transmembrane domains (*4*) and the β subunit to have none (*5*). Although active DHP-sensitive Ca^{2+} channels have been reconstituted in lipid bilayers (*7*) and the microinjection of an expression plasmid carrying the α_1 subunit cDNA restores a DHP-sensitive Ca^{2+} current and excitation-contraction coupling in dysgenic muscle (*8*), it is unknown which subunits are required for a native functional DHP-sensitive Ca^{2+} channel.

To isolate cDNA clones for the γ subunit of the DHP-sensitive Ca^{2+} channel, affinity-purified guinea pig polyclonal antiserum, specific for the gel-purified γ subunit (Fig. 1) (*9*), was used to screen expression libraries constructed from rabbit back skeletal muscle RNA. Overlapping cDNA clones were isolated to determine the nucleotide sequence encoding the protein (Fig. 2A) (*10*).

The 1171-nucleotide (nt) cDNA sequence contains a 666-nt open reading frame coding for 222 amino acids (Fig. 2B). The deduced amino acid sequence indicates a calculated molecular weight of 25,058, which is in approximate agreement with the observed molecular mass of 32 kD for the glycosylated (*1*) and 20 kD for the chemically deglycosylated forms (*11*), determined by SDS-PAGE. The deduced amino acid sequence agrees with the authentic NH_2-terminus of the γ subunit, as determined by protein sequencing of the purified skeletal muscle protein (*12*). An analysis of the predicted amino acid sequence of the γ subunit for local hydrophobicity revealed four putative transmembrane domains (Fig. 2C). These segments are designated I (residues 11 to 29), II (residues 105 to 129), III (residues 140 to 155), and IV (residues 180 to 204). Unlike many of the transmembrane segments in the α_1 subunit (*3*), none of the above predicted segments contain any charged residues. The length of the predicted transmembrane segments varies from 16 to 25 amino acids. The NH_2-terminal sequence does not resemble a hydrophobic signal sequence.

On the basis of the local hydrophobicity and the lack of an NH_2-terminal signal sequence, the predicted secondary structure of the γ subunit includes four transmembrane segments separating intracellularly located NH_2- and COOH-terminals. Consistent with biochemical studies (*1*), the two potential N-linked glycosylation sites (Fig. 2B) reside on the extracellular face of the membrane. Of the six consensus phosphorylation sites (Fig. 2B), only Ser^2 and Ser^{214} are predicted to be intracellular. The observed decreased mobility of the γ subunit on SDS-PAGE in the presence of reducing agents, and the resistance of the protein to proteolytic digestion in the absence of disulfide bond reduction, indicate that disulfide bonds play a major role in determining the secondary structure of the native protein. The deduced primary structure of the γ subunit contains ten cysteine residues, one in the COOH-terminal intracellular segment, five within the hydrophobic transmembrane segments, and the remaining four in the first extracellular loop between

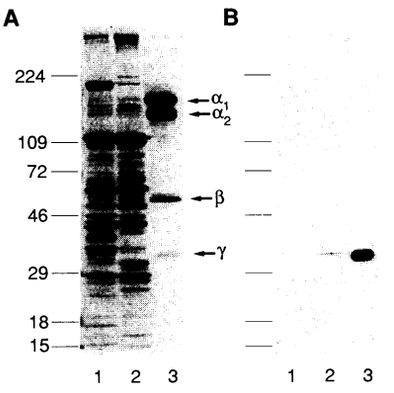

Fig. 1. Rabbit skeletal muscle DHP receptor subunit composition and γ subunit antibody probe specificity. (**A**) Coomassie blue–stained, reduced SDS-acrylamide gel showing crude (100 μg of microsomes) (lane 1), enriched (100 μg of triads) (lane 2), and purified (20 μg of DHP receptor) (lane 3) preparations of DHP-sensitive Ca^{2+} channel (*20*). (**B**) Immunoblot of identical samples as in (A), stained with GP16 polyclonal antiserum that had been affinity-purified (*9*). Arrows show the positions of the four subunits; the positions of the prestained molecular weight standards (BRL) are on the left ($M_r \times 10^{-3}$).

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