and purified from sucrose gradients as described (*10*). Partial Mbo I digests were size-fractionated by PFGE, and 9- to 25-kb DNA was ligated into the Bam HI site of λEMBL3 (StrataGene), and packaged with Gigapak Gold (StrataGene) and plated on *Escherichia coli* P2392. Phage plaques containing human DNA inserts were identified by their ability to hybridize to L1 [L. B. Kole, *J. Mol. Biol.* **165**, 257 (1983)] and Alu [W. R. Jelinek *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 77, 6130 (1980)] repeats and total human DNA.

14. Mouse A9 cells containing a single human chromosome with a translocation between chromosome 11 and a small portion of the X chromosome (Xqter → Xq26:11q23 → 11pter) were constructed by the microcell transfer technique [R. E. K. Fournier and F. H. Ruddle, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 319 (1977); M. Koi *et al.*, *Jpn. J. Cancer Res.* (GANN) **80**, 122 (1989)]. The microcell donor cells were the hybrids formed between human fibroblast GM 03552 (Human Genetic Mutant Cell Repository, Camden, NJ) and hypoxanthine-guanine phosphoribosyltransferase (HPRT)–deficient A9 cells. The hybrids contain several human chromosomes including chromosome 11. A9 cells were again used as recipient cells for a microcell transfer, and cells with a transferred chromosome X/11 containing the *HPRT* gene were selected in medium containing hypoxanthine, aminopterin, and thymidine (HAT).
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22. We thank R. Worton and D. Schlessinger for encouragement and advice; L.-C. Tsui, R. Weksberg, M. Minden, C. Thompson, J. Lowe, M. Tewari, and F. Collins for helpful discussions; S. Bodrug, C. McDowell, N. Jordan, J. Rommens, and M. Olson, for providing genomic libraries; C. Campbell and M. S. McAndrews-Hill for sequencing; M. Glaves for technical assistance; and A. Huntzicker for preparing the manuscript. This work was supported by Clinical Research grant no. 6-551 from the March of Dimes Birth Defects Foundation grant (A.P.F.); the NCIC (B.R.G.W. and H.Y.); a Steve Fonyo NCIC studentship (A.H.); an Ontario Graduate studentship (L.B.); the New Zealand Cancer Society (A.E.R.); and NIH grants CA48932 (A.P.F.) and T32DK07458 (S.E.K.) and T32GM07315 (L.M.K.).

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Regulation of Gene Expression with Double-Stranded Phosphorothioate Oligonucleotides

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Alteration of gene transcription by inhibition of specific transcriptional regulatory proteins is necessary for determining how these factors participate in cellular differentiation. The functions of these proteins can be antagonized by several methods, each with specific limitations. Inhibition of sequence-specific DNA-binding proteins was achieved with double-stranded (ds) phosphorothioate oligonucleotides that contained octamer or κB consensus sequences. The phosphorothioate oligonucleotides specifically bound either octamer transcription factor or nuclear factor (NF)-κB. The modified oligonucleotides accumulated in cells more effectively than standard ds oligonucleotides and modulated gene expression in a specific manner. Octamer-dependent activation of a reporter plasmid or NF-κB–dependent activation of the human immunodeficiency virus (HIV) enhancer was inhibited when the appropriate phosphorothioate oligonucleotide was added to a transiently transfected B cell line. Addition of phosphorothioate oligonucleotides that contained the octamer consensus to Jurkat T leukemia cells inhibited interleukin-2 (IL-2) secretion to a degree similar to that observed with a mutated octamer site in the IL-2 enhancer. The ds phosphorothioate oligonucleotides probably compete for binding of specific transcription factors and may provide anti-viral, immunosuppressive, or other therapeutic effects.

ANALYSIS OF THE FUNCTION OF EUkaryotic transcriptional regulatory proteins in mammalian cells has been limited. Because such transcription factors are often essential to cell viability, mutant cell lines lacking these proteins can be difficult to obtain. An alternative approach is to generate trans-dominant mutants that interfere with the function of transactivators. While this strategy has been successful (*1*), the generation of such mutants is not always possible. Another method utilizes promoter competition, whereby plasmids containing cis-acting elements in common with an indicator gene are introduced in high copy number into cells (*2*). Because these plasmids must be maintained uniformly in large numbers of cells, this approach has also been limiting. Advances in the synthesis of DNA now allows an alternative approach to this problem. Oligonucleotides with modified phosphodiester bonds, such as phosphorothioate, methyl phosphate, phosphoramidite, or methyl phosphonate derivatives can be routinely synthesized in large amounts and are relatively resistant to nucleases (*3, 4*). Because of their increased cell permeability and stability, these compounds have been used as antisense agents (*5*). We sought to ascertain whether double-stranded (ds) phosphorothioates could penetrate cells, bind sequence-specific DNA-binding proteins, and interfere with eukaryotic transcription in vivo.

To determine whether ds phosphorothioates could compete for binding of sequence-specific DNA-binding proteins, we used ^{32}P-labeled ds oligonucleotides that contained the octamer or κB elements in the electrophoretic mobility shift assay (EMSA). An octamer site derived from the IL-2 enhancer and nuclear extracts from Jurkat cells were used for these studies (*6–8*). Protein binding to the ^{32}P-labeled IL-2 octamer probe (unmodified) was inhibited equally well by unlabeled ds oligonucleotide or phosphorothioate octamer sequences, but not by κB sequences of either type (Fig. 1A). Conversely, protein binding to ^{32}P-labeled κB probe (unmodified) was inhibited in an indistinguishable manner by ds oligonucleotide or phophorothioate κB sequences, but not by octamer sequences of either type (Fig. 1B). When phosphorothioates were used as labeled probes, we observed specific binding using octamer (Fig. 1C) or κB (Fig. 1D) in the EMSA. In addition, protein-binding to the phosphorothioates was inhibited equally well by the appropriate unmodified or phosphorothioate oligonucleotides (Fig. 1), but not by oligonucleotides with mutations within the appropriate consensus binding site (*9*). Because phosphorothioates competed with equimolar potency, they may serve as potential antagonists, despite the fact that they differ chemically from standard DNA and may not interact with DNA binding proteins in an identical manner. The phosphorothioates also showed increased resistance to digestion by deoxyribonuclease (DNase) I (*9*), as described for other endonucleases (*10*).

To compare the efficacy of cellular incorporation of ds phosphorothioate and phosphodiester oligonucleotides, we incubated ^{32}P-labeled samples of each type (octamer sequences) with Jurkat T leukemia cells.

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Cell-associated radioactivity was quantitated and analyzed with denaturing polyacrylamide gels. Within 4 hours, ds phosphorothioates accumulated in Jurkat cells (Fig. 2A) at sizes consistent with both monomer and trimer forms (Fig. 2B). Although radioactivity from the unmodified ds oligonucleotides became cell-associated at a comparable rate (Fig. 2A), significantly lower concentrations persisted in cells (Fig. 2B), suggesting that degradation had occurred.

We next examined whether ds phosphorothioates could modulate the transcriptional activity of sequence-specific DNA binding proteins in vivo. To analyze the octamer phosphorothioate, an Epstein-Barr virus–transformed B cell line (clone 13) (*11*) was transfected with a reporter plasmid in which an immunoglobulin gene octamer sequence was inserted upstream of a *fos* promoter-chloramphenicol acetyltransferase (CAT) gene construct (*12*). Four hours after transfection, the ds octamer phosphorothioate was added, and the result was a >90% inhibition of octamer-dependent gene expression. A lesser degree of specific inhibition was seen with the unmodified octamer oligonucleotides. In contrast, when either the ds κB phosphorothioate or κB oligonucleotide was included in the culture medium, a small nonspecific reduction in octamer-dependent transcription was observed (Fig. 3A). Addition of single-stranded phosphorothioates that contained the octamer sequence had no effect on CAT activity (Fig. 3B), suggesting that this effect was not a result of hybridization with the reporter plasmid. Using the HIV enhancer, which contains two κB sites, we tested the ability of ds κB phosphorothioates to inhibit HIV-CAT expression. Inhibition (>80%) of HIV-CAT expression was observed in clone 13 cells (Fig. 3C), which display constitutive NF-κB binding activity. In contrast, the unmodified ds κB oligonucleotides and both types of octamer DNA's inhibited HIV-CAT activity in a nonspecific manner in this cell line. Finally, none of the ds phosphorothioates or oligonucleotides specifically inhibited the Rous sarcoma virus (RSV) enhancer (Fig. 3D), which contains no recognizable octamer or κB regulatory elements.

We tested the ability of ds phosphorothioates to inhibit expression of the endogenous IL-2 gene in Jurkat cells. This gene contains octamer sequences, which contribute, in part, to IL-2 enhancer function (*6–8*). Mutation of a site between −75 to −70 in the IL-2 enhancer abolished binding of an octamer transcription factor (Fig. 1) and reduced IL-2–driven CAT expression to 30 to 50% of control values (Fig. 4A). As a means of determining whether expression of the IL-2 gene could be similarly reduced by ds octamer phosphorothioates, we incubated Jurkat cells with this reagent (0.2 to 7.5 μM) for 2 hours prior to stimulation of IL-2 secretion with phorbol myristate acetate (PMA) and phytohemagglutinin (PHA). Dose-dependent inhibition was observed, and, at the highest concentration of phosphorothioate (7.5 μM), the reduction in IL-2 synthesis was similar to that observed for the mutant octamer IL-2 enhancer (Fig. 4B). No effect was observed with single-stranded, phosphorothioate derivatives, and no cytopathic

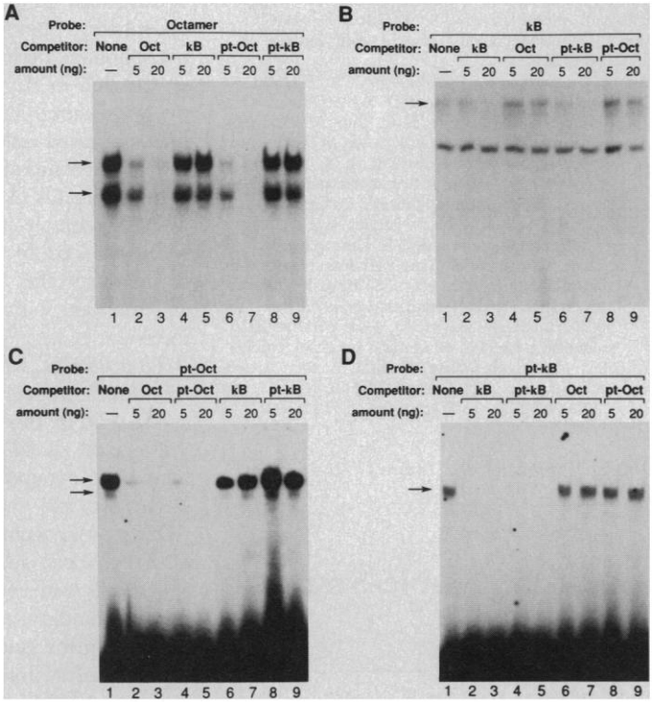

Fig. 1. Binding of octamer and NF-κB transcription factors to ds oligonucleotides and phosphorothioates. Binding site specificity was tested by EMSA with the following ^{32}P-labeled probes: (**A**) unmodified ds oligonucleotides with two copies of the octamer site (Oct) from the IL-2 enhancer; (**B**) unmodified ds oligonucleotides with two copies of κB sites from HIV enhancer (κB); (**C**) ds phosphorothioates with one IL-2 octamer site (pt-Oct); or (**D**) ds phosphorothioates with two copies of κB from the HIV enhancer (pt-κB). Nuclear extracts (8 to 10 μg) from unstimulated (A and C) or induced (B and D) Jurkat cells (40 nM PMA for 4 hours at 37°C) were incubated with labeled octamer or κB probes, either alone or in the presence of the indicated amounts of unlabeled competitors. Specific inducible complexes are indicated by arrows. Unstimulated Jurkat cells contain the Oct-1 but not Oct-2 transcription factor (*16*). Nuclear extracts were prepared (*17*), and the EMSA was performed as described (*18*). The nucleotide sequences and method of synthesis of the probes are as described (*19*).

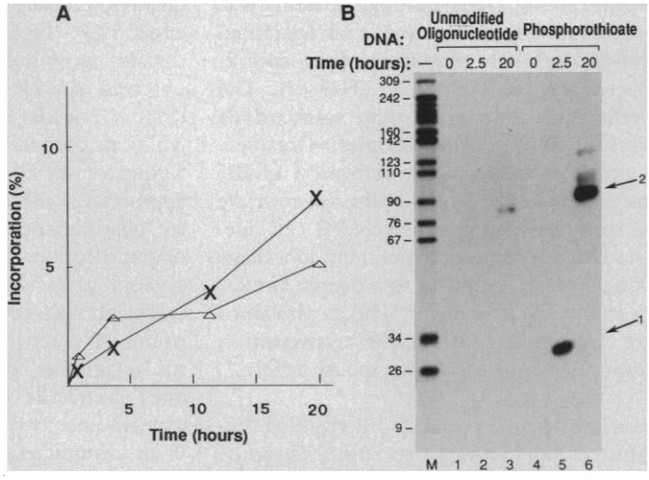

Fig. 2. Analysis of cellular uptake and processing of phosphorothioates. Analysis of (**A**) cellular incorporation of ds oligonucleotides (x) or phosphorothioates (△) and (**B**) denaturing polyacrylamide gel electrophoresis (PAGE) of cell-associated probes. Arrows indicate the position of the 26-bp (1; monomer) or 78-bp (2; trimer) DNA. Jurkat T leukemia cells (2.5 × 10^5) were incubated with ~1 ng of ^{32}P-labeled octamer oligonucleotides (~6 × 10^6 cpm/pmol) or an equivalent amount of ds octamer phosphorothioate (~6 × 10^6 cpm/pmol) in 200 μl of RPMI 1640 medium supplemented with fetal calf serum (1%), glutamine, penicillin, and streptomycin in a 96-well microtiter plate. A 26-bp oligonucleotide that contained one copy of the octamer site was used in this experiment. Probes were labeled in the single-stranded form with T4 polynucleotide kinase and γ-^{32}P-ATP prior to annealing. Cells were incubated at 37°C for 2 to 20 hours and analyzed. Cell-associated radioactivity was determined after three washes in phosphate-buffered saline, and Cerenkov counts in the cell pellets and supernatants were determined. The cell-associated DNA was phenol-chloroform extracted, precipitated, and analyzed by denaturing PAGE and autoradiography. Results are representative of two independent experiments.

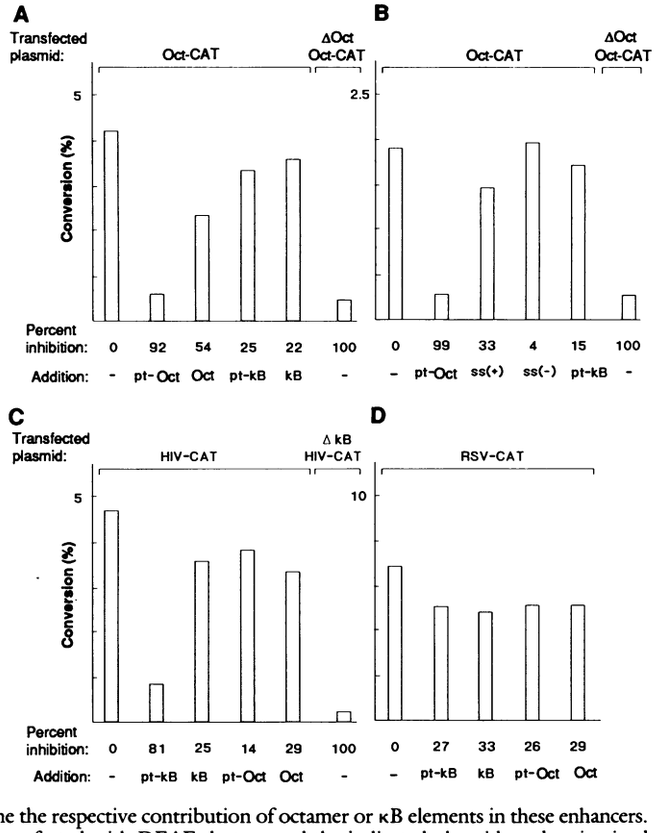

Fig. 3. Inhibition of transcription by phosphorothioates in clone 13 cells. The effect on gene expression of ds unmodified oligonucleotides or phosphorothioates (7.5 μM) was assessed in clone 13 cells after transient transfection with (**A**) an octamer-CAT reporter plasmid (*12*); (**B**) the octamer-CAT reporter plasmid comparing single-stranded and ds phosphorothioates; (**C**) HIV enhancer-CAT reporter plasmid; or (**D**) the RSV enhancer-CAT reporter plasmid. Addition of the relevant ds phosphorothioate (pt-Oct or pt-κB), single-stranded sense [ss(+)] or antisense [ss(−)] pt-Oct, or oligonucleotide (Oct or κB) are indicated, with sequences as in (*19*). The CAT fusion expression plasmids that contained point mutations in the regulatory elements [octamer (Δ Oct) (*12*) or ΔκB HIV-CAT (*18*)] were transfected to determine the respective contribution of octamer or κB elements in these enhancers. Clone 13 cells (10^7) were transfected with DEAE-dextran and the indicated plasmids and maintained as described (*20*) in 0.1 ml aliquots at 1 to 5 × 10^6 cells/ml. Four hours after transfection, the indicated oligonucleotides or phosphorothioates (7.5 μM) were added to ~10^5 cells. After an additional 40 hours, cell extracts were prepared, protein concentrations assayed, and CAT activity determined according to standard methods (*21*). Standard deviations for each CAT assay were less than 10%, and results are representative of 2 to 4 independent transfections.

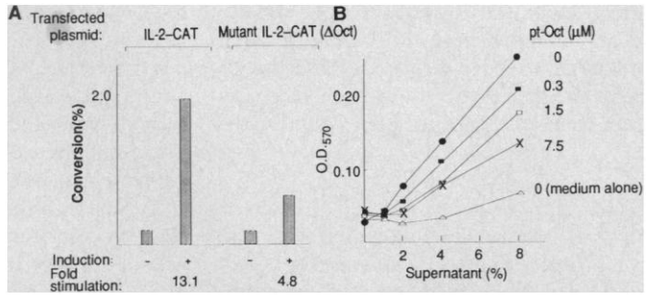

Fig. 4. Effect of octamer phosphorothioate on IL-2 secretion in Jurkat T leukemia cells. Analysis of (**A**) the contribution of the octamer sequence to IL-2 enhancer activity and (**B**) specific inhibition of IL-2 secretion by IL-2 octamer phosphorothioates. IL-2 gene expression was stimulated by the addition of PMA (40 nM) and PHA (1 to 2 μg/ml). (A) Jurkat cells (10^7) were transfected by the DEAE-dextran method (*20*) with IL-2–CAT plasmids with either a wild-type or mutant octamer site. The IL-2 octamer sequence was modified between positions −75 to −70 to change the sequence from AAAACATTTT to CTCGAGTTTT. After transfection and incubation for 24 hours, cells were incubated for an additional 20 hours with PMA and PHA. CAT activity was determined as in Fig. 3. Phosphorothioates were added 1 hour before incubation with PMA and PHA. (B) Supernatant was collected 24 hours after stimulation. Secreted IL-2 in Jurkat culture supernatants was measured with the IL-2–dependent CTLL cell line and a colorimetric assay (*22*). Cell proliferation expressed as OD_{570} is on the ordinate and the concentration of Jurkat supernatant is on the abscissa. Values represent the average of triplicate determinations, and standard deviations were <10%.

effects were observed at these concentrations (*9*). Because other cis-acting regulatory sequences contribute to regulation of the IL-2 gene (*6–7, 13*), the observed partial inhibition of IL-2 gene expression was expected.

Synthetic oligonucleotides have been used as antisense compounds, which selectively inhibit expression of particular gene products by interfering with the translation or processing of specific cellular mRNA's (*14*). Use of antisense agents, however, is limited, often because the gene that encodes the protein of interest has not been cloned. With the method described in this report, function of the DNA-binding proteins themselves can be inhibited if the cis-acting regulatory elements have been characterized. We estimate that 10^7 to 10^8 molecules of exogenous phosphorothioate are found per cell when 10 μM oligonucleotide is incubated in 200 μl of culture medium containing 2.5 × 10^6 cells/ml. With micromolar concentrations of ds phosphorothioates, >75% of the treated cells contained this compound (*9*). If a fraction (10%) of these molecules enter the nucleus, it is likely that this represents a significant excess of binding sites over transcriptional regulatory proteins. If 10^3 to 10^4 such regulatory proteins are present in the nucleus (*15*), this concentration of oligonucleotide represents an excess of 10^3 to 10^4. Our data suggest that some DNA-binding proteins recognize specific bases within the helix, independent of the phosphodiester linkages. This feature of these transcription factors may permit further modification of oligonucleotide phosphodiester linkages for competition studies. For example, to improve aqueous solubility, modified phosphodiester sequences could be interspersed with unmodified phosphodiester bonds on each strand. Because of the limited toxicity of and specific transcriptional regulation by ds phosphorothioates, these agents may be useful in the inhibition of transcription of disease-causing genes.

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19. The ds oligonucleotide probe for the octamer site was 5′-TGTAATATGTAAAACATTGTCTGTAA-TATGTAAAACATT-3′. The sequence of the phosphorothioate sense strand used as competitor was identical, and the antisense strand represented the reverse complement. In Fig. 1C, a single copy octamer phosphorothioate site, 5′-ATATGTGTAA-TATGTAAATTT-3′ was used as a labeled probe. The sequence of the κB probe was 5′- GATCAGG-GACTTTCCGCTGGGGACTTTCC- 3′. The sequence of the phosphorothioate sense strand was 5′-AGGGACTTTCCGCTGGGGACTTTCC-3′. The antisense strand represented the reverse complement. Oligonucleotides were synthesized on the Applied Biosystems DNA Synthesizer Model 380B with nucleoside 3′ hydrogen phosphonates as monomers. Columns with covalently attached oligonucleotides were exposed overnight, after synthesis, to a reagent mix [(0.05 g/ml elemental sulfur in 48% carbon disulfide (CS_2), 48% pyridine, and 4% triethylamine; Aldrich] for sulfurization of phosphodiester bonds. The columns were rinsed twice with CS_2 and three times with acetonitrile and 2% triethylamine before overnight exposure to ammonia (*3*). The crude phosphorothioate DNA analogs were purified by reversed-phase high-performance liquid chromatography (HPLC). The peak eluting with 30% acetonitrile was collected and detritylated, and the DNA was precipitated with ethanol. Purity was assessed by PAGE, which revealed a single band that migrated to the position expected for unmodified oligonucleotides of similar length.
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Hyperkalemic Periodic Paralysis and the Adult Muscle Sodium Channel α-Subunit Gene

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Hyperkalemic periodic paralysis (HYPP) is an autosomal dominant disorder characterized by episodes of muscle weakness due to depolarization of the muscle cell membrane associated with elevated serum potassium. Electrophysiological studies have implicated the adult muscle sodium channel. Here, portions of the adult muscle sodium channel α-subunit gene were cloned and mapped near the human growth hormone locus (*GH1*) on chromosome 17. In a large pedigree displaying HYPP with myotonia, these two loci showed tight linkage to the genetic defect with no recombinants detected. Thus, it is likely that the sodium channel α-subunit gene contains the HYPP mutation.

THE PRIMARY PERIODIC PARALYSES are autosomal dominant disorders displaying episodes of muscle weakness accompanied by slight abnormalities in the concentration of blood potassium: elevated in the hyperkalemic form and reduced in the hypokalemic form (*1–3*). Recurrent paralysis attacks are associated with the eventual development of an irreversible vacuolar myopathy, which may be debilitating. The ultimate pathogenetic step in each form of the disorder is muscle membrane inexcitability due to depolarization (*4*), leading to paralysis.

Hyperkalemic periodic paralysis (HYPP; also known as Gamstorp's disease or adynamia episodica hereditaria) typically begins in the first decade with attacks of muscle weakness precipitated by cold, rest after exercise, fasting, or ingestion of potassium (*5*). The three clinical variants of HYPP (*1–3, 6*) breed true and include HYPP with myotonia (muscle stiffness following contraction), which includes the majority of cases, HYPP without myotonia, and HYPP with paramyotonia (cold-induced myotonia). In vitro studies have demonstrated that in HYPP with myotonia, a tetrodotoxin-sensitive sodium current is triggered in affected muscle by a slight increase in extracellular potassium that would normally not be sufficient to open the voltage-gated sodium channel (*6, 7*). The α subunit of the sodium channel can itself form a voltage-gated sodium channel that can be blocked by tetrodotoxin in vitro (*8, 9*), albeit with abnormal kinetics involving slow inactivation in the absence of an additional low molecular weight factor (*10–14*). We have therefore tested for genetic linkage between HYPP and the locus for the α subunit of the adult muscle sodium channel.

Recently, rat cDNA clones encoding the α subunits of the adult muscle sodium channel (*12*) and of the fetal muscle sodium channel (*15*) were isolated. We took advantage of strong evolutionary conservation to clone two intracellular domains of the human adult muscle sodium channel α subunit (Fig. 1A). Na2 included ~600 bp of the intracellular domain between repeats II and III (nucleotides 2434 to 3099 of the rat coding sequence) and was expected to be highly homologous only to the adult human isoform. Na3 spanned ~300 bp of the carboxyl terminal end (nucleotides 4834 to 5166) and was expected to be homologous to both adult and fetal human α subunits. These regions were amplified by the polymerase chain reaction (PCR) with normal adult human skeletal muscle single-stranded cDNA as the template, and the PCR products of appropriate size were cloned (plasmids h-Na2 and h-Na3) and sequenced (Fig. 1B) (*16*).

DNA blot analysis of human genomic DNA digested with 11 restriction enzymes and probed with h-Na2 showed only a single band for each, consistent with the existence of a single-copy gene. H-Na2 and h-Na3 were localized by somatic cell hybrids

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Table 1. Lod scores for linkage of HYPP to the sodium channel α-subunit gene.

Lod score ($\hat{z}$)	Recombination fraction ($\hat{\theta}$)					
	0.00	0.05	0.10	0.20	0.30	0.40
Two-point lod score: H-Na2 versus *GH1*	9.89	8.96	7.96	5.83	3.52	1.23
Two-point lod score: HYPP versus h-Na2	4.00	3.63	3.23	2.39	1.47	0.53
Two-point lod score: HYPP versus *GH1*	2.40	2.15	1.88	1.32	0.74	0.23
Multipoint lod score: HYPP versus h-Na2/*GH1*	7.02	6.42	5.79	4.43	2.92	1.15