

26. *Onchocerciasis and Its Control*, vol. 852 (World Health Organization, Geneva, 1995).
27. For immunohistochemistry, eyes were enucleated and snap frozen in liquid nitrogen. Sections (5 μ m) were fixed in 4% formaldehyde for 25 min and incubated with rat antibodies to mouse neutrophil (NIMP R14, provided by T. Bianco, Liverpool School of Tropical Medicine; final concentration, 8 mg/ml), rabbit anti-eosinophil major basic protein (1:5000, provided by J. Lee, Mayo Clinic, Scottsdale, AZ), or anti-PECAM-1 (MEC13.3; BD-Pharmingen, CA). Sections were then incubated with fluorescein isothiocyanate-conjugated anti-rat or anti-rabbit im-

- munoglobulin G (Vector Laboratories, Burlingame, CA) and examined by fluorescent microscopy. Cells were counted either throughout the corneal section or in representative 200 $\times$ fields. Images of limbal vessels were captured with a Scion digital camera apparatus (Olympus), and PECAM-1 expression was determined by image analysis (Adobe Photoshop).
28. For KC and MIP-2 ELISAs, corneas were dissected, suspended in 400 ml of RPMI 1640 medium, and sonicated for 90 s at 50 cycles/s (Sonics VibraCell, Danbury, CT). After centrifugation, the concentration of cytokines in supernatants was determined by two-

site enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

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Regulation of Corepressor Function by Nuclear NADH

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The corepressor CtBP (carboxyl-terminal binding protein) is involved in transcriptional pathways important for development, cell cycle regulation, and transformation. We demonstrate that CtBP binding to cellular and viral transcriptional repressors is regulated by the nicotinamide adenine dinucleotides NAD⁺ and NADH, with NADH being two to three orders of magnitude more effective. Levels of free nuclear nicotinamide adenine dinucleotides, determined using two-photon microscopy, correspond to the levels required for half-maximal CtBP binding and are considerably lower than those previously reported. Agents capable of increasing NADH levels stimulate CtBP binding to its partners in vivo and potentiate CtBP-mediated repression. We propose that this ability to detect changes in nuclear NAD⁺/NADH ratio allows CtBP to serve as a redox sensor for transcription.

CtBP was initially identified through its ability to interact with the COOH-terminus of adenovirus E1A. Mutation of the CtBP binding site in E1A decreases its transcriptional repression effects and increases its ability to direct cellular transformation (1, 2). CtBP also participates in the actions of cellular transcription factors involved in growth and differentiation, as demonstrated in *Drosophila melanogaster* (3) and vertebrate systems (4). The recent demonstration that yeast Sir2 utilizes NAD⁺ as a substrate (5–7) and the remarkable sequence conservation of CtBP with the dehydrogenases and reductases (2) (Fig. 1A), enzymes that use nicotinamide adenine dinucleotides as cofactors, led us to investigate whether CtBP might similarly be regulated by NAD⁺ or NADH.

CtBP expressed in bacteria or isolated from HeLa cells was incubated with glycerophosphate, acetoacetate, pyruvate, lactate, acetate, formate, and ethanol in the presence of NAD⁺ or NADH under a variety of experimental conditions. No dehy-

drogenase or reductase activity was detected. We next tested whether CtBP was regulated in some other manner by NAD⁺ or NADH. This hypothesis was suggested by the near-perfect conservation of the NAD⁺/NADH binding signature near the middle of the CtBP sequence (Fig. 1B). One possibility was that NAD⁺/NADH could affect the ability of CtBP to interact with its partners. To test this hypothesis, we examined the interaction of bacterially expressed CtBP with glutathione S-transferase (GST)–E1A fusion proteins at different concentrations of NAD⁺/NADH. To our surprise, CtBP binding was regulated dramatically, with NADH increasing the interaction at concentrations in the nM range (Fig. 1C). NAD⁺ also increased binding, but was 2 to 3 orders of magnitude less effective. NADP⁺, NADPH, and flavin adenine dinucleotide (FAD⁺) had little effect (Fig. 1D) (8). NAD⁺/NADH similarly affected CtBP binding to a prototypical cellular repressor, ZEB (Fig. 1E), which is known to block transcription at least in part via CtBP interactions (9).

Knowing the physiological concentrations of free nuclear NAD⁺/NADH is critical for assessing whether these molecules regulate CtBP function in vivo, the concentration of NAD(P)H can be determined in different cellular compartments using two-photon excitation microscopy (10). This

was done in Cos7 cells by quantitative imaging of the total intensity and lifetime of NAD(P)H fluorescence. For these measurements, NADH and NADPH are indistinguishable, so we measure the sum of both molecules. Comparing the total intensity to a standard curve of free NAD(P)H in solution, we found that the nucleus contained 113 μ M NAD(P)H (Fig. 2A). Precise determination of the concentration is complicated by the fact that the fluorescence of free and bound forms of NAD(P)H differs. Free NAD(P)H has a considerably lower quantum efficiency than that bound to protein. Because the quantum efficiency is associated with the fluorescence lifetime, we can determine the fraction of bound NAD(P)H by fluorescence lifetime imaging (11). The fluorescence lifetime image was homogeneous across all subcellular compartments (Fig. 2B) with a value of 3.41 ns ($n = 6$ cells), as compared to 0.45 ns for free NAD(P)H. This indicates that the vast majority of NAD(P)H is bound and that our estimate of 113 μ M is ~ 7.5 -fold too high (ratio of 3.41 to 0.45). The corrected nuclear NAD(P)H concentration is thus ~ 15 μ M. To quantitate the amount of free NAD(P)H, we performed a multifrequency experiment with phase modulations at 80, 160, and 240 MHz and fit multiple exponential decays to the fluorescence lifetimes. One lifetime component was fixed at 0.451 ns [the lifetime for free NAD(P)H], and the other was allowed to vary with the nonlinear least squares fit. According to this fit, the fraction of fluorescence associated with the free component was $4.4 \pm 2.7\%$. Thus, the upper limit of free NAD(P)H is 660 nM. If we assume that the NADPH/NADH ratio is ~ 4 (12), then the concentration of free NADH in the nucleus is ~ 130 nM (13), well within the range required for stimulating the E1A:CtBP interaction.

Because NAD⁺/NADH affected CtBP binding to multiple transcriptional repressors, we speculated that these cofactors most likely functioned by altering CtBP structure. Support for this idea was obtained from limited proteolysis experiments. In the absence of NAD⁺/NADH, trypsin treatment releases a 10-kD fragment from the CtBP NH₂-terminus, resulting in

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the 30-kD fragment visualized in Fig. 3A. This fragment is not generated if CtBP is incubated with nicotinamide adenine dinucleotides. To confirm these findings, we examined the binding of CtBP containing a mutation in the NAD⁺/NADH interaction site (14). Although the basal interactions between E1A and the CtBP mutant were maintained, the stimulation by NAD⁺/NADH was lost (Fig. 3B). Additionally, NAD⁺/NADH did not protect the CtBP mutant from trypsin digestion (13). We conclude that CtBP binding to E1A, and presumably other repressors, is regulated by NAD⁺/NADH and that NADH is far more effective in regulating binding.

One model consistent with our observations is that CtBP evolved from the dehydrogenases and reductases in a manner that resulted in loss of enzymatic activity, but retention of the capacity to be regulated by NAD⁺/NADH. To test this hypothesis, we investigated whether the association of E1A and CtBP could be regulated by agents that perturb cellular redox state. E1A and FLAG-tagged CtBP were cotransfected into Cos7 cells, which were subsequently treated with 200 μ M CoCl₂, 10 mM Na

azide, or hypoxia (1% O₂). Complexes were isolated using an antibody to a FLAG epitope (anti-FLAG), and Western blots were probed with antibodies to E1A and CtBP. The effects of these treatments on free nuclear NAD⁺/NADH cannot be measured directly, but an estimate of their effects on the free cytoplasmic pools can be derived from the [pyruvate]/[lactate] ratio (13, 15). Assuming no barrier to the diffusion of free NAD cofactors between the cytoplasmic and nuclear compartments, these measurements should reflect the free nuclear NAD⁺/NADH ratios. Each of the treatments decreased the free cytoplasmic NAD⁺/NADH ratio (Fig. 4A), probably via an increase in free NADH. Moreover, each treatment also increased the association of FLAG-CtBP with E1A (Fig. 4B). Absolute levels of E1A and CtBP were not affected.

To confirm that perturbations in NAD⁺/NADH ratios affected CtBP interactions in a manner that could regulate transcription, we performed mammalian two-hybrid assays. Cos7 cells transfected with VP16CtBP and GalZEB, a fusion gene containing the DNA-binding domain of Gal4 fused to the CtBP-binding domain of ZEB

(9), were treated 8 hours after transfection with 200 μ M CoCl₂. ZEB:CtBP interactions were significantly increased by CoCl₂ treatment (Fig. 4C). Western blots showed that the wild-type and mutated VP16CtBP proteins were expressed at similar levels. Mutation of the NAD⁺/NADH binding site in CtBP virtually eliminated reporter activity, indicating that even the basal levels of nuclear NAD⁺/NADH can stimulate the ZEB-CtBP interaction. No interaction was detected when ZEB proteins containing mutated CtBP binding sites were used, and no increase in activity was seen in control interactions (i.e., CREB:CtBP) (13).

To test whether this pathway affects repression of a naturally occurring promoter, we cotransfected Cos7 cells with an E-cadherin reporter gene and truncated ZEB constructs containing an E-box binding domain and wild-type or mutated CtBP-binding motifs (16). As reported previously, ZEB repressed the E-cadherin promoter in a manner that depended on the CtBP interaction sites (Fig. 4D). Treatment with CoCl₂ or hypoxia significantly enhanced the level of CtBP-mediated repression. Neither CoCl₂ nor hypoxia affected expression of the E-cadherin promoter in the presence of a ZEB mutant incapable of binding CtBP (8).

This study suggests that the transcriptional corepressor CtBP is regulated through binding of nicotinamide adenine dinucleotides. The concentration of NAD⁺/NADH required to stimulate the E1A-CtBP interaction in vitro is surprisingly low and reflects the low levels of nicotinamide adenine dinucleotides found in the nucleus (Fig. 2) (10). NAD⁺/NADH should readily pass through nuclear pores, suggesting that cellular perturbations that affect free cytoplasmic levels should also cause changes in the nuclear compartment. Levels of free nuclear nicotinamide adenine dinucleotides

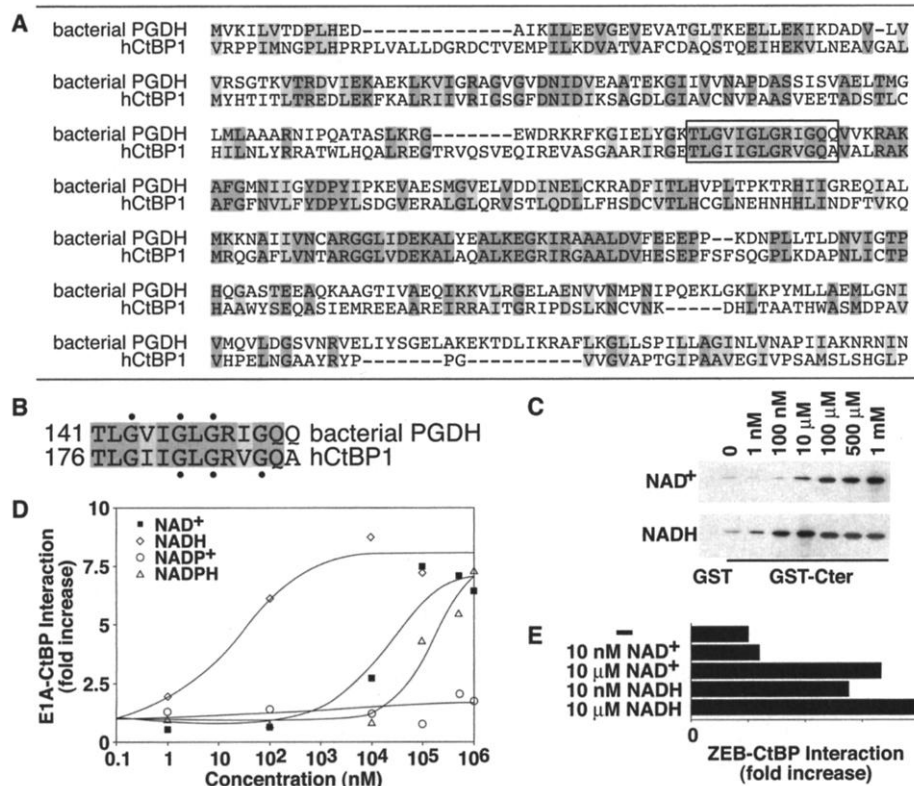


Fig. 1. NAD⁺/NADH regulates CtBP binding. (A) Alignment of bacterial phosphoglycerate dehydrogenase and human CtBP. (B) Alignment of putative NAD⁺/NADH binding sites. Gly residues important for binding are indicated. (C) Binding of recombinant CtBP to GST-E1A at various concentrations of NAD⁺ and NADH. Glutathione beads were coated with GST-E1A (COOH-terminal 67 amino acids of E1A), and bound CtBP was quantified by Western blotting. (D) Relative interactions as a function of nicotinamide adenine dinucleotide concentration (log scale). (E) Binding of recombinant CtBP to GST-ZEB₅₉₅₋₇₂₀ at various concentrations of NAD⁺ and NADH.

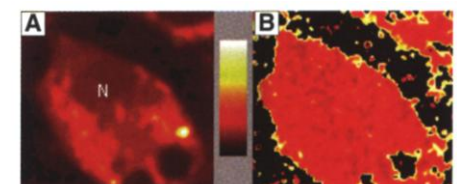


Fig. 2. Determination of nuclear NADH concentration. (A) Two-photon excitation imaging of NAD(P)H shows the autofluorescence intensity from a typical Cos7 cell. The color bar indicates the range of NAD(P)H from 0 (dark red) to 1 mM (white). Note paucity of signal in the nucleus (N). (B) Lifetime image from the same cell, acquired by phase-modulation techniques using the instrument described by French *et al.* (23). The same color bar is used, but for this figure the range represents lifetime from 0 to 10 ns for each pixel in the cell rather than intensity. The average lifetime in the nucleus as well as other subcellular compartments was 3.41 ns.

had not previously been determined, however. The binding constant (Michaelis constant) K_m of Hst2, a cytoplasmic Sir2-like histone deacetylase, for NAD^+ is 70 μM (7), approximately the level required for half-maximal stimulation of CtBP-E1A binding (Fig. 1D). Assuming that this value reflects the concentration of free NAD^+ and that the free $NAD^+/NADH$ ratio is 644 (Fig. 4A), we estimate that the free NADH concentration is about 110 nM. Free nuclear NADH levels measured using two-photon microscopy confirm this estimation. Because free NAD^+ levels greatly exceed those of NADH, large changes in the $NAD^+/NADH$ ratio do not require corre-

spondingly large changes in free NAD^+ . Thus, changes in nuclear redox could be manifested primarily through NADH, which is consistent with the higher sensitivity of CtBP to NADH than NAD^+ . Of interest, Rutter *et al.* (17) recently reported that binding of the transcription factor NPAS2 to DNA is also regulated by the redox state of NAD cofactors. The concentration of NADH and NADPH required in that study for half-maximal binding was, however, ~10 mM, five orders of magnitude higher than the free nuclear concentrations that we have determined. Whether NPAS2 is sensitive to physiologically relevant levels of nicotinamide adenine

dinucleotides thus remains to be determined. Large changes in cellular redox state occur at birth (18), in response to ethanol (19), and in certain metabolic abnormalities such as diabetes (15). Thus, the mechanism described in this report could influence multiple transcriptional repressor pathways. The best-characterized target promoter for CtBP in mammalian cells is probably the E-cadherin gene (16, 20), and loss of E-cadherin expression in tumors correlates with metastasis, invasion, and poor clinical prognosis (21, 22). Our studies indicate that CtBP-mediated repression of the E-cadherin promoter is enhanced by hypoxia. Because tumor cells are frequently hypoxic and thereby would be expected to have an increased NADH/ NAD^+ ratio, we predict that the concomitant increase of ZEB-CtBP binding may contribute to tumor invasiveness.

Fig. 3. $NAD^+/NADH$ induces a conformational change in CtBP. (A) Limited proteolytic digestion of CtBP without (control) or with 100 μM NAD^+ or 100 μM NADH using various doses of trypsin. Western blots were developed using antibodies to CtBP or a COOH-terminal His-epitope (Qia-gen), as indicated. The 30-kD fragment (indicated by arrows) generated in the absence of $NAD^+/NADH$ results from the loss of a 10-kD NH_2 -terminal fragment. (B) The $NAD^+/NADH$ binding site in CtBP is required for the stimulated interaction. CtBP proteins, either wild-type (WT) or mutated (G183A), were tested for their ability to bind to E1A in the absence (con) or presence of increasing concentrations (100 nM to 100 μM) of NAD^+ or NADH by GST pull-down assays.

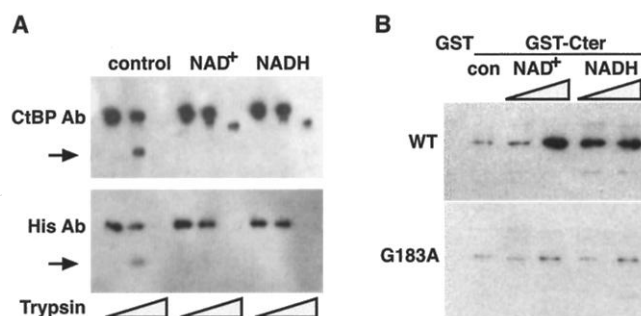
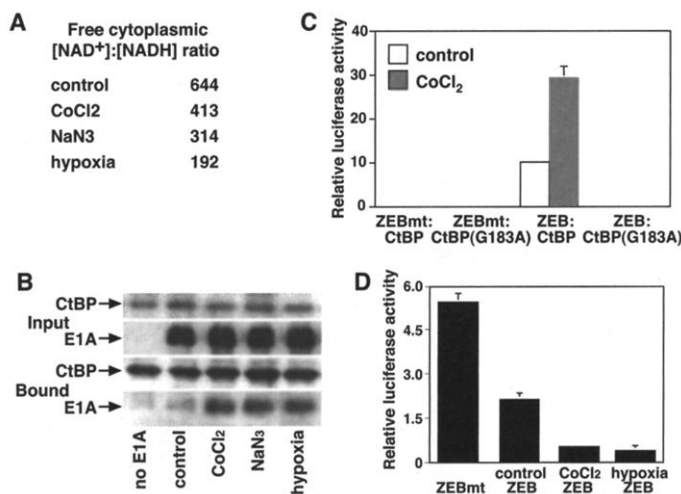


Fig. 4. Redox state regulates CtBP interaction in vivo. (A) Effects of various treatments on the free cytoplasmic $NAD^+/NADH$ ratio. Cells were treated with 200 μM $CoCl_2$, 10 mM azide, or exposed to hypoxia for 16 hours. Cellular lactate and pyruvate were determined, and the free cytoplasmic $NAD^+/NADH$ ratio was calculated as described by Williamson *et al.* (15). (B) $CoCl_2$, azide, or hypoxia (1% O_2) increase the amount of E1A associated with CtBP. E1A and FLAG-tagged CtBP were cotransfected into Cos7 cells, complexes were isolated using anti-FLAG M_2 matrix (Sigma), and Western blots were probed with antibodies against E1A (M73, Santa Cruz) and CtBP. Cells were treated with 200 μM $CoCl_2$ or 10 mM azide for 1 hour, or exposed to hypoxia for 3 hours. Input panels show that these treatments did not change the levels of CtBP and E1A. (C) $CoCl_2$ increases the interaction of ZEB and CtBP in a mammalian two-hybrid assay. Cos7 cells were cotransfected with pairs of interacting components in the presence of a 5 $\times$ Gal-E1b-luc reporter. The ZEB component is fused to the Gal DNA binding domain and the CtBP component to VP16. G183A mutation ablates the NADH/ NAD^+ binding site in CtBP. ZEBmt represents ZEB₇₀₀₋₇₇₆ with all three CtBP-binding sites mutated (9). (D) Hypoxia and $CoCl_2$ enhance CtBP-mediated repression. Cos7 cells were cotransfected with an E-cadherin reporter gene and truncated ZEB constructs containing an E-box binding domain and wild-type or mutated CtBP-binding motifs. Cells were treated with 200 μM $CoCl_2$ or exposed to hypoxia for 16 hours.



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