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# Survival of Male Patients with Incontinentia Pigmenti Carrying a Lethal Mutation Can Be Explained by Somatic Mosaicism or Klinefelter Syndrome

The International IP Consortium*

**Incontinentia pigmenti (IP), or "Bloch-Sulzberger syndrome," is an X-linked dominant disorder characterized by abnormalities of skin, teeth, hair, and eyes; skewed X-inactivation; and recurrent miscarriages of male fetuses. IP results from mutations in the gene for NF-κB essential modulator (*NEMO*), with deletion of exons 4–10 of *NEMO* accounting for >80% of new mutations. Male fetuses inheriting this mutation and other "null" mutations of *NEMO* usually die in utero. Less deleterious mutations can result in survival of males subjects, but with ectodermal dysplasia and immunodeficiency. Male patients with skin, dental, and ocular abnormalities typical of those seen in female patients with IP (without immunodeficiency) are rare. We investigated four male patients with clinical hallmarks of IP. All four were found to carry the deletion normally associated with male lethality in utero. Survival in one patient is explained by a 47,XXY karyotype and skewed X inactivation. Three other patients possess a normal 46,XY karyotype. We demonstrate that these patients have both wild-type and deleted copies of the *NEMO* gene and are therefore mosaic for the common mutation. Therefore, the repeat-mediated rearrangement leading to the common deletion does not require meiotic division. Hypomorphic alleles, a 47,XXY karyotype, and somatic mosaicism therefore represent three mechanisms for survival of males carrying a *NEMO* mutation.**

## Introduction

Incontinentia pigmenti (IP), or "Bloch-Sulzberger syndrome" (MIM 308310), is an X-linked dominant disorder characterized by abnormalities of ectodermal tissue. Typically, IP presents in four dermatologically recognized stages: (1) early blistering with eosinophilia, (2) eruption of hyperkeratotic lesions, (3) hyperpigmentation along the lines of Blaschko and, finally, (4) dermal scarring. Abnormal tooth eruption, malformed tooth crowns, and patchy alopecia are commonly seen, and retinal dysplasia can sometimes lead to visual problems. In ~10% of cases, there may be neurological problems such as seizures, spasticity, or mental retardation (Landy and Donnai 1993). The vast majority of female patients with IP exhibit skewed X-inactivation patterns in blood and fibroblasts, following selective loss of cells expressing the mutated IP gene (Parrish et al. 1996). In these tissues, cell loss coincides with birth, since neonatal skin and cord blood may still possess cells expressing the mutated X chromosome.

We have shown that IP results from mutations in the gene for NF-κB essential modulator (*NEMO*) (The International Incontinentia Pigmenti Foundation 2000). Moreover, an intrachromosomal rearrangement that deletes exons 4–10 of *NEMO* (*NEMOΔ4–10*), accounts for ~83% of new mutations. This mutation is readily detectable either by the appearance of a novel 8-kb *Hin*dIII band in Southern blot analysis or by a diagnostic PCR that yields a 2-kb product from the *NEMOΔ4–10* allele. NEMO is a component of the IκB kinase (IKK) complex, which is indispensable for activation of the NF-κB transcription factor (Rothwarf et al. 1998; Yamaoka et al. 1998). One function of NF-κB activity is the protection of cells against apoptosis induced by tumor necrosis factor (TNF) (Van Antwerp et al. 1996). Skewed X inactivation in female patients is therefore assumed to result from the apoptosis of cells

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that have no protection against cell death and the survival and proliferation of cells expressing the healthy X chromosome. IP is classically considered a male-lethal disorder, and many women with IP have recurrent early miscarriages. Extrapolating from studies of *NEMO* knockout mouse lines, in utero lethality of hemizygous male fetuses is likely to be due to a lack of protection against apoptosis, particularly in the liver, where TNF levels are high (Rudolph et al. 2000). Surviving male patients with IP have not been described in families carrying either the common deletion or other mutations predicted to inactivate NEMO protein function. However, less deleterious (hypomorphic) mutations can give rise to surviving males. These patients present with a phenotype quite distinct from IP, which includes ectodermal dysplasia and immunodeficiency with or without lymphedema and osteopetrosis (Zonana et al. 2000; Aradhya et al. 2001; Doffinger et al. 2001; Jain et al. 2001; Mansour et al. 2001). Female carriers of these mild mutations may be asymptomatic or have clinical signs of IP. Male patients with a clinical presentation that resembles that of female patients with IP—that is, the characteristic four dermatological stages coupled with dental and ocular abnormalities—are rarely reported. Several such patients have been found to have a 47,XXY karyotype and Klinefelter syndrome. However, male patients with IP and a normal karyotype have also been described (reviewed by Scheuerle [1998]).

In this article, we describe four boys who presented with a clinical picture typical of female IP. In all four patients, the presence of the common *NEMO* deletion normally associated with male lethality is detectable. In one patient, a 47,XXY karyotype and skewed X inactivation provides protection against the morbid effects of the mutation. The other three karyotypically normal (46XY) patients acquired the *NEMOΔ4–10* deletion postzygotically and are somatic—and, potentially, germ cell—mosaics. Hypomorphic mutation, abnormal karyotypes, and mosaicism, therefore, provide three mechanisms for survival of males carrying a mutation at the IP locus.

## Methods

### Patient Samples

All patient samples and clinical details were obtained after consent and local ethical-committee approval.

### Fluorescence In Situ Hybridization (FISH)

A lymphoblast culture was prepared from a peripheral blood sample, and the cells were harvested for FISH. FISH was performed according to the manufacturer's suggestions (Vysis). The X and Y whole-chromosome paints were directly labeled in Spectrum Orange and Spectrum Green, respectively.

### X-Inactivation Analysis

DNA from peripheral blood was analyzed to determine X-inactivation status, using a method reported elsewhere (Parrish et al. 1996). In brief, 250 ng of genomic DNA from patients was digested overnight with the methylation-sensitive *Hpa*II enzyme or with a control enzyme, *Rsa*I. Fifty nanograms of the digest was used in a PCR to amplify the polymorphic $(CAG)_n$ repeat of the human androgen receptor (*HUMARA*) locus. The *Hpa*II restriction site is within the PCR amplicon and prevents amplification, if digested. Thus, only the methylated, inactive X chromosome is detected, and the polymorphism allows determination of which X chromosome is active or inactive.

### Detection of NEMOΔ4–10 by PCR and Southern Analysis

Detection, by PCR, of the *NEMOΔ4–10* rearrangement was performed using the protocol described elsewhere (The International Incontinentia Pigmenti Foundation 2000). Long-range PCR (EXPAND, Roche Molecular Biochemicals), with primers 3FH (GACCAGCTC-CCCTCCACTGTC) and JF3R (CTCGGAGACACAGG-AACCAGCA), produces a 2-kb product only if the rearrangement is present. The wild-type fragment of ~13 kb is seen in male or female samples that do not carry the mutation but is rarely seen in heterozygotes, since amplification of the 2-kb product predominates. A modified protocol that utilizes a forward primer closer to the boundary of rearrangement was used for detection of the rearrangement in patient 4, in whom DNA was refractory to amplification of the 2-kb product by 3FH and JF3R. The details of this method are in preparation and are available from the authors on request. For Southern analysis, 7 μg of DNA was digested with *Hin*dIII and was resolved on 0.7% agarose in 1 × Tris-borate/EDTA. DNA was transferred in 20 × saline sodium citrate to immobilon NY+ (Millipore) membrane and was UV cross linked at 40,000 joules with a Stratalinker (Stratagene). *NEMO* gene fragments were detected by hybridization to *NEMO* probes specific to exon 2 or exon 3 of the gene (GenBank accession number AJ271718). Exon 2 is not present in the pseudogene. Exons 2 and 3 are contained on a novel 8-kb *Hin*dIII fragment produced as a result of the *NEMOΔ4–10* rearrangement.

### PCR Detection of the Wild-Type NEMO Gene

For specific detection of the undeleted *NEMO* gene, amplification between intron 2 (GAG GAC CAA TAC CGA GCA TC) and exon 4 (ACC CTC CAG AGC CTG GCA TTC ) or between exon 2 (CCCTTGCCCTGTTG-

GATGAA) and intron 4 (AACCCTGGAAGGGGTCTC-CGGAG) was performed. Amplification was performed using system 3 of the long-range PCR EXPAND kit, with annealing at 65°C.

## Results

### Patient 1 (XL409-05, United States)

Patient 1 is a 1-year-old male child (fig. 1*a*) who developed, between the ages of 10 d and 4 wk, vesicles on the right arm, on the dorsum of each leg, and in the interdigital areas on the right hand and right foot. A skin biopsy confirmed marked dyskeratosis and eosinophilic infiltration consistent with IP. All lesions progressed to become papules, warty hyperkeratosis, and linear hyperpigmentation. A small area of alopecia was noted on the left parietotemporal area of the scalp. The other affected members of this family, XL409, are all female, and they demonstrated similar skin abnormalities. Southern blot analysis revealed that all affected individuals, including XL409-05, carried the *NEMO*Δ*4–10* male-lethal deletion associated with 83% of IP cases (indicated by the appearance of the 8-kb band in fig. 1*b*). Interestingly, XL409-05 also showed a normal band (12 kb) on this blot, suggesting that he had two X chromosomes. FISH confirmed a 47,XXY karyotype (fig. 1*c*). Finally, polymorphism analysis revealed that XL409-05 had complete skewing of X inactivation, similar to that in his affected female relatives (fig. 1*d*). The same polymorphism also indicated that the proband's extra X chromosome was inherited from his father and originated from a nondisjunction event during the first meiotic division in the paternal germline (fig. 1*d*).

### Patient 2 (IP114m, United Kingdom)

Patient 2, a boy with no family history of IP, had fluid-filled blisters at birth, on the ankles, chin, and in the groin, that were negative for bacteria or viruses. At age 1 mo the blisters had resolved, and he had developed white, plaque-like lesions on his arms, legs, and face (fig. 2). A skin biopsy showed massive epithelial cell proliferation, dyskeratotic cells, and infiltration of eosinophils, leading to a diagnosis of IP. Fibroblasts from this biopsy were used to determine a 46,XY karyotype. His hyperkeratotic lesions persisted for >1 year but became confined to the feet. Walking was delayed, because of the lesions on his soles. Hyperpigmentation was evident at age 1 mo and became extensive, following Blaschko's lines, particularly on the back and legs, over the next 5 years. This condition faded, but at age 9 years he has retained areas of hyperpigmentation and extensive scarring (fig. 2). He is severely hypodontic, with several mal-

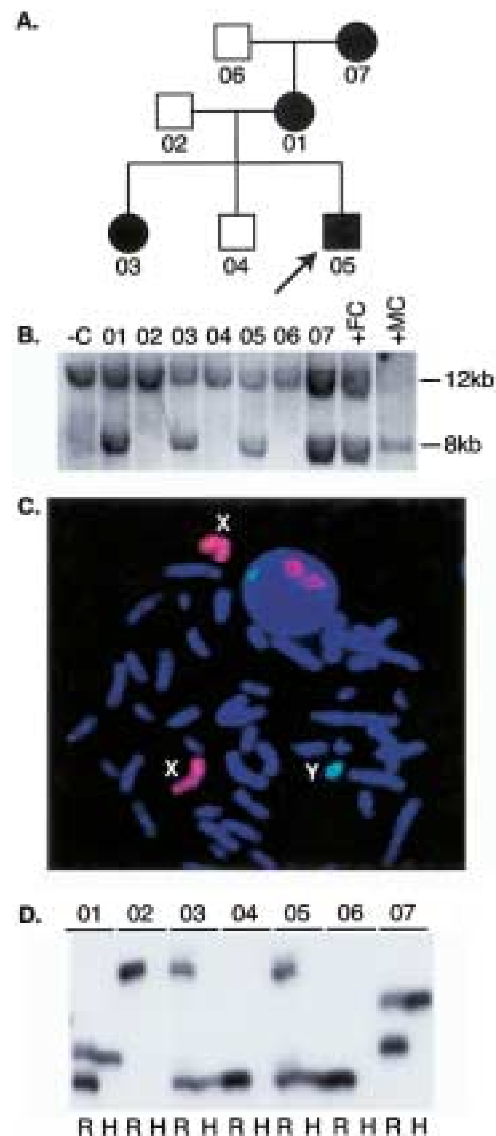


**Figure 1** Analysis of family XL409. *A,* Three-generation pedigree, with the arrow indicating the proband. *B,* Southern blot analysis showing the mutant 8-kb fragment in affected members and only a normal 12-kb band in unaffected individuals. The blot is a *Hin*dIII digest hybridized with a probe unique to exon 2 in the functional copy of *NEMO* (GenBank accession number AJ271718). An unaffected negative control (-C) is included on the blot, in addition to positive controls for the female patient with an IP deletion (+FC) and a spontaneous male abortus (+MC). *C,* Chromosome painting using X- and Y-specific probes, which confirms a 47,XXY karyotype in the proband. The X and Y whole-chromosome paints are directly labeled in Spectrum Orange and Spectrum Green, respectively (Vysis). *D,* X-inactivation analysis of DNA extracted from peripheral blood, using the *HUMARA* $(CAG)_n$ polymorphism, showing complete skewing in affected individuals. The assay detects the inactive X chromosome because it is methylated; two bands in the *Hpa*II (H) lanes reflect random X-inactivation, and a single band indicates complete skewing. The *Rsa*I (R) digest serves as a control.

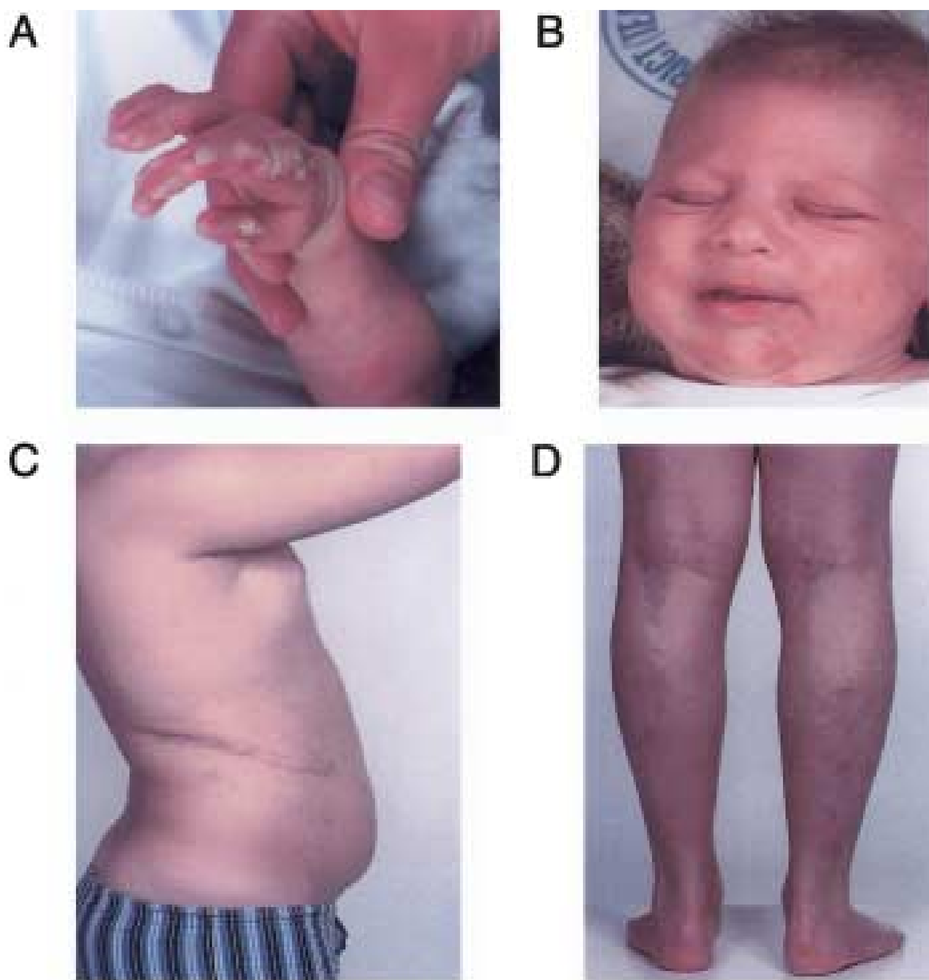


**Figure 2** Clinical presentation of IP114m. Blistering and plaque-like lesions on the hands (*A*) and face (*B*), at age 1 mo. Streaks of hyperpigmentation and scarring on the trunk (*C*) and legs (*D*) at age 10 years.

formed crowns, and he has sparse hair and eyebrows and some atrophic skin on his scalp. His neurological development is normal. At age 9 years, he presented with painless loss of vision in the left eye, which was due to spontaneous vitreous hemorrhage. His vision returned after 6 d, and fundoscopy revealed preretinal scarring, fibrosis in the temporal periphery, telangiectasia, and pigment epithelial mottling. Bilateral corneal opacity was also noted.

A PCR diagnostic for the *NEMO*Δ*4–10* rearrangement clearly showed the presence of this mutation in DNA extracted from the proband's blood (taken at age 9 years) and neonatal fibroblast line (fig. 3*A*) but not in DNA from his mother. Advantage was also taken of the fact that *NEMO*Δ*4–10* deletes exons 4–10 and the observation that the *NEMO* pseudogene does not contain exon 2. Through use of primers in exon 2 and in intron 4 of the *NEMO* gene, a 6.7-kb fragment specific to wild-type *NEMO* was obtained, confirming the presence of an intact *NEMO* gene in the patient (fig. 3*B*). Southern blot analysis of DNA from neonatal fibroblasts showed the 8-kb *Hind*III band representing the *NEMO*Δ*4–10* rearrangement to be present at approximately the same intensity as the wild-type 12-kb fragment (fig. 3*C*). Note that the lower band of the 12-kb doublet is part of the *NEMO* gene, and the upper band belongs to the X-linked homologous *NEMO* pseudogene that contains exons 3–10. Thus, IP114m is heterozygous—and, therefore, mosaic—for the *NEMO*Δ*4–10* mutation. Interestingly, the rearrangement was not detectable by Southern analysis of blood DNA. X chromosomes carrying the mutation are therefore present at a much lower level in the blood sample taken at age 9 years (detectable only by PCR), compared to level in the neonatal fibroblast line. This is consistent with the hypothesis that cells possessing the mutation undergo uncontrolled apoptosis.

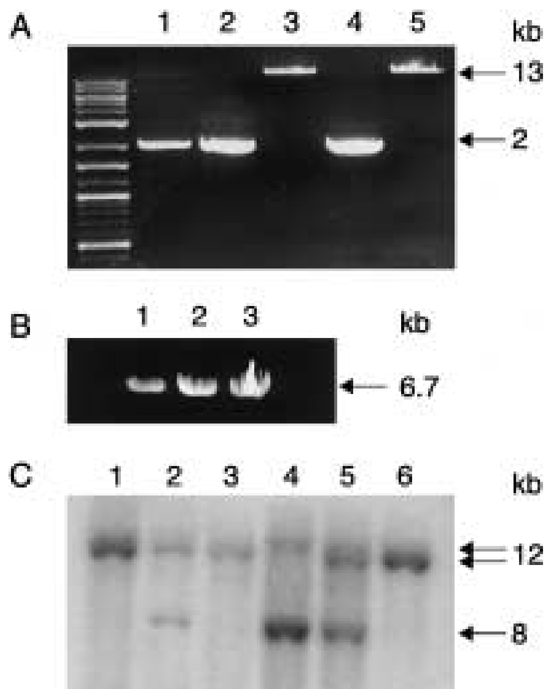


**Figure 3** *A,* PCR to detect *NEMOΔ4–10,* using primers that span the deletion breakpoint, as described in the Methods section. Template DNAs are as follows: *lane 1,* blood DNA from 9-year-old IP114m; *lane 2,* IP114m neonatal fibroblast line; *lane 3,* unaffected mother of IP114m; *lane 4,* a known female carrier of *NEMOΔ4–10;* and *lane 5,* a healthy female control. *B,* PCR detection of the wild-type NEMO gene, using primers in exon 2 and intron 4 to give a 6.7-kb band. *Lane 1,* IP114m; *lane 2,* mother of IP114m; and *lane 3,* healthy female control. *C,* Detection of *NEMO HindIII* fragments by Southern blot analysis. *NEMO* fragments are detected by hybridization to a probe encoding exon 3. *Lane 1,* blood DNA from 9-year-old IP114m; *lane 2,* IP114m neonatal fibroblast line; *lanes 3 and 6,* DNA from healthy female controls; *lane 4,* DNA from a spontaneous male abortus carrying only the *NEMOΔ4–10* allele; *lane 5,* DNA from a female IP carrier of the *NEMO* deletion. Note that there is a doublet at ∼12 kb, representing digestion products from the *NEMO* bona fide (*lower band*) and pseudo-genes (*upper band*). For the male subject in lane 4, who is hemizygous for the *NEMOΔ4–10* mutation, the lower 12-kb band, representing the wild-type gene, has disappeared, whereas IP114m (*lanes 1 and 2*) still has the wild-type allele and is therefore mosaic.

### *Patient 3 (IP-LN, Italy)*

Patient 3 was born after a normal pregnancy. Family history was negative for IP, skin and neurological disorders, and miscarriages. At birth, erythematous blisters were scattered over the body surface, particularly on the upper and lower extremities and on the back and axillae in a swirled pattern, according to the distribution of Blaschko's lines. His mother was carefully examined, and no dental, hair, or skin anomalies were seen. Mucosae, C-reactive protein, blood culture, and skin swabs were all normal, and skin treatment with local antibiotics was not successful. Complete blood counts showed eosinophilia of 790/$mm^3$. At 10 d, a skin biopsy suggested a diagnosis of IP. Subsequent cardiac, head, and abdominal ultrasound, electroencephalogram, chest X-rays, and eye examinations were normal, and his karyotype was 46,XY. At 30 d, several skin lesions resolved without scars, and some warty lesions, similar to those observed for case 1, were evident on the hands. At age 3 mo, the infant was feeding and growing well, neurological development was normal, and the warty lesions were no longer evident. At age 5 mo, linear yellowish streaks were present in those areas previously marked by vesicular lesions. At age 23 mo, the child is growing well, shows very light yellowish streaks on the skin, has a conic tooth in his mouth, and shows normal neurological development.

DNA from patient peripheral blood lymphocytes was analyzed by PCR for the presence of *NEMOΔ4–10* (The International Incontinentia Pigmenti Foundation 2000). DNA from the male patient yielded the specific 2-kb PCR product diagnostic for this mutation (fig. 4*A*), whereas his parents' DNA was negative for the *NEMOΔ4–10* rearrangement. To explain the survival of this patient, the presence of the normal X chromosome was confirmed. Through use of primers in intron 2 and in exon 4 of the *NEMO* gene, a 3.3-kb fragment specific for wild-type *NEMO* was obtained from peripheral blood DNA (fig. 4*b*). Moreover, in DNA from an Epstein-Barr virus–transformed lymphocyte cell line, only the normal X chromosome was detected. This suggests negative selection against the cells carrying the mutation in cell culture.

### *Patient 4 (IPTC, France)*

Patient 4 had no antecedents with IP but presented at birth with a vesicular rash on the trunk and limbs. Biopsy revealed typical IP histology, with hypereosinophilia but a normal 46,XY karyotype. He went on to develop areas of hyperpigmentation. At age 8 wk, he had epileptic episodes that were controlled by medication. A brain scan revealed an ischemic lesion. At age 8 mo, subsequent to a viral infection, he developed a second episode of blistering, with lesions located on the primary scars. At age 5 years, he is severely mentally retarded.

The presence of the common mutation in DNA from the proband's blood was not detectable when the previously described diagnostic assay was used, but it was confirmed using a new protocol diagnostic for the *NEMOΔ4–10* rearrangement. This yields a smaller product (1 kb) in the presence of the mutation (fig. 5). Even when this protocol was used, detection of *NEMOΔ4–10* required inclusion of 1–2 $\mu$g of DNA in the reaction. This suggests that the level of mosaicism

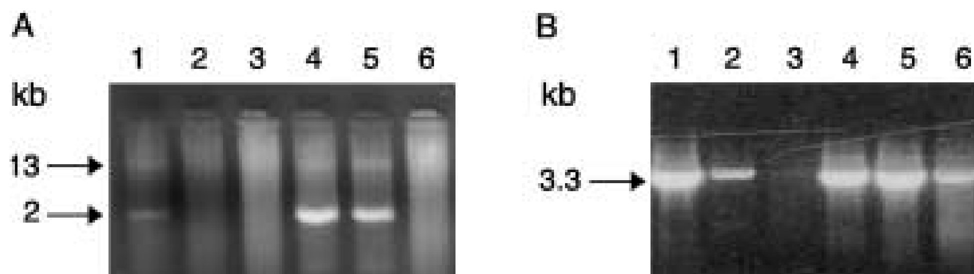


**Figure 4** *A,* Diagnostic PCR yields a 2-kb band in patients with IP and a 13-kb band in controls. DNA templates are as follows: ***lane 1,*** patient peripheral blood lymphocytes; ***lane 2,*** patient immortalized lymphocyte cell line; ***lane 3,*** mother's lymphocytes; ***lanes 4 and 5,*** female patients with IP; ***lane 6,*** healthy sample. *B,* Amplification between the intron-2 forward primer and a primer from exon 4, giving a band at 3.3 kb only from wild-type *NEMO.* DNAs are from patient's immortalized lymphocyte cell line (***lane 1***), patient's peripheral blood lymphocytes (***lane 2***), healthy sample (***lane 3***), mother's DNA (***lane 4***), and female patients with IP (***lanes 5 and 6***).

in the blood of this patient was low. The available DNA was insufficient for Southern analysis.

## Discussion

Although IP is classified as a male-lethal X-linked dominant disorder, a small number of surviving male patients have been reported, some within families with typically affected female patients (Scheuerle 1998). A 47,XXY karyotype is one mechanism by which males may survive the effects of inheriting a lethal IP mutation, since this establishes a heterozygous genotype that is compatible with survival. Several cases of IP have been reported in 47,XXY patients (Kunze et al. 1977; Ormerod et al. 1987; Prendiville et al. 1989; Garcia-Dorado et al. 1990; Fowell et al. 1992; Kirchman et al. 1995; Scheuerle 1998). Here we provide the first molecular confirmation that IP in these cases can result from inheritance of a mutation in *NEMO,* and we provide the first description of skewed X inactivation in a 47,XXY male patient. In this patient, only the paternal X is active in most cells, since the maternal X carries the IP mutation. If the X chromosome carries imprinted genes, this may have a bearing on the eventual Klinefelter syndrome–associated phenotype in this patient, since the skewed X inactivation leads to expression from the same X chromosome in all of his affected cells.

Three surviving 46,XY male patients possessing a *NEMO* mutation as well as a wild-type gene had typical sequential IP skin stages. Interestingly, in all three cases the postzygotic mutation is the common deletion, *NEMO*Δ*4–10,* caused by rearrangement between tandem repeats in intron 3 and downstream of the *NEMO* gene (The International Incontinentia Pigmenti Foundation 2000). A predominantly paternal origin for this mutation in families with IP (The International Incontinentia Pigmenti Foundation 2000; authors' unpublished observations) suggests that the mutation occurs primarily through intrachromosomal exchange rather than through misalignment of repeats during meiosis (a copy of *NEMO* is not found on the Y chromosome). The finding that this mutation is not restricted to meiotic divisions but can occur in mitotic cells supports this conclusion. Furthermore, the mutational mechanism is unlikely to involve half-chromatid (single DNA strand) exchange, as proposed by Lenz (1975) to explain IP in male subjects, since recombination between repeats requires the interaction of DNA duplexes (whole chromatid). Interestingly, postzygotic inversion between repeat sequences in a female carrier of a *factor VIII* mutation has also recently been described (Oldenburg et al. 2000).

Postzygotic mutation and somatic mosaicism is becoming increasingly recognized as a mechanism for explaining unusual haplotype segregation or the phenotype of male subjects carrying X-linked dominant mutations. For example, somatic mosaicism was recently reported for surviving male patients carrying mutations in the genes for the X-linked dominant diseases

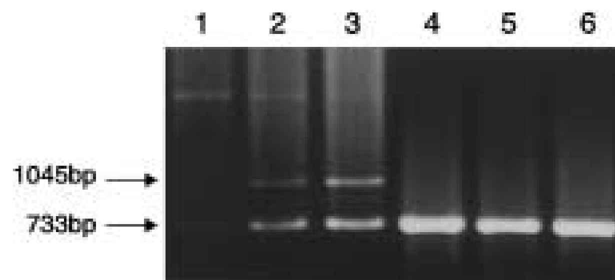


**Figure 5** Presence of *NEMO*Δ*4–10* in DNA from the blood of patient 4, using a new protocol that yields a 1.04-kb product in the presence of the mutation. Lanes 1–3 contain the products obtained from using 500–2,000 ng of template DNA. Lanes 4–6 show absence of the mutation-specific band in samples from healthy subjects. The lower 733-bp band is an internal control for DNA quality.

Rett syndrome and Conradi-Hünermann-Happle syndrome (CDPX2) (Clayton-Smith et al. 2000; Has et al. 2000). Interestingly, both CDPX2 and IP have been described as displaying "anticipation," a term used to describe an increase in disease severity with the number of generations (Carney 1976; Traupe et al. 1992). Somatic and gonadal mosaicism in individuals at the top of pedigree provides an explanation for these observations. Moreover, given that the mutational mechanism giving rise to most cases of IP is repeat-mediated deletion, mosaicism is likely to be quite a common occurrence and to account for some of the extremely mild presentation in some female IP obligate mutation carriers (Woffendin et al. 1999). Somatic and/or germ line mosaicism in parents of patients with apparently sporadic IP should therefore always be considered when investigating families with IP.

In summary, karyotypic analysis and examination of the *NEMO* gene are, therefore, strategies for the investigation of male patients with IP symptoms. Since the *NEMO*Δ*4–10* rearrangement is the most likely mutation, this should be sought initially. Furthermore, the subsequent selection against mutation-bearing cells indicates that, where possible, neonatal DNA samples from blood as well as from affected tissue should be used for DNA analysis.

The boys described in this report presented with florid signs of IP, probably because the mutational event occurred early in development. It is likely that, depending on the timing and location of somatic rearrangement of *NEMO,* male patients with very localized skin lesions will also exist. Since this article was prepared, three additional male carriers of *NEMO*Δ*4–10* with clinical signs of IP have been identified through diagnostic screening (J. Whittaker, unpublished data).

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *NEMO* [accession number AJ271718])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for IP [MIM 308310])

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