Growth Factors Regulate Transin Gene Expression by c-*fos*–Dependent and c-*fos*–Independent Pathways

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The rapid induction of the proto-oncogene c-*fos* by growth factors and other bioactive agents, and the recent evidence that the c-*fos* protein (Fos) is associated with transcriptional complexes, suggests that Fos may represent an integral part of an intracellular messenger pathway that triggers changes in gene expression and ultimately phenotypic alterations. This report examines the role of c-*fos* in growth factor stimulation of transin, a matrix-degrading secreted metalloproteinase. Platelet-derived growth factor (PDGF) stimulation of transin RNA was blocked by a selective reduction in Fos synthesis with antisense c-*fos* mRNA, whereas epidermal growth factor (EGF) stimulation of transin occurred despite an equivalent inhibition of Fos levels. The stimulatory effect of both PDGF and EGF on transin transcription involved factors recognizing the sequence TGAGTCA, which is found in the transin promoter and is reported to be a binding site for the transcriptional factor Jun/AP-1 and for associated Fos and Fos-related complexes. Thus both Fos-dependent and Fos-independent pathways exist for growth factor regulation of gene expression, and both effects may be mediated through the same cis-acting transcription element.

THE COMPLEX CHANGES IN CELL growth rate and phenotype elicited by growth factors are believed to be initiated by the transcriptional induction of a set of "immediate early" response genes (*1*), of which the best characterized is the nuclear proto-oncogene c-*fos* (*2*). Several studies have suggested that c-*fos* induction is essential for the growth-promoting activities of certain growth factors and serum (*3–5*). Recently, a role has been suggested for Fos as a trans-acting transcription factor for the adipocyte gene *aP2* (*6, 7*), the mouse α1-(III) collagen gene (*8*), collagenase (*9*), several viral promoter regions (*6*), and the c-*fos* gene itself (*10*). We have exploited the use of antisense RNA to examine the possibility that c-*fos* may play a role in the intracellular pathway used by growth factors to stimulate transin gene expression.

Transin was originally cloned as a cellular gene differentially expressed in polyoma virus–transformed FR3T3 cells (*11*). Subsequent studies revealed that transin RNA is induced in Rat-1 cells by several oncogenes, including v-*src*, H-*ras*, and K-*ras*, and by the growth factor EGF (*11, 12*). In mouse NIH 3T3 fibroblasts, transin RNA is induced by EGF, PDGF, and the phorbol ester tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Fig. 1). The observations that these agents all induce a rapid stimulation of c-*fos* that precedes the observed increase in transin RNA levels and the knowledge that protein synthesis is required for induction of transin by growth factors (*13, 14*) suggested that c-*fos* may act as a "third messenger" and directly alter transcription of the transin gene.

To investigate the role of c-*fos* in transin gene activation, we have utilized NIH 3T3 fibroblasts bearing a fragment of human c-*fos* in either the sense (S2) or antisense (AS1) orientation under the steroid-inducible mouse mammary tumor virus (MMTV) promoter (*3*). This antisense c-*fos* construct has previously been shown to inhibit cell growth (*3*). Total cytoplasmic RNA was extracted from PDGF-, EGF-, and TPA-stimulated cells in the presence and absence of dexamethasone and analyzed for transin RNA. PDGF, EGF, and TPA are capable of inducing transin mRNA in S2 and AS1 cells (Fig. 1). The addition of dexamethasone to S2 cells had no effect on transin induction by any of these agents. In AS1 cells, however, where addition of dexamethasone results in an excess of antisense RNA complementary to c-*fos* RNA (*3*), PDGF stimulation no longer resulted in transin induction. These results suggested that PDGF induction of c-*fos* was required for transin expression. Surprisingly, EGF induction of transin under the same circumstances was unaffected by antisense c-*fos*. TPA-treated cells displayed approximately a 50% decrease in transin in the presence of dexamethasone. The level of RNA hybridizing to the plasmid pD5 [a cDNA clone demonstrating no differential response with the growth state of rat fibroblast cells (*15*)] was unaffected by growth factor or dexamethasone treatment. Similar results were obtained with another NIH 3T3 antisense c-*fos* clone, AS2 (*3, 14*).

Immunofluorescent labeling of PDGF-, EGF-, and TPA-treated AS1 cells with antiserum to a synthetic peptide based on the Fos sequence (*16*) confirmed the induction of Fos by these agents (*17*). Dexamethasone induction of antisense c-*fos* resulted in an 81% and 90% reduction, respectively, in the number of cells expressing PDGF- and EGF-induced Fos (*17*). Expression of antisense c-*fos* also reduced the ability of EGF and PDGF to stimulate DNA synthesis as measured by nuclear incorporation of [^{3}H]thymidine (Table 1). Dexamethasone induction of antisense c-*fos* reduced PDGF-stimulated DNA synthesis to an average of 23% of control values in three distinct antisense-containing NIH 3T3 cell lines and to an average of 19% of control values after

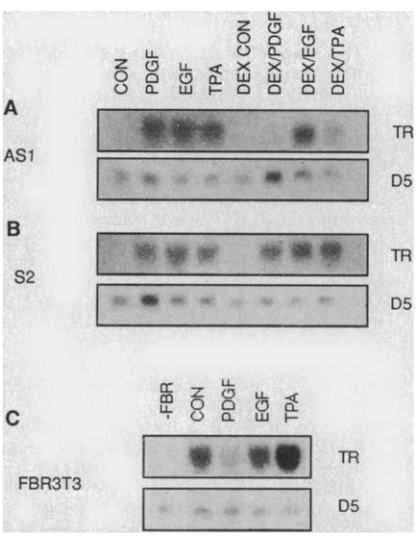

Fig. 1. Effects of *fos* on growth factor and TPA-induction of transin RNA. NIH 3T3 fibroblasts bearing a human 5′ c-*fos* gene fragment in either the antisense (3′ to 5′) (**A**) or sense (5′ to 3′) (**B**) orientation under the steroid-inducible mouse mammary tumor virus (MMTV) promoter (*3*) were deprived of serum for 24 hours before stimulation with PDGF (200 ng/ml), EGF (20 ng/ml, Gibco), or TPA (100 ng/ml, Sigma) or no addition (CON) both in the presence of dexamethasone (1 μ*M*, Sigma) or vehicle alone (0.1% ethanol). Total cytoplasmic RNA was collected 8 hours after stimulation, and 20-μg samples were subjected to RNA hybridization analysis as previously described (*11*). A purified rat transin cDNA insert (1.6-kb Eco RI fragment from pTR1) (*11*) was labeled by random priming (Boehringer Mannheim) with [α-^{32}P]dCTP (deoxycytidine 5′-triphosphate) to a specific activity greater than 5×10^8 cpm per microgram. Filters were stripped of radiolabeled DNA and reprobed with D5, a cDNA corresponding to mRNA unaffected by alterations in growth rates (*15*). Results similar to those for the AS1 cells were obtained with the independently isolated clone AS2 (*3*). (C) NIH 3T3 cells were cotransfected with FBR viral DNA (*18*) and SV2neo and selected for neomycin resistance; a clone expressing v-*fos* was grown to confluence and deprived of serum for 24 hours. Cells were stimulated with PDGF, EGF, or TPA for 8 hours at concentrations described above, or with no addition (CON). −FBR = NIH 3T3 cells, no addition.

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Table 1. Effect of antisense c-*fos* on growth factor–stimulated DNA synthesis. The indicated cells were deprived of serum for 3 days, then treated with dexamethasone (+DEX) or ethanol vehicle (−DEX) for 1 hour before addition of EGF (20 ng/ml) or PDGF (200 ng/ml). [^{3}H]Thymidine (6.7 Ci/mmol, 1 mCi/ml) was added 12 hours after the addition of growth factors. Cells were processed for autoradiography after an additional 12 hours of thymidine labeling. Percentage labeled nuclei was determined by taking the mean of duplicate counts of 200 cells (+DEX/−DEX × 100 = % of CON). Duplicates varied by 10% or less. Background labeling (no stimulation) was less than 5%.

Cell line	PDGF			EGF		
	−DEX	+DEX	% of CON	−DEX	+DEX	% of CON
AS1	51	11	21	33	8	24
AS2	33	4	12	29	5	17
AS3	39	14	36	33	5	15
S2	55	54	98	76	74	97
S3	49	49	100	38	39	102

EGF stimulation. No effect of dexamethasone treatment was observed in NIH 3T3 cell lines containing the c-*fos* fragment in the sense orientation. Despite the equivalent reduction of PDGF- and EGF-induced Fos and DNA synthetic activity after antisense induction, the antisense c-*fos* transcript was selective in eliminating PDGF- but not EGF-induced transin RNA (Fig. 1).

To determine if *fos* expression is sufficient for transin induction, NIH 3T3 cells were transfected with an FBR v-*fos*–containing plasmid (*18*) and analyzed for transin expression by RNA hybridization analysis. Expression of v-*fos* (confirmed by DNA and RNA hybridization) resulted in growth factor–independent transin expression (Fig. 1C). The similarities in sequence between c-*fos* and v-*fos* (*19*) had suggested that c-*fos* expression would also be sufficient for transin induction. Alternative mechanisms for transin induction must also exist, however, as c-*fos* induction was not required for EGF induction of transin.

The PDGF attenuated v-*fos* induction of transin RNA (Fig. 1C). Although the reason for this effect is unclear, it is possible that PDGF decreases long terminal repeat (LTR)–regulated transcription of v-*fos*. EGF, however, had no effect on v-*fos*–stimulated transin, whereas TPA treatment of v-*fos*–transfected cells resulted in a twofold increase in transin RNA.

EGF regulates transin gene expression in Rat-1 fibroblasts at the transcriptional level as determined by nuclear run-on analysis (*11*). To determine if the effects of PDGF, EGF, TPA, and antisense c-*fos* on transin RNA levels in NIH 3T3 cells are also consistent with transcriptional regulation, transfection experiments with transin promoter-reporter gene constructs were performed. Recombinant DNA containing 750 bp of the transin promoter (p750TRCAT) driving the heterologous reporter gene chloramphenicol acetyltransferase (CAT) (*20*) was transfected into AS1 cells, and the effect of growth factors in the presence and absence of c-*fos* was assayed. PDGF, EGF, and TPA stimulation resulted in a 2.2-, 3.3-, and 4.6-fold induction of CAT activity, respectively (Fig. 2). Dexamethasone treatment reduced PDGF-induced activity to 15% of control, whereas no effect was observed on EGF-induced CAT activity. TPA-induced CAT activity was reduced to 55% of control. The effect of antisense c-*fos* on transin transcription as assayed by CAT expression was therefore consistent with that observed at the level of RNA accumulation as determined by RNA hybridization.

Fos has been shown to associate with the trans-acting transcription factor, AP-1 (*7, 21*). A consensus DNA-binding sequence recognized by AP-1, TGAGTCA, is also reported to confer TPA-responsiveness to reporter genes and is referred to as the TPA-responsive element (TRE) (*22, 23*). This sequence is present in the transin promoter at position −71 (*13*). To determine whether PDGF stimulation of the transin promoter may be mediated by the interaction of soluble factors with the TRE of transin, we synthesized two complementary 19-nucleotide oligomers containing the TRE consensus sequence from the transin gene (TRE oligo) (*24*). The oligonucleotides were annealed and used to compete with soluble TRE-binding factors by cotransfection with p750TRCAT into AS1 cells. The cells were deprived of serum and then stimulated with PDGF, EGF, or TPA in the presence or absence of dexamethasone, and CAT activity was determined. In addition, a simian virus 40 (SV40) promoter–β-galactosidase plasmid (*25*) was cotransfected and β-galactosidase activity assayed to normalize for possible differences in transfection efficiency. We observed no significant effect of the oligonucleotides on SV40 promoter–mediated transcription. [Note that the TRE consensus sequence is present in the SV40 enhancer, and that previous experiments have demonstrated minimal effect of these sequences on SV40-mediated transcription (*26*)]. The TRE oligonucleotide reduced PDGF-, EGF-, and TPA-stimulated CAT activity, whereas little or no inhibitory effect on growth factor–induced CAT activity was observed with a control oligonucleotide containing sequences from the c-*myc* gene [Table 2 and (*14*)]. In addition, an oligonucleotide differing from the TRE oligo in only two positions [1 and 6, demonstrated to be essential for TPA inducibility (*22*)] was ineffective in reducing PDGF-, EGF-, or TPA-stimulated CAT activity (Table 2). Soluble factors recognized by the TRE were therefore apparently involved in EGF and TPA stimulation of transin expression, as well as being needed for PDGF stimulation.

The use of inducible antisense mRNAs to block specific proteins has been effective in demonstrating the role of c-*fos* in growth factor–induced and serum-induced DNA synthesis (*3, 5*). We were initially concerned about the use of the dexamethasone-inducible MMTV promoter for antisense RNA production because of the recent report of dexamethasone repression of the homologous rabbit stromelysin gene (*27*). In our system, however, dexamethasone had no significant effect on basal transin RNA levels or growth factor– or TPA-induced transin

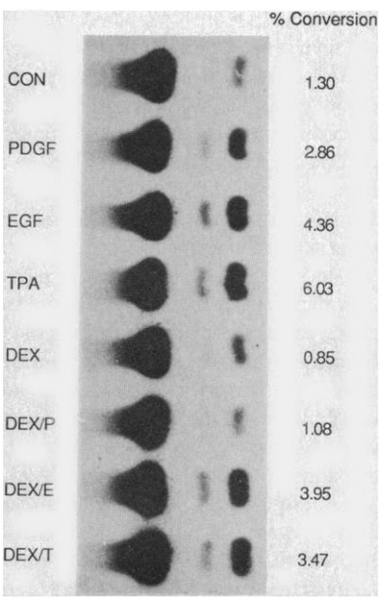

Fig. 2. The effect of antisense c-*fos* RNA on growth factor and TPA-induction of transin promoter–mediated CAT expression. AS1 cells were plated at a density of 6.5 × 10^5 cells per 100-mm plate 1 day before transfection. Calcium phosphate–DNA precipitates containing 15 μg of p750TRCAT were prepared by the method of Graham and van der Eb (*29*). Four hours after the addition of the precipitates, cells were glycerol-shocked for 3 minutes (10% glycerol) and refed with Dulbecco's minimum essential medium containing 10% calf serum. One day after transfection, cells were deprived of serum for 24 hours and stimulated with PDGF, EGF, or TPA with or without dexamethasone as described in the legend to Fig. 1. Cell extracts were prepared 8 hours after stimulation (*30*). Acetylated products of [^{14}C]-chloramphenicol were separated with chloroform-methanol (95:5, ascending) on polysilicic acid glass fiber sheets (Gelman Sciences, Inc.) (*30*). After autoradiography, spots were excised, and radioactive counts were determined by scintillation counting. Data are the mean of two experiments; results varied by no more than 15%.

Table 2. Role of the TRE in modulation of transin expression by growth factors and TPA. AS1 fibroblasts were transfected with: 10 μg of p750TRCAT (no competitor); 10 μg of p750TRCAT and either 1 μg [TRE(1), 25*M* excess] or 10 μg [TRE(10), 250*M* excess] of a double-stranded oligomer containing the TRE from the rat transin gene (*24*); or 10 μg of the TRE oligonucleotide with nucleotide substitutions in positions 1 and 6 of the TRE (*24*) [mTRE(10)]. Transfections also included 2 μg of pCH110 BEM-gal, containing the β-galactosidase gene under control of the SV40 promoter (*25*). Total DNA was adjusted to 22 μg with plasmid DNA in all samples. After exposure to $CaPO_4$-precipitated DNA for 4 hours, cells were glycerol-shocked and refed with serum-containing medium as described in the legend to Fig. 2. Cells recovered for 24 hours, were deprived of serum for 20 hours, and were then treated with PDGF, EGF, or TPA for 8 hours at the concentrations described in the legend to Fig. 1. Cell lysates were analyzed for CAT (*30*) and β-galactosidase activity as described (*25*). CAT results (percent conversion) were normalized to β-galactosidase results to control for slight variations in transfection efficiencies and possible oligonucleotide toxicity. The percentage of control (no competitor) is presented as the mean and range (in parentheses) of two or three experiments.

Competitor	CAT activity (% of control)			
	CON	PDGF	EGF	TPA
None	100	100	100	100
TRE(1)	58 (37–79)	13 (11–16)	14 (12–17)	33 (27–39)
TRE(10)	17 (8–33)	5 (4–6)	5 (2–8)	5 (3–8)
mTRE(10)	88 (38–137)	145 (62–227)	101 (82–120)	128 (117–138)

RNA levels in control sense-containing NIH 3T3 cells (Fig. 1B) and only a slight effect on basal transin promoter–regulated CAT levels (Fig. 2). In addition, the disparate effects of dexamethasone on PDGF- and EGF-induced transin RNA and CAT levels further suggest dexamethasone was not itself affecting transin expression in this system.

TPA induction of Fos levels was reduced 51% by dexamethasone-induced antisense c-*fos*. The reason for partial blocking of c-*fos* by antisense in this case is unclear, although possible explanations include a stimulatory effect of TPA on the host RNA duplex unwindase activity, which is believed to inhibit antisense RNA effectiveness (*28*), or the possibility that TPA induces Fos-related antigens that are not blocked by antisense c-*fos* but are recognized by the antibody (*6, 16*). The reduction in TPA-induced transin mRNA and CAT activity by c-*fos* antisense stimulation was quantitatively similar to the reduction in Fos protein, suggesting that TPA, like PDGF, may also induce transin through a c-*fos*–dependent pathway. Alternatively, TPA-induction of transin may occur through both c-*fos*–dependent and c-*fos*–independent pathways. Support for this concept is obtained from the observation that the combined effect of v-*fos* and TPA on transin induction is greater than that observed with v-*fos* alone (Fig. 1C).

Transin gene expression provides a valuable tool for dissecting intracellular pathways used in mediating oncogene and growth factor effects on gene expression. The observation that serum inhibits transin RNA induction (*11*) and the differing effects of antisense c-*fos* on EGF-induced DNA synthesis and EGF-induced transin expression (Figs. 1 and 2 and Table 1) dissociate transin expression from effects modulated by the growth state of the cell. The identification of transin as a Fos-inducible gene also suggests that c-*fos* is a convergent point for the action of some growth factors and oncogenes on gene transcription, since many growth factors and oncogenes induce c-*fos* (*19*). Recent studies examining the role of c-*fos* in oncogene and phorbol ester induction of the collagenase promoter also support this conclusion (*9*).

These studies suggest that both c-*fos*–dependent and independent pathways exist for growth factor induction of transin gene expression. Recent evidence from our laboratory suggests that the independent pathway may require the action of protein kinase C (PKC), since attenuation of PKC activity eliminates EGF induction of transin RNA (*14*). An oligonucleotide containing the TGAGTCA element in the transin promoter competed for factor binding and inhibited growth factor stimulation by both pathways in vivo. These results and the lack of inhibition by the mutated TRE sequence suggest that the −71 TRE sequences are necessary for growth factor and TPA regulation of transin expression, although we cannot rule out the possibility that this oligonucleotide is competing for trans-acting factors operating through other transin promoter sequences represented in the p750TRCAT construct. This element is identified as a binding site for Jun/AP-1 (*7, 21*), and for associated Fos and Fos-related proteins (*6, 7*). Growth factors may affect transin gene expression, therefore, either by modulating c-*fos* levels (the c-*fos*–dependent pathway) or by altering levels or the phosphorylation state of other factors such as AP-1 or Fos-related antigens that recognize the TRE consensus sequence.

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17. AS1 fibroblasts were grown to confluence, deprived of serum for 24 hours, and stimulated for 1 hour with PDGF, EGF, or TPA either in the presence or absence of dexamethasone. Cultures were then washed three times in cold phosphate-buffered saline (PBS), fixed in 3.0% formaldehyde in PBS (2 × 10 min), treated with methanol, and rinsed well before air drying. After blocking with normal goat serum, a 1:1000 dilution of rabbit polyclonal antiserum directed against the M-peptide of Fos (*16*) was added to the cells and incubated at 4°C overnight. After appropriate PBS washes the cells were incubated with a fluoresceinated goat antibody to rabbit for 60 min. Control studies demonstrated that the observed nuclear fluorescence was dependent on the presence of both the primary and secondary antibodies. The percentages of nuclei that could be scored definitely positive for Fos were as follows (percent positive after antisense induction are in parentheses): PDGF, 48% (9%); EGF, 30% (3%); and TPA, 90% (44% but of decreased intensity). Background immunofluorescence (no stimulation) was less than 2%. Results represent the mean of duplicate 200 cell counts.
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24. *Oligomers*: The TRE double-stranded oligomer was constructed by annealing the following two oligomers: 5′ aattcatgagtcagatctg 3′ and 5′ ttaacagatctgactcatg 3′. This sequence also contains cohesive Eco RI ends and a Bgl II site. A control double-stranded oligomer containing sequences from the c-*myc* gene was constructed by annealing the following two oligomers (*31*): 5′ aacgttgaggggcat 3′ and 5′ atgcccctcaacgtt 3′. The mTRE oligonucleotide was identical to the TRE oligo except that the T in position 7 [position 1 of the TRE as described in (*22*)] was changed to a C, and the C in position 12 (6 of the TRE) was changed to a G. The corresponding changes were also made in the complementary oligonucleotide. All oligomers were synthesized by standard techniques (*32*).
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Mitochondrial DNA Mutation Associated with Leber's Hereditary Optic Neuropathy

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Leber's hereditary optic neuropathy is a maternally inherited disease resulting in optic nerve degeneration and cardiac dysrhythmia. A mitochondrial DNA replacement mutation was identified that correlated with this disease in multiple families. This mutation converted a highly conserved arginine to a histidine at codon 340 in the NADH dehydrogenase subunit 4 gene and eliminated an Sfa NI site, thus providing a simple diagnostic test. This finding demonstrated that a nucleotide change in a mitochondrial DNA energy production gene can result in a neurological disease.

LEBER'S HEREDITARY OPTIC NEUropathy (LHON) is a maternally inherited form of central optic nerve death associated with acute bilateral blindness. Cardiac dysrhythmia is also common and peripapillary microangiopathy is frequently seen in presymptomatic individuals. The age of onset ranges from adolescence to late adulthood; median age of onset is 20 to 24 years (*1–3*).

In numerous large pedigrees, LHON patients have been found to be related exclusively through the maternal lineage (*4*). No case of paternal transmission has been demonstrated. However, in most pedigrees, expression is variable and there is a bias toward males exhibiting ophthalmological problems (*1–5*).

The human mitochondrial DNA (mtDNA) is also maternally inherited (*6*). It codes for a large and small rRNA, 22 tRNAs, and 13 polypeptides of oxidative phosphorylation; ND1 to ND6 of complex I, cytb of complex III, COI to COIII of complex IV, and adenosine triphosphatase (ATPase) subunit 6 and subunit 8 of complex V (*1, 7–9*). Further, chronic exposure of primates and rodents to low levels of respiratory inhibitors results in optic neuropathy and basal gangliar lesions (*1, 10*). Thus, both the symptoms and inheritance of LHON suggest that this disease is the product of an mtDNA mutation.

Restriction analysis of lymphoid and muscle mtDNA samples ruled out deletions (*11*) as the cause of LHON. Therefore, candidate mutations for LHON were sought by cloning and sequencing the mtDNA from the blind proband of a large, black LHON pedigree from Georgia (Fig. 1). Since most reported LHON pedigrees are Caucasoid, analysis of a black LHON mtDNA minimized the chances of associating the disease with a rare ethnic-specific sequence polymorphism. Eighty-five percent of the LHON mtDNA coding region was sequenced. Comparison with the "normal" Cambridge mtDNA sequence (*7*) revealed 25 base substitutions, eight of which altered amino acids (Table 1).

A replacement mutation in the LHON mtDNA sequence would be implicated in the disease if it (i) changed a highly evolutionarily conserved amino acid (Table 1), (ii) was frequently found in LHON patients, and (iii) and was not found in normal

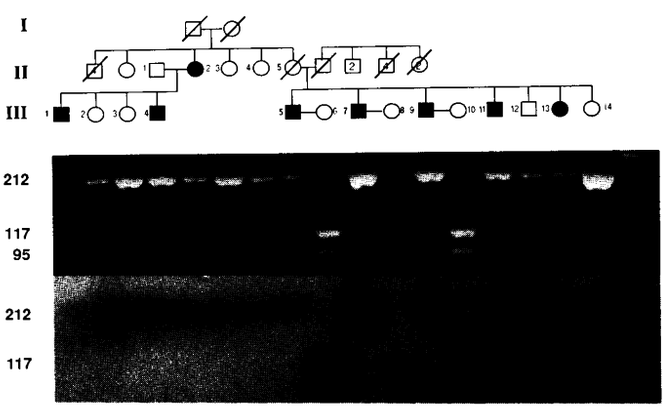

Fig. 1. Black LHON pedigree from the Georgia family. Filled symbols indicate central vision loss, open indicate no reported vision loss. Roman numerals indicate generations. Numbers in symbols indicate multiple siblings. Numbers outside symbols indicate family members studied. The mtDNA of proband III5 was cloned and sequenced. The middle panel shows the Sfa NI digestion patterns of a 212-bp PCR product that includes the nt11778 mutation. DNA for amplification was prepared from fractionated blood platelets (*29*), from white blood cells, or from transformed lymphoblasts. The samples were enriched for white blood cell and lymphoblast mtDNAs by Hirt extraction (*30*). The PCR reaction included 1 ng of target DNA and 2.5 units of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) (*20*). Approximately 200 ng of DNA were digested overnight at 37°C with 1 unit of Sfa NI. Digestion products were separated on 2.5% NuSieve plus 0.9% SeaKem agarose (FMC, Rockland, ME) gels. The lower panel shows the DNA blot hybridization of the Sfa NI digestion products with a 5′ end-labeled internal oligonucleotide. Sizes are expressed in base pairs.

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