MOLECULAR AND CELLULAR BIOLOGY, Oct. 1984, p. 1961–1969
0270-7306/84/101961-09$02.00/0

Vol. 4, No. 10

Molecular Cloning and Characterization of Mutant and Wild-Type Human β-Actin Genes

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Received 23 April 1984/Accepted 19 June 1984

There are more than 20 β-actin-specific sequences in the human genome, many of which are pseudogenes. To facilitate the isolation of potentially functional β-actin genes, we used the new method of B. Seed (Nucleic Acids Res. 11:2427–2446, 1983) for selecting genomic clones by homologous recombination. A derivative of the πVX miniplasmid, πAN7β1, was constructed by insertion of the 600-base-pair 3′ untranslated region of the β-actin mRNA expressed in human fibroblasts. Five clones containing β-actin sequences were selected from an amplified human fetal gene library by homologous recombination between library phage and the miniplasmid. One of these clones contained a complete β-actin gene with a coding sequence identical to that determined for the mRNA of human fibroblasts. A DNA fragment consisting of mostly intervening sequences from this gene was then used to identify 13 independent recombinant copies of the analogous gene from two specially constructed gene libraries, each containing one of the two types of mutant β-actin genes found in a line of neoplastic human fibroblasts. The amino acid and nucleotide sequences encoded by the unmutated gene predict that a guanine-to-adenine transition is responsible for the glycine-to-aspartic acid mutation at codon 244 and would also result in the loss of a *Hae*III site. Detection of this *Hae*III polymorphism among the fibroblast-derived clones verified the identity of the β-actin gene expressed in human fibroblasts.

Two actin isoforms, β and γ, are coexpressed in dividing nonmuscle cells and undifferentiated myoblasts (26–28, 30). These two nearly identical polypeptides polymerize into microfilaments which form highly polymorphic aggregates (bundles and cables) within the soluble and cytoskeletal portions of the cytoplasm (5, 21). Although the specific structural and functional roles of cytoskeletal actins have not been determined, it seems certain that polymeric actin is a major determinant of cytoarchitecture, cytoskeletal rearrangement, movement of organelles, and cell motility.

Electrophoretic variants of β-actin have been found in neoplastically transformed human and mouse fibroblasts (2, 13–15, 26). These variants appear to have arisen as a consequence of somatic mutations in the structural β-actin gene (13, 26). Two human variants were induced or selected in succession after mutagenesis in a fibroblast cell culture (13, 15), and their expression was accompanied by enhanced tumorigenic potential (13). The first of the two mutations was presumably induced by the carcinogen 4-nitroquinolin-1-oxide (11) and has been defined as an exchange of glycine for aspartic acid at residue 244 in the polypeptide (26). Our knowledge of the nucleotide sequence for the mRNA derived from a β-actin cDNA clone (23) and the amino acid exchange that occurred (26) predicts that a guanine-to-adenine transition has occurred in the second base of the codon, which in turn should result in the loss of a *Hae*III restriction site spanning that codon. A mutation at a different site, superimposed upon the same mutant gene, is predicted by the occurrence of a second charge alteration of the mutant β-actin found in a subclonal derivative (HUT-14T) of the original mutant cell line HUT-14 (13).

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To define both mutations and assess their role in the changing phenotypes of the human fibroblast transformants, we cloned the expressed β-actin gene of human fibroblasts and copies of mutant and wild-type genes from the HUT-14 and HUT-14T cell lines. The cloning and identification of these genes were complicated by the presence of a large multi-pseudogene subfamily for β-actin (22). A 600-base-pair (bp) sequence which is the 3′ untranslated region (3′ UTR) of the β-actin mRNA, although β-actin specific in sequence (22), did not distinguish the expressed gene from many of the pseudogenes in hybridization and duplex melting experiments (22). We therefore turned to a method of cloning (25) that involves homologous recombinaiton between λ phage containing β-actin genomic sequences and the 3′ UTR sequence of the β-actin cDNA carried by a small πVX-like plasmid (25), πAN7 (H. Huang, personal communication). This method preferentially selected, by recombination, the clones with the least divergent sequence, i.e., the functional gene and closely related pseudogenes.

MATERIALS AND METHODS

General methods. Growth and transformation of *Escherichia coli*, colony hybridization (8), and purification of plasmid DNA were done as described previously (3). Preparation of Charon 4A and λgtWES phage recombinant DNA, agarose gels, and hybridization blots and the conditions used for hybridization were as described previously (22). Genomic DNA preparation from mammalian cells, DNA digestion with restriction enzymes, and hybridizations on nitrocellulose blots with dextran sulfate were done as described by Ponte et al. (24). The human cell strains were grown and maintained as previously described (15).

Construction of the KD, HUT-14, and HUT-14T human gene libraries. Purified λ Charon 4A (1) vector DNA (*Eco*RI arms), λgtWES.λB′ (16) vector DNA (full-length phage

genome), and packaging extracts prepared from *E. coli* BHB2688 and BHB2690 were purchased from Amersham Corp., Arlington Heights, Ill. Two size classes of fully or partially *Eco*RI-digested fragments from genomic DNA, 2 to 14 or 10 to 23 kilobases (kb), were purified from 0.5% agarose gels [Seakem HGT(P)] by adsorption to glass powder (31). The *Eco*RI DNA fragments 2 to 14 kb long were ligated to λgtWES DNA arms that were generated by *Eco*RI and *Sac*I digestion of λgtWES.λB′ DNA (16). The *Eco*RI fragments (full or partial digests) 10 or 12 to 23 kb long were ligated into λ Charon 4A *Eco*RI arms. The ligation reaction mixture consisted of one part human insert DNA and three parts vector DNA, 66 mM Tris-hydrochloride (pH 7.4), 5 mM $MgCl_2$, 1 mM ATP, 5 mM dithiothreitol, 100 μg of bovine serum albumin (fraction 5), and T4 ligase (a gift from R. Simoni, Stanford University). Ligation reactions (13°C, overnight) were always tested for completion by agarose gel analysis of samples taken at the beginning and end of the reaction. A 4-μl sample of the ligation reaction products was mixed with the two packaging extracts, and phage assembly was allowed to occur for 2 h at room temperature. The packaging reaction mixtures were then diluted with 0.5 ml of phage dilution buffer (10 mM Tris-hydrochloride [pH 7.4], 10 mM $MgSO_4$, 0.01% gelatin), followed immediately by 10 μl of chloroform, and stored at 4°C. Packaging titers were determined by infection of *E. coli* LE392.

Construction of the πAN7β1 miniplasmid. A 600-bp *Eco*RI-*Bam*HI fragment of the cDNA (β-actin 3′ UTR sequence) insert in pHFβA-3′ UT (22) was purified by gel electrophoresis and adsorption to glass powder (31) and then ligated to the *Eco*RI-*Bam*HI large fragment (alkaline phosphatase treated) of plasmid πAN7. *E. coli* W3110(p3) was transformed with the ligation mixture (4), and plasmid DNA from individual ampicillin-resistant (Amp^r) and tetracycline-resistant (Tet^r) colonies was amplified. The structure of πAN7β1 (see Fig. 1) was confirmed by restriction analysis and DNA blotting experiments.

Selection of πAN7β1 recombinant λ phage. We first performed a recombination screen (26, 4) to isolate phage containing DNA homologous to the 3′ UTR sequence miniplasmid πAN7β1 from a highly amplified gene library (17) prepared by ligating partial *Eco*RI digests of DNA derived from a human fetus to the Charon 4A vector. Phage stocks were prepared by infecting bacteria carrying πAN7β1 with 10^6 PFU of the Charon 4A library (17). Phage able to form plaques on *E. coli* W3110 (Su^-) bacteria were present in the lysate at frequencies between 10^{-7} and 10^{-9} (Table 1). The presence of actin coding sequences as well as the 3′ UTR and plasmid vector sequence in these rare clones was confirmed by blotting experiments on Southern transfers of restriction endonuclease-digested DNA isolated after propagation of phage from individual plaques.

TABLE 2. Components of the human HUT-14 and HUT-14T gene libraries

Source of human DNA	Vector	Size range (kb) of *Eco*RI fragments cloned	Human haploid genome equivalents	Recovery of clones with β-actin sequences (no. per 10^5 phage recombinants)
HUT-14	λgtWES	2–14	3.8	8.3[a]
HUT-14T	λgtWES	2–14	9.0	7.1[a]
HUT-14	Charon 4A	12–23	30.0[b]	4.0[a]
HUT-14T	Charon 4A	10–23	30.0[b]	5.0[a]
HUT-14T	Charon 4A	10–23[c]	0.6	5.9[d]

[a] Selected by πAN7β1 recombination.
[b] Estimated from the frequency of recovery of the β-actin gene.
[c] Partial digest fragments.
[d] Selected by in situ plaque hybridization with the 3′ UTR probe.

Recombination screens were then performed as described above on unamplified phage in packaging reactions that were generated by ligating *Eco*RI-digested HUT-14 and HUT-14T DNA ligated to the λgtWES vector arms (16) and phage packaging reactions that were generated by ligating *Eco*RI-digested KD, HUT-14, and HUT-14T DNA ligated to the Charon 4A vector arms. The frequencies of recovery, by recombination selection, of library phage clones that contained the β-actin sequences are shown in Table 2.

RESULTS

The 600-bp 3′ UTR, which was derived from cDNA of the β-actin mRNA, hybridizes specifically to mRNA which codes for β-actin, but not to mRNA which codes for γ-actin or muscle-specific α-actins (22). Also, as expected, we established by mRNA selection-translation experiments that this sequence hybridized to mRNA coding for the mutant β-actin (data not shown). However, Southern blotting experiments with this probe do not distinguish the expressed (i.e., functional) β-actin gene from other members of its numerous pseudogene subfamily (22). Although it can be assumed that the 3′ UTR sequences of the β-actin pseudogenes have diverged from those of the expressed gene, thermal elution experiments with hybrids of *Eco*RI-digested genomic DNA did not allow us to distinguish candidate β-actin gene sequences by their higher T_m (22). We therefore attempted to select library phage by homologous recombination between β-actin gene sequences in recombinant phage DNA and the 3′ UTR incorporated into a πVX-like miniplasmid (25). Our

TABLE 1. Selection of phage clones containing actin sequences by πAN7β1 recombination

Recombination trial	Clone	Frequency of recovery	Genomic *Eco*RI coding fragment[a]				Other *Eco*RI fragments with only 3′ UTR sequences (kb)
			Size (kb)	Hybridization with:			
				5′ Coding sequence	3′ Coding sequence	3′ UTR sequence	
1	M1(β1)-1	1.3×10^{-7}	5.0	−	+	+	1.4, 1.5
	M1(β1)-2	3.3×10^{-9}	6.6	+	+	+	7.1, 1.5
2	M4(β9)-1	3.8×10^{-8}	2.2	−	+	+	1.0
	M4(β9)-2	7.4×10^{-8}	5.8	−	+	+	1.1
	M4(β9)-3	1.8×10^{-8}	2.0	−	+	+	0.7

[a] The 5′ coding probe was an *Ava*I restriction fragment for amino acids 1 through 98 of human skeletal actin (10), and the 3′ coding probe was a *Kpn*I restriction fragment for amino acids 301 through 374 and part of the 3′ UTR sequence of a chicken β-actin cDNA (7).

expectation was that the β-actin gene would be selected preferentially from β-actin pseudogenes by the greater similarity of the 3′ UTR sequence.

Isolation of the β-actin gene from a human fetal DNA library. The structure of the 1,500-bp miniplasmid πAN7β1, which was constructed by inserting an *Eco*RI-*Bam*HI fragment containing the 600-bp 3′ UTR of human β-actin cDNA into sites within the polylinker of πAN7, is shown in Fig. 1. The 3′ UTR sequence is oriented so that the *Sal*I site in the miniplasmid is placed near the junction of the 3′ terminus of the 3′ UTR and the miniplasmid. Recombinant insertion of the miniplasmid into a λ phage carrying the human β-actin gene should thereby insert a reference *Sal*I site at the end of that 3′ UTR which is adjacent to the coding portion of the clone. The only *Sal*I site within the coding region of the human β-actin cDNA is located at codon 10 (22). Therefore, after selection of a recombinant clone, determination of the size of the *Sal*I fragment bearing the actin coding sequence should help distinguish candidate genes with introns from pseudogenes without introns.

Our first attempt to clone the β-actin gene made use of a λ phage library of human fetal DNA (12). The phage in this library had been amplified on numerous occasions. We were already aware that *Eco*RI restriction fragments carrying β-actin 3′ UTR sequences were present in the library in greatly different amounts from their equimolar representation in the genome (unpublished data). The recovery frequencies of phage isolated after πAN7β1 recombination would therefore be the product of their skewed concentrations in the library and their independent recombination frequencies. We nevertheless proceeded to isolate β-actin-carrying clones selected by πAN7β1 recombination.

Two separate recombination trials were performed in which 10^6 PFU of library phage were amplified by infection of either the recombination-proficient *E. coli* WoP3 (πAN7β1) or the nonproficient *E. coli* WoP3 (πAN7). Lytic progeny phage from each amplification were then used to infect a host strain (WoP3Sup0) in which Charon 4A phage do not propagate; consequently, no lytic plaques were produced with the phage derived from the WoP3 (πAN7) infection. Lytic phage derived from infection of strain WoP3 (πAN7β1) produced plaques at a consistent frequency, between 10^{-7} and 10^{-9} of the true titer [in the WoP3 (πAN7β1) and LE392 hosts]. All phage that were isolated contained actin coding sequences and had undergone recombination with the πAN7β1 plasmid.

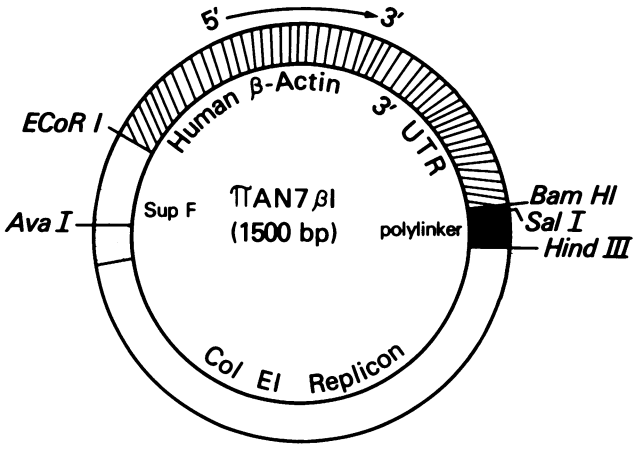

FIG. 1. πAN7β1 plasmid. Construction of this chimeric plasmid is described in the text.

Five distinct phage clones were selected (Table 1). Each contained β-actin sequences, and they are described in terms of the sizes of *Eco*RI fragments that contained either coding or noncoding 3′ UTR sequences. In the first recombination trial, 51 plaques were isolated and the properties of the human DNA insert were examined with three actin-specific probes. Of the 51 plaques, 50 were identical and were designated M1(β1)-1. In addition to three *Eco*RI fragments that contained actin sequences (5.0, 1.4, and 1.5 kb), one *Eco*RI fragment (3.5 kb) which lacked an actin sequence was common to all 50 isolates. This first trial consisted of three independent infections. Thus, the 50 identical phage were derived from a minimum of three separate recombination events but not necessarily from more than three events. A single additional plaque [M1(β1)-2] contained a different phage with a different set of *Eco*RI fragments: three fragments (6.6, 7.1, and 1.5 kb), contained actin sequences, and two fragments (2.0 and 1.2 kb) lacked actin sequences. A second recombination trial produced three more different recombinant clones (Table 1). We interpreted the variable recovery of different plaque types during independent trials as a reflection of the skewed nature of the human λ library as well as the degree of sequence similarity between the πAN7 β-actin insert and the various genomic β-actin sequences.

Recombination with the 3′ UTR sequence leads to insertion of the entire πAN7β1 plasmid, beginning at the 3′ side of the crossover site (25). Thus, two chimeric 3′ UTR sequences that are separated by the πAN7 sequence were created by the recombination event. Two of the clones, M1(β1)-1 and M1(β1)-2, had an additional *Eco*RI fragment precisely the size of a unit-length πAN7β1 plasmid (1.5 kb), which was inserted into the phage genome as a tandem repeat of the πAN7β1 plasmid. This explanation was verified by demonstrating that the same unit-length plasmid fragment containing a 3′ UTR sequence could be generated by digestion with two additional restriction enzymes (*Sal*I and *Ava*I) whose restriction sites occurred only once within the plasmid sequence.

M1(β1)-2 was further distinguished from M1(β1)-1 and the three isolates of the second recombination trial in that it was the only clone that hybridized to a probe that contained the 5′ actin coding sequence (codons 1 through 98). Because M1(β1)-2 contained both 5′ and 3′ sequences of β-actin, it was likely to contain the entire coding region of the β-actin gene. A map of key restriction sites (Fig. 2) disclosed that *Sal*I digestion of M1(β1)-2 generated a 2,500-bp fragment that contained most of the coding sequences for β-actin plus the 3′ UTR sequence. If indeed this fragment contained 1,098 bp of coding sequence (codons 10 to 375) and 600 bp of the 3′ UTR sequence, then the additional 800 bp might be accounted for by the four predicted intron sequences (20) within the coding region. The nucleotide sequence of this gene (S.-Y. Ng et al., manuscript in preparation) confirmed the position of the *Sal*I site at codon 10 and the existence of four intron regions, the sum of whose lengths was 731 bp. Furthermore, the nucleotide sequences of the coding regions of M1(β1)-2 were identical to the β-actin cDNA sequence.

Isolation of a β-actin gene-specific probe and assessment of the frequency of β-actin genes and pseudogenes. A probe derived from intron I of the β-actin gene (Fig. 2) hybridizes to only two discrete bands (ca. 12 to 14 and 6.4 kb) in blotting experiments with *Eco*RI digests of human genomic DNA (Ng et al., in preparation). By contrast, the 3′ UTR probe hybridizes to more than 20 bands (22). Restriction mapping

showed that the λ clone M1(β1)-2 contained the β-actin sequence corresponding to a 12.2-kb genomic fragment which was divided into two *Eco*RI fragments of 6.6 and 7.1 kb by πAN7 recombination.

Detection of the β-actin mutation at codon 244 (26) in a genomic clone would verify the identity of the functional (expressed) β-actin gene in human fibroblasts. Therefore, we constructed libraries of *Eco*RI fragments derived from KD, HUT-14, and HUT-14T cell DNA. The construction of these libraries is described above and shown in Table 2. The critical features of these libraries are as follows. (i) Smaller *Eco*RI restriction endonuclease digest fragments (2 to 12 kb) were found exclusively when the λgtWES vector was used, and larger *Eco*RI restriction digest fragments were found almost exclusively when the Charon 4A vector was used. This method of dividing the human genome proved advantageous in that scrambling of larger fragments with smaller fragments was minimized and most clones derived from either library component contained only a single human *Eco*RI fragment. This feature helped to ensure that after πAN7β1 recombination the resulting recombinant phage would not become less viable or resist packaging. (ii) A total of 10^4 recombinant phage generated by the packaging reaction were amplified as a unit within *sup*$^+$ bacteria on a single agar plate, but the progeny phage of this amplification step were never combined with other amplification aliquots; thus, isolated clones of genes and pseudogenes could be considered the products of independent cloning events when isolated from different amplification aliquots. Accordingly, we could test for the presence of the β-actin gene homologs in each aliquot by πAN7β1 selection. If the β-actin gene were present, we could use the intron probe to recover, from the original aliquot, genomic clones without πAN7 insertion. (iii) The frequency of gene and pseudogene isolation from such libraries is an assessment of the complexity of β-actin-related sequences within the human genome. Conversely, the ratio serves as a criterion for the randomness and completeness of the library itself.

β-Actin clones from human 10- to 23-kb *Eco*RI fragments. When size-fractionated *Eco*RI fragments ranging from 10 or 12 to 23 kb from HUT-14 and HUT-14T cell DNA were used to prepare recombinant phage, the frequency of recovery of β-actin clones per total recombinant phage screened ranged from 4×10^{-5} to 5×10^{-5} for the KD, HUT-14, and HUT-14T libraries (Table 2). Amplification aliquots (10^4 packaging events) were first screend by πAN7β1 recombinant selection to determine which library aliquots contained β-actin genes or pseudogenes. Library aliquots that contained β-actin 3′ UTR sequences were rescreened by conventional in situ plaque hybridization to select clones that hybridized to the 3′ UTR probe. This probe was used to collect a spectrum of the pseudogene family in addition to multiple isolates of the putative functional gene. After purification, each β-actin clone was recombined with πAN7β1. Both the nonrecombinant and πAN7β1 recombinant forms of each clone were then examined by *Eco*RI and *Sal*I restriction endonuclease

TABLE 3. Clones containing β-actin sequences isolated from libraries of 10- to 23-kb genomic *Eco*RI fragments

Library source[a]	Clone	Genomic *Eco*RI fragment size (kb)	Distance (kb) from 5′ *Eco*RI site to 3′ end of the 3′ UTR (*Sal*I site)[b]	2.5-kb *Sal*I fragment present[c]	Hybridization to intron I probe
HUT-14T, fully digested, Charon 4A	14Tβ-15	13.8	6.6	+	+
	14Tβ-16	13.8	6.6	+	+
	14Tβ-17	13.8	6.6	+	+
	14Tβ-18	11.0	4.3	−	+
	14Tβ-19	13.8	6.6	+	+
	14Tβ-20	13.8	6.6	+	+
	14Tβ-21	13.8	6.6	+	+
	14Tβ-22	14.2	8.5	−	+
	14Tβ-23	13.8	6.6	+	+
	14Tβ-24	13.8	6.6	+	+
HUT-14, fully digested Charon 4A	14β-25	18.5	14.6	−	−
	14β-26	14.4	6.0	+	+
	14β-27	13.8	6.6	+	+
	14β-28	ND[d]	14.1	−	−
	14β-29	13.8	6.6	+	+
	14β-30	13.8	6.6	+	+
	14β-31	13.8	6.6	+	+
	14β-32	13.8	6.6	+	+
KD, fully digested, Charon 4A	KDβ-1	13.8	6.6	+	+
HUT-14T, partially digested, Charon 4A	14Tβ-1	5.3	4.3	−	−
	14Tβ-2	10.5	7.9	−	−
	14Tβ-4	4.3	3.8	−	−
	14Tβ-5	8.1	ND	−	−
	14Tβ-12	2.9	ND	−	−

[a] Cell line, degree of *Eco*RI fragment digestion, and vector.

[b] Plasmid πAN7β1 recombinant phage clones were constructed with plaque-pure clones selected by in situ plaque hybridization with the 3′ UTR probe. Before recombination, each clone contained a single human *Eco*RI fragment, whereas after recombination two *Eco*RI fragments were generated by insertion of πAN7β1 into the genomic *Eco*RI fragment; the sizes of the two *Eco*RI fragments generated and identification of the fragment containing the coding and intron I sequences showed the position of the β-actin sequence within the genomic *Eco*RI fragment.

[c] The 2.5-kb *Sal*I fragment is generated by πAN7β1 insertion during recombination and is characteristic of the β-actin gene (Fig.1).

[d] ND, Not determined.

digestion, and the resulting DNA fragments were hybridized with the intron I, 3′ UTR, and coding probes to fully assess their identity and relatedness (Table 3). In total, 8 of 10 isolates from HUT-14T DNA and 5 of 8 isolates from HUT-14 DNA contained a β-actin gene similar to that found in M1(β1)-2, because each of these separate clones hybridized strongly to the intron probe. In addition, their πAN7β1 recombinants contained the characteristic 2.5-kb *Sal*I fragment that carries the β-actin coding, intron, and 3′ UTR sequences. The size of the uninterrupted genomic fragment for these clones was about 13.8 kb. Three other clones hybridized only weakly to the intron probe. The πAN7β1 recombinants of two of these, 14Tβ-18 and 14Tβ-22, had unique *Eco*RI and *Sal*I digestion products as well as an uninterrupted *Eco*RI fragment of a different size. It therefore seems likely that these two clones were unrelated and were dissimilar to the 13 common clones. Another clone, 14β-26, had a 14.4-kb *Eco*RI fragment and gave only weak hybridization to the intron probe, but did yield a 2.5-kb *Sal*I fragment after πAN7β1 recombination.

The results with libraries constructed with genomic *Eco*RI DNA fragments of 10 to 23 kb are in contrast to the results obtained when we constructed libraries of 2- to 14-kb genomic DNA *Eco*RI fragments cloned in the λgtWES vector (Table 2). This library yielded no β-actin clones carrying either a 12.2- or a 13.8-kb *Eco*RI restriction fragment as well as the characteristic 2.5-kb *Sal*I fragment of the β-actin–πAN7 recombinants. Instead, we isolated 61 clones carrying β-actin sequences at frequences of 7×10^{-5} to 8×10^{-5} (Table 2). All of these clones probably carry pseudogenes, as determined by their lack of hybridization to the β-actin intron I probe and comparison of detailed restriction maps (data not shown). In addition, we performed a limited screening of another library, constructed from partially *Eco*RI-digested genomic DNA fragments (Table 2). The clones derived from this library all contained pseudogenes since they lacked key restriction sites indicative of the expressed β-actin gene and failed to hybridize with the intron I probe (Table 3).

***Eco*RI polymorphism at the 5′ flank of the β-actin gene.** The *Eco*RI fragment carrying the β-actin gene, including its introns, in the πAN7β1 KD, HUT-14, and HUT-14T recombinants (Table 3) was 8.2 kb long. By contrast, the *Eco*RI fragment bearing the β-actin gene in M1(β1)-2, derived from a human fetal DNA library, appeared to be only 6.6 kb long (Fig. 2). Both clone types contained a common 7.1-kb *Eco*RI fragment in addition to a fragment of either 8.2 or 6.6 kb which hybridized to the intron I probe. The 7.1-kb fragment appeared to hybridize only with the 3′ UTR probe. We conclude that this 7.1-kb fragment was generated by recombination of πAN7β1 with either a 12.2- or 13.8-kb genomic *Eco*RI DNA fragment.

We attempted to determine whether this difference in fragment length (8.2 versus 6.6 kb) was due to a simple 5′ *Eco*RI restriction site polymorphism neighboring orthologous genes or whether the different fragment sizes represented paralogous alleles. Accordingly, the *Eco*RI digestion fragments of three of the πAN7β1 recombinant β-actin clones from HUT-14 DNA [14β-27(β1), 14β-29(β1), and 14β-30(β1)] and the fetal gene clone M1(β1)-2 were subcloned into plasmid pBR322. We digested these subclones with *Eco*RI and separated the resulting fragments by agarose gel electrophoresis. Each blot was hybridized first to the β-actin intron I probe and then to the β-actin 3′ UTR probe (Fig. 3). The intron probe hybridized to the 8.2-kb *Eco*RI fragment of 14β-27(β1), 14β-29(β1), and 14β-30(β1) and to the 6.6-kb *Eco*RI fragment of M1(β1)-2. By contrast, the 3′ UTR probe hybridized to the 7.1-kb *Eco*RI DNA fragment common to all four clones as well as to the 8.2- or 6.6-kb *Eco*RI fragments containing the intron I sequences. This result indicates that the genes isolated from HUT-14 and HUT-14T cell DNA differ from the fetus-derived gene in M1(β1)-2 in the location of an *Eco*RI site in the genomic DNA flanking the 5′-region of the actin gene. All 13 independent πAN7β1 recombinant clones derived from both HUT libraries and one additional clone derived from the KD cell DNA library (Table 3) had an identical arrangement with respect to the positions of flanking *Eco*RI sites. Furthermore, these clones were derived from both alleles (see below). The uninterrupted *Eco*RI fragment in the corresponding non-πAN7 recombinant clones was 13.8 kb long (Table 3). We therefore conclude that the β-actin gene resides on a 13.8-kb genomic *Eco*RI fragment. The different length of the restriction fragment in the genomic DNA of clone M1(β1)-2 most likely arises from an *Eco*RI polymorphism in human genomes, although, the possibility that it is an artifact incurred during

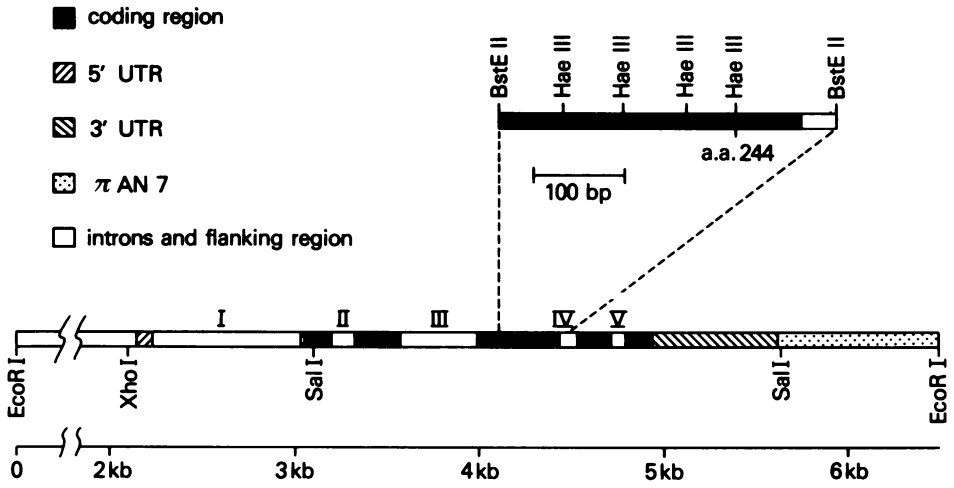

FIG. 2. Restriction endonuclease map and structure of the human β-actin gene–πAN7β1 recombinant clone M1(β1)-2. Inset, 366-bp *Bst*EII fragment.

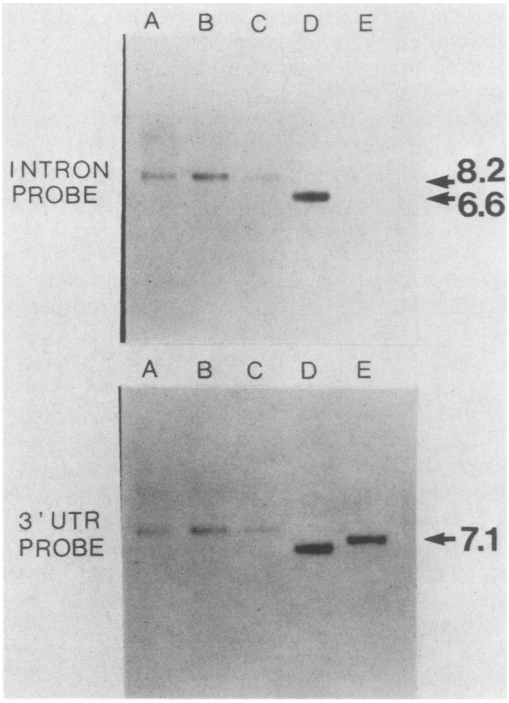

FIG. 3. Southern blot of *Eco*RI-digested pBR322 subclones of *Eco*RI fragments derived from separate bacteriophage clones of human β-actin genes. Lanes: A, 14β-27(β1), 8.2-kb fragment; B, 14β-29(β1), 8.2-kb fragment; C, 14β-30(β1), 8.2-kb fragment; D, M1(β1)-2, 6.6-kb fragment; E, M1(β1)-2, 7.1-kb fragment. A single nitrocellulose blot from an 0.8% agarose gel was first hybridized to the ^{32}P-labeled intron I probe (top) and then rehybridized to the ^{32}P-labeled 3′ UTR probe (bottom).

λ phage clone construction or propagation cannot be excluded.

Discrimination between mutant and wild-type allelic clones of the β-actin gene by a *Hae*III cleavage test for the mutation at codon 244. The sequences derived from the gene in M1(β1)-2 (Ng et al., in preparation) and from a cDNA clone (23) show that codons 243, 244, and 245 (Asp-Gly-Gln) were encoded by GAC GGC CAA. Since the first β-actin mutation in HUT-14 cells resulted in an exchange of the glycine (codon 244) for an aspartic acid residue, the predicted sequence for codon 244 after the mutation is GAC. The unmutated sequence GGCC (codons 244 and 245) is a restriction site for the endonuclease *Hae*III, a site which should be absent in mutant copies of the gene from HUT-14 and HUT-14T cells. *Bst*EII sites flank the mutation site, cleaving between codons 158 and 159 and at a site 38 bp into intron IV, respectively (Fig. 2). This *Bst*EII fragment (366 bp) was isolated from the DNA of three plasmid subclones of the HUT-14 πAN7β1-derived β-actin genes [the 8.2-kb *Eco*RI fragment from 14β-27(β1), 14β-29(β1), and 14β-30(β1)] and three additional plasmid subclones from non-πAN7-derived HUT-14T β-actin genes (the 13.8-kb *Eco*RI fragment from 14Tβ-17, 14Tβ-21, and 14Tβ-24). Within this *Bst*EII fragment, there were *Hae*III sites at codons 182, 204, and 228 and at codon 244, the site of the mutation (Fig. 2). Digestion of the *Bst*EII fragment from the wild-type β-actin gene with *Hae*III generated five restriction fragments, of 71, 65, 72, 52, and 106 bp, whereas the mutated gene missing the *Hae*III site at codon 244 should be cleaved into only four restriction fragments, of 71, 65, 72, and 158 bp. The 158-bp *Hae*III fragment diagnostic of the HUT-14 mutation at codon 244 was predicted from the sum of the 52- and 106-bp segments. Figure 4 shows the *Hae*III digestion products for this 366-bp *Bst*EII fragment from the six clones tested. Four of six clones from HUT-14 [clones 14β-27(β1) and 14β-29(β1)] and HUT-14T (clones 14Tβ-21 and 14Tβ-24) had the 158-bp *Hae*III-*Bst*EII fragment indicative of copies of the gene mutated at codon 244. The two remaining clones, 14β-30(β1) and 14Tβ-17, showed the wild-type digestion pattern indicative of the normal, unmutated gene. The β-actin genes cloned from the HUT-14 and HUT-14T cell DNA libraries therefore represented both the wild-type and mutant alleles. Furthermore, the presence of the predicted mutation in one of the alleles formally proves that these genes, and not the other *Eco*RI β-actin coding fragments, are the expressed β-actin genes in these human fibroblast strains. Sequence analysis of the genes carrying the mutations confirmed that these genes contain the predicted point mutation and therefore are expressed (C.-S. Lin et al., manuscript in preparation).

Recombination frequencies within 3′ UTR sequences of clones of β-actin genes and pseudogenes. Our original premise was that πAN7β1 would preferentially recombine with the real β-actin gene, since its sequence should have the highest degree of base matching compared with that of pseudogenes. Accordingly, we measured the recombination frequency between πAN7β1 and 13 of the nonallelic β-actin clones isolated by in situ plaque hybridization (Table 4). A true β-actin gene clone, 14Tβ-16 (Table 3), gave the highest recombination frequency, as expected. Clones HRL25 (22) and 14Tβ-13 (HRL25-like), both of which show weak intron-specific hybridization and seem to correspond to the 6.4-kb *Eco*RI fragment detected in genomic blots (Ng et al., manuscript in preparation), were lower in recombination frequency by 1 order of magnitude, indicating sequence divergence within the 3′ UTR.

The 10 remaining clones listed in Table 4 showed further

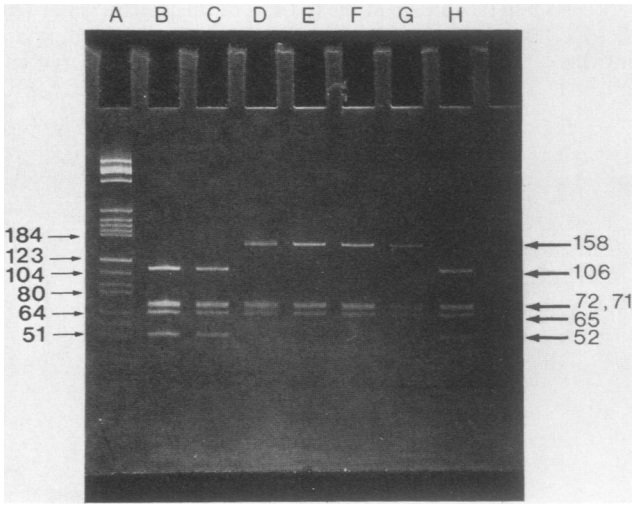

FIG. 4. A 10% polyacrylamide gel of ethidium bromide-stained *Hae*III restriction digest fragments of a 366-bp *Bst*EII fragment spanning the β-actin codon 244. Lanes: A, *Hae*III pBR322 DNA digest markers; B, M1(β1)-2; C, 14β-30; D, 14β-29; E, 14β-27; F, 14Tβ-24; G, 14Tβ-21; H, 14Tβ-17. Sizes (in bp) of marker fragments are indicated to the left. The precise sizes of the β-actin DNA fragments as determined by DNA sequencing are indicated to the right.

TABLE 4. Frequency of recombination between λ clones containing β-actin or pseudogene sequences and πAN7β1

Clone[a]	Frequency of πAN7β1 recombinants[b]
14Tβ-16[c]	3.3×10^{-3}
14Tβ-5	9.4×10^{-4}
HRL25	2.4×10^{-4}
14Tβ-13[d]	1.5×10^{-4}
14Tβ-4	1.4×10^{-4}
HRL51	1.0×10^{-4}
14Tβ-1	$>7.6 \times 10^{-5}$
14Tβ-2	3.6×10^{-5}
14Tβ-12	$>1.6 \times 10^{-5}$
HRL24	1.5×10^{-5}
HRL35	7.1×10^{-6}
HRL21	6.2×10^{-6}

[a] Clones 14Tβ-16, 14Tβ-1, 14Tβ-2, 14Tβ-4, 14Tβ-5, 14Tβ-12, and 14Tβ-13 were isolated from libraries described in Table 2, and the properties of these clones are summarized in Table 3; clones HRL21, -24, -25, -35, and -51 are described by Ponte et al. (22).

[b] Large plaques of each clone were produced by infection of the recombination-proficient strain *E. coli* W3110 (p3 πAN7β1); bacteriophage were then eluted from the plaques and titrated on *sup*$^+$ and *sup*^{o} hosts to determine the frequency of πAN7β1 recombination.

[c] β-Actin gene.

[d] HRL25-like.

reductions in recombination frequency with πAN7β1. These clones have been analyzed by restriction site mapping (22; unpublished data), by limited sequence analysis (J. Engel, Ph.D. thesis, Stanford University, Stanford, Calif., 1982), and by blotting experiments with probes of intron sequences, 5′ and 3′ coding sequences, and 3′ UTR sequences (6, 7, 22; J. Engel, Ph.D. thesis; unpublished data). From the results of these studies, all 10 of these clones appear to contain β-actin pseudogenes. The πAN7 recombination system therefore seems to be a way to preferentially select true genes from among divergent pseudogene members of a multigene family.

DISCUSSION

The πVX-πAN7 recombination method for selecting recombinant library clones allowed first-trial selection from the β-actin multigene family of a clone that eventually proved to contain the expressed gene. This gene was cloned from a highly amplified human fetal gene library previously screened at random for the β-actin gene by in situ plaque hybridization without success (7, 22; J. Engel, Ph.D. thesis). In retrospect, the reason for the previous failures (18, 19, 22) must be related to the skewed representation of the β-actin gene in the human fetal library DNA relative to that of other members of the β-actin gene and pseudogene families. The β-actin gene recombines between threefold and ca. 2,000-fold more frequently than any other member of the β-actin family of sequences (Table 4).

We identified recombinant phage M1(β1)-2 as containing the likely β-actin gene from among more than 100 other independent recombinant clones that contained β-actin sequences. We determined that it carried both a *Sal*I site at codon 10, as predicted from the cDNA sequence (22), and enough extra bp to accommodate the several predicted introns. As a consequence of the homologous recombination event, a reference *Sal*I restriction site in the plasmid polylinker was inserted into the recombinant phage genome adjacent to the 3′ UTR of the β-actin gene. The sizes of liberated *Sal*I fragments of clones indicated the coding potential of the clone as well as the cumulative size of the introns between codon 10 and the 3′ UTR sequence. Since most of the β-actin clones lacked additional sequences between the *Sal*I sites and appeared to contain processed pseudogenes (18, 19), and since more than half lacked a *Sal*I site within the entire human DNA insert, many clones were easily disqualified as candidates.

The arrangement of *Eco*RI fragments in πAN7 recombinant clones was easily deduced by identifying the fragments that contained a coding sequence adjacent to the 3′ UTR sequence and those that contained only the 3′ UTR sequence. This information allowed us to predict the position of the gene sequence within a map of the genomic *Eco*RI fragment uninterrupted by the plasmid insertion. Once this map was constructed, the likelihood that the clone contained the complete gene could be evaluated on the basis of reasonable assumptions about the size of the introns.

Clones containing the correct β-actin gene in their πAN7 recombinant form had a characteristic 2.5-kb *Sal*I fragment which accounted for 365 codons, a 600-bp 3′ UTR, and ca. 800 extra bp, the cumulative intron size predicted from the sizes of introns of the β-actin gene from rats (20). This finding led us to begin sequencing M1(β1)-2 as the prime candidate over many other clones that had been isolated. Determination of the nucleotide sequence of the β-actin gene in clone M1(β1)-2 (Ng et al., in preparation) has shown that this gene contains a coding sequence identical to the sequence of a full-length β-actin cDNA isolated from a cDNA library of human fibroblast mRNA (10, 23). We conclude that M1(β1)-2 contains the human β-actin gene expressed in human fibroblasts.

Because of the complexity of the human β-actin gene-pseudogene family, however, there remained a formal possibility that the gene cloned from the fetal gene library was not the gene that had undergone mutation in HUT-14 cells and their progeny. Since a mutation in codon 244 is expressed in one of two functional alleles in the human fibroblast cell line HUT-14 (26), the presence of this mutation in approximately half the HUT-14 DNA segments analogous to M1(β1)-2 would confirm the identity of the fetus-derived β-actin gene as the gene expressed in human fibroblasts. Because the predicted size of the genomic *Eco*RI fragment containing the β-actin gene was 12.2 kb, based on the fetus-derived gene, we initally assumed that this clone could be isolated by using the λgtWES vector that can produce viable clones with inserts of up to 14 kb (16). Repeated screening of λgtWES libraries (derived from both HUT strains) by in situ plaque hybridization with the intron-I β-actin-specific probe or selection of πAN7β1 recombinants failed to produce the gene analogous to the fetus-derived β-actin gene (data not shown). Instead, 61 pseudogenes were isolated from the λgtWES libraries, including two distinct copies of a pseudogene on a 6.4-kb *Eco*RI genomic fragment that gave weak hybridization to the intron-specific probe. On the other hand, Charon 4A libraries containing *Eco*RI DNA fragments between 10 and 23 kb long were found to be enriched for a 13.8-kb *Eco*RI fragment which showed strong hybridization reactivity with the intron-specific probe. Clones bearing this fragment had identical restriction maps and πAN7 recombinant structure with the fetus-derived gene with but one exception: the 5′ genomic *Eco*RI site flanking the HUT-derived genes was ca. 1.6 kb more distal from the β-actin gene than was the *Eco*RI site in the fetus-derived gene. This restriction fragment length difference is probably due to an *Eco*RI polymorphism in the human population or, less likely, may have been introduced as an artifact during the construc-

tion or propagation of the fetal DNA library. Since identical *Eco*RI fragments were isolated from the three related libraries (KD, HUT-14, and HUT-14T) and reproduced in 14 discrete recombinants derived from separate alleles, it seems reasonable to conclude that the 13.8-kb *Eco*RI fragment is a naturally existing form. This conclusion is further supported by our isolation of two copies of the β-actin gene from an unrelated cell line that also have a 13.8-kb *Eco*RI fragment (unpublished data). The codon 244 mutation was found in some, but not all, of the clones from the HUT-14 and HUT-14T cell lines, and these clones also had a 2.5-kb *Sal*I fragment (after πAN7β1 recombination). Since these same clones share equal specificity with the fetus-derived gene for the intron-specific probe, we conclude that these genes from the HUT cell lines are alleles and are the homologs of the gene in M1(β1)-2, which was cloned from the human fetal DNA library.

The fetus-derived clone HRL25 and the two clones from the HUT-14 and HUT-14T libraries (14β-13 and 14Tβ-14) appear to be independent isolates of the same β-actin pseudogene because πAN7 recombinant derivatives produced identical restriction maps. These clones also show uniformly weak hybridization with the intron-specific probe. The β-actin sequence in this clone type, which lacks a *Sal*I site at codon 10 and therefore contains a pseudogene, may have diverged from that of the expressed β-actin gene after a gene duplication event. It may therefore be representative of a class of pseudogenes with introns which are distinct from the majority of β-actin pseudogenes that appear to be of the processed type.

The human β- and γ-actins of the cytoskeleton differ from each other by only 4 amino acids (26, 28) and by only 17 or 18 amino acids from the abundant actin isoform of the lower eucaryotic unicellular amoeba *Physarum polycephalum* (28, 29). Such strong selective pressure is probably related to critical functions of the actins as major components of the cytoskeleton. We have now isolated cloned copies of the wild-type and two mutant β-actin genes, one with a codon 244 mutation and the other with a currently undefined second mutation (13). Additional mutant β-actin genes can be constructed by site-directed mutagenesis. Since normal actin genes are expressed after gene transfer (9), we should now be able to examine the relationship between mutational defects in β-actin, cytoskeletal dysfunction, and altered cellular phenotype.

ACKNOWLEDGMENTS

We thank Tom Maniatis for providing bacterial strains and technical advice and Henry Huang for providing the πAN7 plasmid and their unpublished data.

This work was supported by an American Cancer Society grant to J.L. and by Public Health Service grants from the National Institutes of Health and the Veterans Administration to L.K.

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