In the *Hox-1.5* and *-1.6* mutants there was no evidence for transformations of structures, in contrast to the transformations of cervical vertebrae seen when *Hox-1.1* is ectopically expressed in somites[24]. Because the development of the head involves interactions between rhombomeres, neural crest, surface ectoderm, paraxial mesoderm and pharyngeal endoderm, the alterations of *Hox* expression in a subset of these might not generate a simple transformation, particularly as the role of *Hox* genes in patterning the mesoderm and endoderm is not clear. Furthermore, removal of one *Hox* gene may alter expression of multiple *Hox* genes and other components of the head specification network.

The *Hox* code of the branchial region is different from that of the trunk, where anterior boundaries of subfamily members can be offset from each other. There are many morphological grounds for believing that the head and trunk have distinct developmental mechanisms[1,18,28,29], which we believe has resulted in the use of the same genes in different ways in the two contexts. *Antennapedia* class *Hox* genes are not expressed in more anterior parts of the head, which must therefore employ other genes[30–32] and patterning mechanisms[28]. In an analogous way the patterning of anterior parts of the head in *Drosophila* is also thought to involve molecular mechanisms independent of *Antennapedia* class genes[33,34]. □

Received 1 August; accepted 26 September 1991.

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ACKNOWLEDGEMENTS. We would like to thank T. Lufkin and P. Chambon for sharing data before publication on the *Hox 1.6* mutants, D. Duboule for the gift of mouse *Hox 1* probes, P. Sharpe for information on the lack of paralogues in the mouse *Hox 3* complex, D. Noden, P. Thorogood and A. Lumsden for discussions on head development, members of the Boncinelli lab for sharing information on human *HOX* clusters, L. Stubbs for chromosomal localization of *Hox 4.9* and J. Brock for graphic illustration. This work was funded in part by the UKMRC, the 3rd AIDS project and the Italian Association for Cancer Research. P.H. is in receipt of an MRC studentship.

A *de novo Alu* insertion results in neurofibromatosis type 1

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NEUROFIBROMATOSIS type 1 (NF1) is a common autosomal dominant disorder with a high mutation rate and variable expression, characterized by neurofibromas, *café-au-lait* spots, Lisch nodules of the iris, and less frequent features including bone deformities and learning disabilities[1]. The recently cloned *NF1* gene encodes a transcript of 13 kilobases from a ubiquitously expressed locus on chromosome 17 (refs. 2–4). Most NF1 patients are expected to have unique mutations, but only a few have so far been characterized, restricting genetic and functional information and the design of DNA diagnostics. We report an unusual *NF1* mutation, that of a *de novo Alu* repetitive element insertion into an intron, which results in deletion of the downstream exon during splicing and consequently shifts the reading frame. This previously undescribed mechanism of mutation indicates that *Alu* retrotransposition is an ongoing process in the human germ line.

The 31-year-old male patient (D.D.) exhibits several features of NF1, including one cutaneous neurofibroma, axillary freckling, Lisch nodules, cervical nerve root tumours, and macrocephaly. *Café-au-lait* spots are not present. His parents show no signs of NF1, and DNA fingerprinting analysis found no evidence of nonpaternity. Part of the *NF1* complementary DNA detected an abnormal Southern blot pattern in the patient's DNA after digestion with several restriction enzymes[2]. This was consistent with a small insertion (300–500 basepairs (bp)) in a 3.8-kilobase (kb) *Eco*RI fragment which contains six *NF1* exons

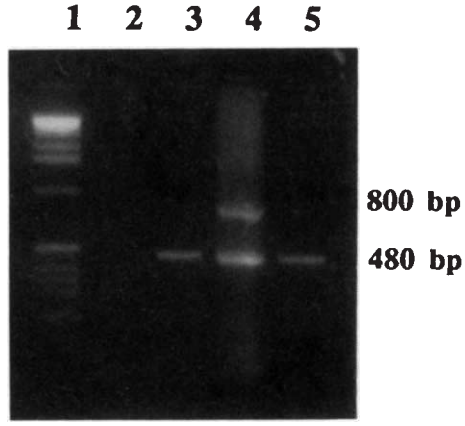

FIG. 1 Ethidium bromide staining of a 1.0% agarose gel demonstrates the insertion in the exon 6 PCR product. Lane 1 contains the BRL 1-kb ladder, lane 2 contains a water (negative) control, lanes 3 and 5 are products from the patient's father and mother, respectively. and the patient's PCR products are shown in lane 4. All show the normal fragment of 480 bp, but the patient also has an abnormal fragment of ~800 bp. DNA from both the patient's leukocytes and from an established lymphoblastoid line gave the same result (data not shown).

METHODS. Genomic DNA from the patient and his parents was extracted as described[2]. Genomic DNA (100–500 ng) was amplified using the exon 6 primers already described[3], with 35 cycles (each cycle entailed 1 min each at 94°C for denaturation, 65°C for annealing, and 72°C for extension) using standard buffers and reagents recommended by Cetus. One-tenth of each PCR reaction was loaded per lane.

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  1  GGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCGGGCGGA
 61  TCACGAGGTCAAGAGATCGAGACCATCCCGGCTAAAACGGTGAAACCCCGTCTCTACTAA
121  AAATACAAAAAAATTAGCCGGGCGTAGTGGCGGGCGCCTGTAGTCCCAGCTACTTGGGAG
181  GCTGAGGCAGGAGAATGGCGTGAACCCGGGAGGCGGAGCTTGCAGTGAGCCGAGATCCCG
241  CCACTGCACTCCAGCCTGGGCGACAGAGCGAGACTCCGTCTC(A)n
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FIG. 2 Sequence of the *Alu* insertion in the exon-6 PCR product from NF1 patient D.D. The entire sequence of the PCR product was obtained and the exon and other intron sequences were found to be normal, with the exception of the *Alu* insertion. This *Alu* sequence is identical to the PV or HS-1 *Alu* subfamily consensus[12-14], with the exception of the two bases underlined (a substitution of A for G at position 72, and an additional A in the string of As at position 128). A poly(A) tract over 40 nucleotides long was found at the 3′ end.
METHODS. The PCR product was cloned into the *Bam*HI site of Bluescript I (KS-) at cloning sites built into the PCR primers, and transformed into BRL DH5-α cells as recommended by the supplier. Plasmid DNA from two independent clones was extracted[18] and sequenced on both strands by direct sequencing of plasmid DNA (Sequenase, US Biochemicals).

in the 3′ third of the *NF1* coding region[3]. These six exons were individually examined by polymerase chain reaction (PCR) amplification of the patient's DNA, using primers derived from surrounding introns[3]. One exon, termed number 6 in current nomenclature[3], showed a pattern consistent with a 320-bp insertion (Fig. 1, lane 4); the remaining exons were normal. The parents' DNA showed only the normal pattern for exon 6 (Fig. 1, lanes 3 and 5). Analysis of 50 other NF1 patients failed to show this abnormality. The abnormal PCR product was cloned and sequenced; it consisted of the normal exon 6 sequence with an *Alu* repetitive element inserted 44 bp upstream of the exon. Sequencing of this element (Fig. 2) showed that the *Alu* is inserted in the opposite orientation of the *NF1* gene, that a substantial poly(A) tract is present, and that the *Alu* is flanked by 3–13-bp direct repeats (exact length was indeterminate owing to the poly(A) tract) (Fig. 3). The site of insertion was immediately adjacent (upstream) to an A/T stretch of 26 bp, consistent with the proposed sites preferred for *Alu* integration[5].

As the exonic sequence is undisturbed, we thought that the *Alu* insertion might disrupt normal splicing of the transcript. It was suspected that exon 6 might be lost during splicing, because the insertion was closest to this exon and could interfere with branch-point recognition. A PCR experiment was designed to examine the patient's *NF1* RNA from exons 5 through to 7 (Fig. 3). The normal product, from the beginning of exon 5 through to the middle of exon 7, was expected to be 515 bp long, whereas a corresponding product lacking exon 6 would be 235 bp. RNA PCR consistently gave products of both lengths in the patient's RNA; normal controls showed only the normal band (Fig. 4*a*). Both products hybridized with exon 5 (Fig. 4*b*), and exon 6 hybridized with only the normal 515-bp fragment (Fig. 4*c*). The 235-bp product was cloned and sequenced and found to contain the sequences expected from exons 5 and 7 precisely spliced together (data not shown). This results in a shift in the translational frame when the exon 7 sequence is encountered, with a predicted subsequent premature truncation of the NF1 protein. Thus, the patient's *NF1* allele containing the *Alu* insertion should produce an NF1 protein that lacks the portion encoded by exon 6 and terminates within the exon 7-encoded region. This would result in a protein missing the C-terminal 771 amino acids of the predicted 2,818 for the entire NF1 protein[6].

Two somatic cell hybrids containing only the mutant *NF1* allele were constructed on a hamster cell background. RNA PCR detected the abnormal band in the hybrids, with no evidence for production of the normal allele from the mutant gene by hybridization (data not shown). In addition, Southern blot analysis of hybrid and parental DNA using the polymorphic probe THH59 (17q23–25.3) showed that the allele receiving the *Alu* insertion was paternal in origin (data not shown). This agrees with a previous genetic analysis which indicates a predominance of paternal origin in new-mutation NF1 cases[7].

To our knowledge, this is the first report of a disease-causing mutation consisting of a *de novo Alu* insertion: *Alu* elements

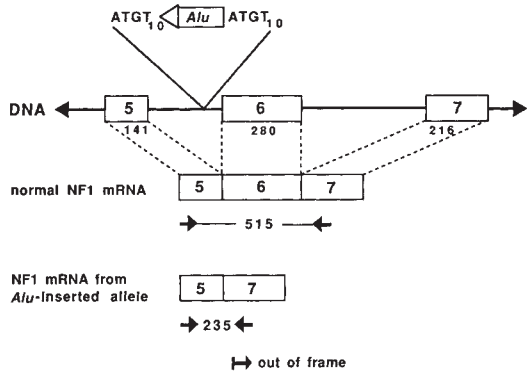

FIG. 3 Schematic of the *Alu* insertion and experimental design for RNA PCR analysis. The *Alu* resides in the intron in the opposite orientation from the *NF1* gene, flanked by direct repeats, 44 bp upstream of exon 6. PCR primers in exons 5 and 7 were chosen to analyse whether exon 6 is present in all of the patient's *NF1* transcripts, or whether the *Alu* insertion causes exon 6 to be lost during splicing of the mutant allele.

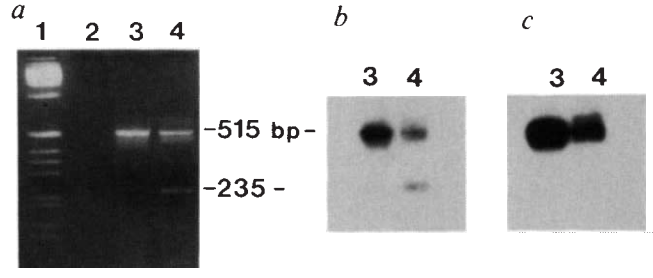

FIG. 4 *a*, Ethidium bromide staining of PCR products from RNA PCR analysis. Lane 1 contains the BRL 1-kb ladder; lane 2, a water control; lane 3, a normal lymphoblastoid control; and lane 4 shows the products from the patient's analysis. The normal product is 515 bp, and the abnormally small band in the patient's lane is 235 bp. (Other non-NF1 RNA sources analysed showed only the normal fragment (data not shown).) Hybridization with exon 5 (*b*) indicates that the 235-bp fragment is a product from the *NF1* gene including exon 5. But the 235-bp fragment fails to hybridize to the exon 6 probe (*c*), indicating that exon 6 is lost during splicing.
METHODS. RNA extracted from lymphoblastoid cell lines[18] was reverse-transcribed with oligod(T)[19]. The PCR consisted of 35 cycles of 94 °C denaturation, 65 °C annealing, and 72 °C extension under conditions recommended by Cetus. The exon-5 primer sequence used was 5′-CAGATCTGCTTGATGTTGTACTAAG-3, and the exon-7 primer sequence was 5′-TGACAGCAGCTGACTTGACTTTGC-3′. The gel was transferred to two membranes (bidirectionally) with standard Southern blotting methods. The filter in *b* was hybridized to a radiolabelled exon-5 PCR product, and the other membrane (*c*) was hybridized to a radiolabelled exon-6 PCR product, using standard random priming, hybridization and wash conditions[2].

have been involved in the generation of disease mutations by recombination[8-10] or point mutation[11], but not as new element. The mechanism of the splicing effect of the inserted element is unclear, but exon skipping is indicative of a defect in branch-point recognition.

Comparison of this *Alu* sequence with known subsets of the *Alu* family revealed that this particular element most closely matches the subfamily known as PV (refs 12, 13) or HS-1 (ref. 14), with the differences noted in the Fig. 2 legend. This subfamily is considered the most recently inserted *Alu* subgroup, many members of which are polymorphic in the human population[15]. Consistent with recent origin is the finding that this subfamily is transcriptionally active[12]. The close agreement of the *de novo* *Alu* element sequence reported here with this subfamily strongly supports the concept of one or a few 'master' *Alu* elements capable of providing new retrotransposons[13,15-17]. The presence of the long poly(A) tract (a presumed remnant from an RNA intermediate) and the direct repeats (which presumably arose during integration), as well as the *de novo* appearance of this *Alu*, indicate that it probably arose by retrotransposition in the father's germline. The possibility of insertion into the paternal chromosome during early embryogenesis must also be considered, though no evidence for mosaicism was encountered. If *Alu* retrotransposition is an ongoing process in human biology, it is likely that there will be other examples of this mechanism of mutation in human genetic disease. □

Received 19 August; accepted 10 September 1991.

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ACKNOWLEDGEMENTS. We thank A. Brereton, D. Wood and B. Hall for technical assistance, and G. Matera for critically reading the manuscript. This work was supported by the National Neurofibromatosis Foundation and the NIH. F.S.C. is an investigator of the Howard Hughes Medical Institute.

Complementation of transforming domains in E1a/*myc* chimaeras

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THE *myc* oncogene is functionally similar to adenovirus E1a in its ability to collaborate with activated *ras* oncogenes to transform primary fibroblasts[1,2]. The transforming functions of E1a and *myc* have been mapped to two distinct regions in each protein[3,4]. I investigated the functional similarities between E1a and *myc* by constructing E1a/*myc* chimaeras to discover whether the individual transforming domains of E1a could complement individual *myc*-transforming domains. Transformation assays in rat embryo fibroblasts demonstrated that the N-terminal transforming domain of E1a (CR1; ref. 5) could complement the C-terminal transforming domain of *myc* in *cis*, and that the reciprocal chimaera (N-terminal *myc*/C-terminal E1a) was also active. Chimaeras constructed using domains from transformation-defective mutants of either E1a or *myc* were inactive, indicating that both E1a and *myc* domains contribute to function. These experiments suggest that transformation by *myc* and E1a may involve interactions with common substrates.

To test complementation of the v-*myc* C-terminal domain by E1a, I constructed a series of chimaeras (Fig. 1*a*) and tested their ability to transform rat embryo fibroblasts (REFs) in collaboration with EJ-*ras*. These experiments showed that E1a/v-*myc* chimaeras containing CR1 could fully transform REFs to tumorigenesis in syngeneic rats (Table 1*a*). This observation was explored further by construction of chimaeras using a series of deletion mutants in E1a. As summarized in Table 1*b*, the chimaera EMCCΔ37-51, which lacks the portion of CR1 with homology to human papilloma virus-16 (HPV-16) protein E7 (ref. 6), was inactive. Complementation in *trans* (ref. 7) was not observed (Table 1*b*), although this could have been due to inefficiency in either cotransfection or complementation.

To determine whether a functional *myc* C-terminal domain was required for complementation of CR1, I constructed E1a/c-*myc* chimaeras using the transformation-defective c-*myc* mutant IN-373 (ref. 4). The E1a/IN-373 chimaera was transformation-defective, whereas the E1a/c-*myc* chimaera was competent (Table 1*c*). It seemed possible that if the activity of the E1a/*myc* chimaeras arose through functional similarities between E1a and *myc*, then the reciprocal chimaera (N-terminal *myc*/C-terminal E1a) also should be active. Two v-*myc*/E1a recom-

TABLE 1 E1a N-terminal domains containing CR1 can complement the *myc* C-terminal domain

	Oncogenes	Foci per plate (experiments 1, 2, 3)	Growth in soft agar	Tumours in rats
(*a*)	EJ-*ras* +13EMXC	60	+	NT
	EJ-*ras* +12EMXC	120	+	NT
	EJ-*RAS* +EMCA	22	+	2/2
	EJ-*ras* +EMCC	16, 11	+	2/2
	EJ-ras +EMNC	10	+	2/2
	EJ-*ras* +LTR-*myc*	27, 22, 42	+	2/2
	EJ-*ras* +E1a-243	148	+	2/2
	EJ-*ras* +E1a-289	140	+	2/2
	EJ-ras alone	0, 0, 0	−	NT
(*b*)	EJ-*ras* +MLV-EMCC	57, 46, 70	NT	NT
	EJ-*ras* +MLV-EMCCΔ37-51	0, 0	NT	NT
	EJ-*ras* +MLV-EMCCΔ54-72	22, 31	NT	NT
	EJ-*ras* +MLV-EMCCΔ73-82	108	NT	NT
	EJ-*ras* +MLV-EMCCΔ58-81	104	NT	NT
	EJ-*ras* +MLV-EMCCΔ37-51 +E1aΔ120-139	0, 0	NT	NT
	EJ-*ras* alone	0, 0, 0	NT	NT
(*c*)	EJ-*ras* +SV-LTR-*myc*	42	+	NT
	EJ-*ras* +SV-LTR-IN373	0	−*	NT
	EJ-*ras* +SV-EhMCC	23	+	NT
	EJ-*ras* +SV-EM(IN373)	0	−	NT
	EJ-*ras* alone	0	−	NT

a, Constructs shown in Fig. 1*a* were tested for their ability to transform REFs in collaboration with EJ-*ras*. Focus formation was assayed as described[1]. Average number of foci per plate is shown (four plates per experiment). Results of repeated experiments also are shown. Where indicated, transformation was characterized further by picking foci and assaying their ability to grow in soft agar and to produce tumours in syngeneic rats. Soft agar colonies were produced by seeding about 10^4 cells into 0.35% agar. Transformed cells were cloned from soft agar colonies and used to test tumorigenesis in syngeneic rats by injecting 10^7 cells subcutaneously. Tumours of 2 cm typically were produced within 1 week. *b*, EMCC deletion mutants are shown in Fig. 1*b*. E1aΔ120-139 was constructed by ligation of blunted *Cla*I and *Fok*I sites on pJN20 (ref. 20). *c*, To test the contribution of the *myc* C-terminal domain to transformation by EMCC, the chimaera EM(IN373) was constructed using the human c-*myc* mutant IN-373 which contains a 4-serine-codon insertion at position 373 (ref. 4). The E1a/c-*myc* chimaera EhMCC, containing the wild-type human c-*myc* C-terminal domain was used as a control. The expression vector used for these studies contained the SV40 enhancer and origin of replication. Expression of c-*myc* and IN-373 was driven by a Mo-MLV long terminal repeat. EMCC and EM(IN373) were expressed from the E1a promoter. NT, Not tested.
* One small colony observed which could not be established in culture.