There are approximately 20 actin genes in the human genome

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SUMMARY

By three different lines of evidence there are approximately 20 copies of actin genes in the human genome. Firstly, the rate of hybridisation of a mouse actin probe to human DNA indicates that there are a minimum of 20 complementary copies of the actin sequence per genome. Secondly, this probe hybridises to 17-20 bands in Southern blots of restriction enzyme digests of total human DNA. Most of these bands hybridise with both 3' and 5' fragments of the cDNA and are therefore likely to contain the entire gene sequence. Thirdly, we have picked 12 actin recombinants from a genomic library, and at the level of restriction enzyme mapping these represent nine different genes. Probability calculations indicate that these recombinants were picked from a pool of at least 20 different genes.

INTRODUCTION

Actins are extremely old evolutionarily and are found in all eukaryotes (for review see 1). They can be separated by isoelectric focusing into three main groups (2), though they are >90% homologous in amino acid sequences (3). Beta and gamma actins are cytoplasmic actins involved in the cytoskeleton and in internal cell mobility phenomena (4). The most acidic form, the alpha actins are the predominant actin form in muscle tissue (3). Actins are highly conserved and the cytoplasmic actins from Physarum and mammals have only 4% (17/374) amino acid differences (5). In mammals four different muscle actins have been sequenced, from fast muscle, cardiac muscle, aorta and stomach (3). These vary only by four to six amino acids from each other, and by about 25 amino acids from the beta and gamma actins.

The actin genes have now been analysed in many different organisms. In yeast there is one actin gene (6, 7) that has a single 304 bp intron in the coding sequence (8). Drosophila have six actin genes, widely dispersed through the genome with at least one intron (9, 10). However, other organisms contain larger numbers of actin genes. Dictyostelium has about 17 actin genes none of which contain introns (11, 12), and sea urchins have at least 11 different actin gene (13), each with several introns (14). Chicken DNA has at least 5-11 actin

genes by kinetic analysis (15). The chick muscle actin gene contains at least three small introns (16).

From the protein data it would thus be expected that mammals contain at least six gene sequences coding for actin. Since the actins are highly conserved, actin probes from one species can be used to detect actin genes in another (6, 13). We have used probes from the mouse to detect actin genes in human DNA. However by three different criteria there appear to be many more than six different actin genes in the human genome.

MATERIALS AND METHODS

Recombinant DNA work was carried out under CI containment conditions in accordance with the GMAG (U.K.) guidelines for recombinant DNA research. Plasmids were grown and purified as previously described (17, 18). A partial AluI and HaeIII genomic library (a kind gift of Tom Maniatis, 19) was plated and screened (20). Actin containing recombinants were picked and put through three rounds of plaque purification. "Mini preparations" of DNA were purified from plate lysates (21). Agarose plates with confluent lysis were overlaid with 5 mls of 10mM Tris pH 7.5; 10mM EDTA, and left at 37° for 4 hours. To 4 ml of supernatant 0.4 ml 0.5M EDTA pH 8.5; 0.2 ml 2M Tris Base (not pHed) and 0.2 ml 10% SDS were added. 10 µl of diethylpyrocarbonate were added and the mixture heated at 65° for 30 mins in a fume cupboard. 1.0 ml of 5M potassium acetate (not buffered) was added and the mixture left on ice for 60 mins. The white precipitate was spun out at 12K for 10 mins, and the supernatant extracted once with phenol, once with ether, and precipitated with ethanol overnight at -20°. The DNA was dissolved in 300 µl of 10mM Tris; 1mM EDTA, and 15 µl digested with restriction enzyme and run on one slot of an agarose gel.

Restriction endonucleases were purchased from New England Biolabs or prepared in this laboratory. Human DNA was prepared from lymphocytes (22) and digested at 37° in 6mM Tris pH 7.8; 6mM MgCl₂; 6mM beta-mercaptoethanol; 200 µg/ml nuclease-free bovine serum albumin (Bethesda Research Labs.) supplemented with NaCl to 100mM for EcoRI, or buffers recommended by New England Biolabs. The digested DNA was fractionated by agarose gel electrophoresis using a horizontal slab gel apparatus (22). DNA was transferred from agarose gels to Schleicher and Schuell PH79 (0.1 µm) nitrocellulose filters (23) and hybridised with radioactive probes using the modifications of Jeffreys and Flavell (22). Hybridisation was carried out at 65° in 3 x SSC (1 x SSC is 0.15M NaCl; 0.015M Na Citrate), 0.1% sodium dodecyl-sulphate, 10 µg/ml Herring sperm DNA, 10 µg/ml poly A (Sigma).

After hybridisation the filters were treated as described by Jeffreys and Flavell (22) with a final wash of 1 x SSC, 0.1% SDS 65° 30 mins or 0.1 x SSC, 0.1% SDS 65° 30 mins. Washed filters were exposed to preflashed Fuji X-ray film for 2-5 days at -70° using an Ilford Tungstate intensifying screen.

Hybridisation Probes

Mouse muscle actin plasmid was digested with the appropriate

restriction enzymes and the fragments separated by agarose gel electrophoresis. The fragments containing the actin sequences were cut from the gel and DNA purified by a modification of a freeze-squeeze method (24). DNA was labelled in vitro by nick translation (25) using ^{32}P α dCTP and α dTTP (400 Ci/mM) purchased from Amersham. E.coli DNA polymerase I was purchased from Boehringer-Mannheim and DNase I from Sigma.

Kinetic Analyses

Human DNA or pAL41 DNA was sonicated to give an average fragment size of 1,000 bases and 500 bases for the human and plasmid DNAs respectively. Hybridisation was carried out in 0.3M NaCl; 100mM Tris pH 7.5; 1mM EDTA, at 70° and the single stranded sensitivity to S1 nuclease (Sigma) analysed as previously described (26).

RESULTS

Mouse and Human Actin cDNA Plasmids

Several probes have been used to analyse the actin genes found in human DNA. The recombinant plasmid pAM91 (Figure 1) was isolated from a cDNA library made from mouse muscle RNA, and sequence analysis has shown that the insert is complementary to skeletal muscle actin (27). This plasmid was used to generate probes specific for the 5' and 3' portions of the gene, by double digestion of the plasmid with both PvuII and PstI (Figure 1). A recombinant plasmid containing a DNA sequence complementary to one of the non-muscle actin mRNAs (beta or gamma), was isolated by screening a mouse lymphocyte cDNA library using pAM91. This plasmid, designated pAL41 contains an inserted sequence of 1,150 nucleotides, corresponding to 100 bp of 3' non-coding sequence and 90% of the coding sequence (S.Alonso and A.Minty, unpublished results).

Figure 1 - A restriction enzyme map of the mouse muscle actin cDNA clone pAM91. Digestion with PstI generates a 1,150 bp coding fragment. Double digestion with PstI and PvuII generates fragments from the 5' and 3' portions of the coding region.

pAM91 was also used to identify a human muscle actin from a muscle cDNA library (pHM1) and a non-muscle actin from a human lymphocyte library (pSL3). The size of the inserted DNA in these plasmids is about 300 bp (S.Humphries, unpublished). These plasmids have not been characterised further.

Kinetic Estimate of Number of Actin Genes

A kinetic analysis was performed to estimate the number of actin genes in human DNA (Figure 2). The nick-translated 1,150 bp PstI fragment (mostly coding sequence) of pAL41 hybridises to human DNAs with a $Cot_{1/2}$ of 150 moles per l sec. By comparison with the rate of hybridisation of mouse alpha and beta globin cDNA (29) this would indicate about 10 copies per genome. A more accurate estimate can be obtained by a reconstruction experiment. Sonicated homologous (pAL41) actin plasmid DNA was added to the driver DNA in an amount estimated to represent 20 genes/haploid genome. Addition of the homologous sequences accelerates the reaction 2 fold (Cot of 75 moles per l sec) suggesting an estimate of 20 actin genes per genome. Furthermore, addition of pAL41 DNA equivalent to five genes per haploid genome increased the $Cot_{1/2}$ by 1.25-fold, as expected (Cot

Figure 2 - Kinetic analysis of the hybridisation of an actin probe to human DNA. Hybridisation mixtures contain sonicated human DNA at a concentration of 4 mg/ml, with or without the addition of sonicated plasmid pAL41 DNA at 200 ng/ml (20 parts actin sequence/ 10^7). The nick-translated probe was the 1,150 bp PstI insert from pAL41, specific activity 1×10^8 dpm/ μ g. 1,000 cpm per point were used and analysed after the appropriate length of incubation using S1 nuclease. About 100 bp of the 3' end of the insert are from the 3' non-coding region of the mRNA (S.Alonso and A.Minty, unpublished data). These sequences from a mouse probe would not be expected to hybridise the human DNA because of divergence (28). $Cot_{1/2}$ values indicated have been corrected for this.

= 120 moles/litre sec).

Southern Blot Analysis of Human Actin Genes

Nick-translated 1,150 bp PstI fragment from pAM91 was used as a probe for actin genes in Southern blots of human DNA digested with a variety of restriction enzymes (Figure 3). When the filters are washed at low stringency (1 x SSC) (Figure 3A) 17-20 bands of varying intensity can be seen. In the RI digest, two major bands of 2.5 and 3.8 kb are seen. When filters are washed at

Figure 3 - Detection of actin gene sequences in human DNAs. 1.5×10^6 dpm (40 ng) of nick-translated mouse muscle actin DNA (1,100 bp fragment of pA91) was incubated in 10 mls of hybridisation solution at 65° for 68 hours. The filters were washed as described to a stringency of 1 x SSC (A) or 0.1 x SSC (B) and exposed for 2 days, or 7 days. Lanes R, H and B, human DNA digested with EcoRI, HindIII and BglII respectively. The sizes of the major bands are shown, and were calculated by comparison with the position of restriction fragment size markers (lambda DNA digested with EcoRI and HindIII) run on the agarose gel (not shown).

high stringency ($0.1 \times \text{SSC}$) (Figure 3B) only the intensely hybridising bands are seen, though others remain at low intensity. Notably a 18 kb BglIII fragment is absent after high stringency washes, even after long exposure times.

The simplest interpretation of these results is that there are 15-20 actin genes in humans. However it is possible that the large number of hybridising bands is generated by the presence of internal restriction enzyme sites within the actin coding sequence. Thus each band would represent only a portion of the gene. This was investigated by using probes derived from pAM91 homologous to either the 5' or 3' part of the coding sequence. When these probes were hybridised to Southern blots of human DNA (Figure 4), the pattern of hybridisation is similar to that seen with the whole pAM91 insert. The majority

Figure 4 - Hybridisation of EcoRI restricted human DNA with 5' and 3' actin probes. 1.5×10^6 cpm (40 ng) of nick-translated 5' or 3' pAM91 probe was incubated with filters in 10 mls of hybridisation solution at 65° for 68 hours. The filters were washed as in Figure 3. The migration of fragments of Lambda DNA digested with HindIII is shown.

of the bands are detected with both probes, indicating that the 15-20 bands seen represent at least one gene each.

Purification of Actin Genomic Recombinants

About 150,000 recombinant plaques of a partial *AluI*/*HaeIII* library in Charon 4A were screened using a human non-muscle actin plamid probe (19, 20). From this screen (approximately 1 genome equivalent of DNA), more than 50 plaques gave positive signals of varying intensity. Twelve of these were selected at random, purified, DNA prepared (21) and digested with *EcoRI*.

Figure 5 shows the pattern of bands observed, and shows the hybridisation of a blot of this gel with actin probe. It can be seen that recombinant numbers 4 and 20 contain the same three *EcoRI* fragments and probably represent the same isolate picked twice. All the other recombinants differ in their *EcoRI* band pattern and are therefore independent isolates of human actin genes. Several of these recombinants contain *EcoRI* fragments of similar size that hybridise with the probe (Figure 5 and Table 1); notably a 1.8 kb fragment is common to recombinants 8 and 12, a fragment of 2.5 kb is seen in 7 and 11, and one of 6.5 kb in 4 and 26. However with the exception of 8 and 12, these recombinants have different *BglII* size fragments (Table 1) and are thus probably different genes.

It is possible however that these recombinants each contains only fragments of the actin genes. This was again tested using probes for the 3' and 5' end of the actin coding sequence. These probes were hybridised to Southern blots of *EcoRI* restricted recombinant DNA (Figure 6). It can be seen that recombinants 1, 2, 5, 9 and 10 hybridise equally with both probes and therefore contain a complete actin coding sequence. 8 and 12 also contain a complete coding sequence but the gene is cleaved by *EcoRI* such that the 1.8 kb fragment contains the 5' end of the gene, while the larger hybridising fragment contains

TABLE I The size of the fragment hybridising to nick translated pSL3 is shown in kilobases for *EcoRI*, *BglII* and *XbaI* digests.

<u>RECOMBINANT</u>	<u>RI (kb)</u>	<u>BglII (kb)</u>	<u>XbaI (kb)</u>
1	5.05	5.0	7.1
2	5.2	15.4	7.1/8.8
4	6.5	6.7	13.5
5	7.2	5.0	20
7	2.5	1.9	20
8	1.8/16.0	2.0	20
9	4.8	2.5	20
10	4.3	1.9	18.0
11	2.5	20	20
12	1.8/6.2	2.0	9.0
26	6.5	20	-

Figure 5 - EcoRI analysis of recombinant DNA. Aliquots of each recombinant DNA (25 μ l for recombinant 5, 15 μ l for all others) were digested with EcoRI, and run on a 1% agarose gel (5A). The gel was blotted and hybridised with a nicktranslated human lymphocyte actin probe (pSL3). 150 ng of actin sequence, specific activity 1×10^7 cpm/ μ g were incubated in 50 mls for 24 hours. Filters were washed to $1 \times$ SSC; 0.1% SDS, and exposed for 2 days (5B).

Figure 6 - Hybridisation of recombinants with 5' and 3' specific actin probes. Aliquots of each recombinant were digested with EcoRI and the DNA run on a 1% agarose gel and blotted. Hybridisation was carried out in 40 mls with 100 ng of either actin probe prepared from pAM91 (specific activity 1.5×10^7 cpm/ μ g) for 24 hours. Filters were washed to $1 \times$ SSC and exposed for 2 days.

the 3' end of the gene. Recombinants 4 and 7 hybridise predominantly with the 3' probe and 11 and 26 with the 5' probe. Thus it would appear that of the 11 different recombinants picked, six contain the sequence for a complete actin gene and four contain sequences for only part of the gene.

The size of the EcoRI fragment is not diagnostic for different genes because of the method of production of the library. EcoRI linkers were ligated onto 20 kb fragments of human DNA (19) and thus an actin gene sequence located near to either end of the recombinant DNA fragment may be detected as an artefactually small EcoRI fragment. The clones were therefore analysed using the restriction

enzymes *Msp*I and *Alu*I (Figure 7). These enzymes cleave at tetrameric recognition sites (32) and would be expected to generate many fragments within the actin gene sequence. Recombinants that contain identical actin genes on different RI fragments, or genes that are related in sequence would be expected to contain fragments of similar size. This is the case with 4 and 20, the identical isolates. Both generate one *Msp*I and three identical *Alu*I bands. Recombinants 8 and 12 also appear identical in this analysis, indicating that they are different isolates of the same gene. Of the other recombinants only 5 and 26 show overlap of fragments, with the 1.8 kb *Msp* fragment and 2.0 *Alu* fragment of 26 being also present in 5. It is thus possible that 26 represents the independent isolation of the 5' portion of the complete actin gene of recombinant 5.

At this level of analysis it would appear that of the twelve actin isolates picked at random from a genomic library, at least nine are from different genes (six complete plus another three partial genes).

Figure 7 - Digestion of recombinants with *Alu*I and *Msp*I. Aliquots of each recombinant DNA were digested with A) *Msp*I, or B) *Alu*I, and run on a 1% agarose gel. The gel was blotted and hybridised with a nick-translated actin probe (pSL3), as described in Figure 5.

DISCUSSION

In humans, our analysis by three different methods indicates that there are approximately 20 actin gene sequences in the genome. The estimate derived from rate of reannealing depends upon a reconstruction, where addition of an amount of actin DNA equivalent to 20 genes homologous to the probe doubles the observed rate of reaction. The simple interpretation of this is that there are at least 20 gene sequences in the human genome. This is a minimum estimate since the sequence of the actin genes has diverged from the mouse cytoplasmic actin sequence used as a probe, and sequence divergence would be expected to reduce the rate of hybridisation considerably (30, 31). The extent of this reduction is hard to estimate. The most diverged actin protein sequences known differ by 7% between alpha and beta actins (3). Similarly by Tm analysis the chick muscle and non-muscle actins show a 4-8% divergence in the coding sequences at the DNA level (33). If this degree of divergence occurs between the probe and most of the actin genes, it can be calculated that the rate of hybridisation would be reduced at least ~1.5 fold. This would significantly increase the estimated gene number.

The second estimate of actin gene number comes from Southern blots. 17-20 hybridising bands of varying intensity can be detected using each of three different restriction enzymes. Most of these bands hybridise with both a 3' and a 5' probes and are thus candidates for whole genes. Because the mRNAs for actins are between 1,600-2,000 bases (34), several genes could be arranged on some of the larger hybridising fragments in tandem array. This would mean that the 17-20 hybridising bands is an under-estimate of actin gene number, and could explain why some fragments give hybridisation signals of greater intensity than others. However we believe this possibility is unlikely since none of the recombinants picked from a genomic bank appear to contain more than one actin gene, indicating that the genes are not closely linked.

An alternative explanation for the different intensity of hybridisation is that fragments giving a strong signal represent the probe hybridising to homologous sequences, and weak bands represent hybridisation to non-homologous sequences. We favour this explanation since Southern blots of mouse DNA incubated with a muscle or a non-muscle actin probe also have about 15 hybridising bands, but show the same fragments hybridising with different intensity with the different probes (A.Minty, unpublished observations). Since the probe used to hybridise to the human blots was a muscle actin probe, this interpretation would suggest that the two strongly hybridising bands at 2.5 kb and 3.8 kb are muscle actin sequences. These bands hybridise with both 5' and 3' specific probes (Figure 4), but this interpretation must be considered speculative as the extent of divergence between the DNA sequence of human and mouse actin genes is unknown, and the differential hybridisation observed with the mouse probe cannot necessarily be extrapolated to human DNA.

Cleveland et al (35) have used a chick non-muscle actin sequence to probe Southern blots of HindIII digested human DNA, and reported 6-10 hybridising fragments. However other faintly hybridising bands are visible in their autoradiographs, and we believe the difference in our observations can be

explained in our use of washes of varying stringency after hybridisation (as seen in Figures 3A and 3B).

Twelve actin recombinants have been picked from a human genomic library. Restriction enzyme mapping indicates that two of these recombinants are identical, and two are isolates of the same gene. Hybridisation with a 5' or 3' specific probe demonstrates that six of the recombinants contain an entire gene sequence, and four others contain sequences that hybridise only to either the 3' or 5' probe. Further analysis using restriction enzymes that cleave DNA frequently, reveals a widely different pattern of bands in the different recombinants. It also indicates that recombinant 26 may be an independent isolate of the 5' end of the gene cloned in recombinant 5. Calculations based on the probability of randomly picking 9 out of 11 different genes, indicate that there is a >90% chance of there being over 20 actin genes per haploid genome.

It should be noted that only one of the nine different recombinants generates two fragments that hybridise with an actin probe and thus contains an internal EcoRI site. This strengthens the interpretation that most of the hybridising bands in the genomic blots represent whole actin genes and thus that the gene number is approximately 20. The four recombinants containing only parts of an actin sequence may have been generated by the partial AluI/HaeIII digestion of the original DNA to construct the library. Further analysis of the location of these sequences in the cloned DNA is required, since the prediction would be that the partial actin sequences should be located at either end of the cloned human DNA.

The function of the 20 or so actin genes is unknown. It is possible that recent gene duplication for specific actin genes may have occurred, to give (for instance) two closely related muscle actin genes. Another possibility is that many of the sequences we detect may represent pseudogenes. Hybridisation with heterologous probes alone cannot distinguish pseudogenes and diverged real genes (36), although pseudogenes could be distinguished definitively by sequencing or by expression systems. Another possibility is that the other sequences are uncharacterised actin genes that are expressed in low amounts in cells, or are only expressed in specialised cells. Most protein analyses would not detect minor actins present at the 1% level. Several recent reports indicate that different actins may be present in different cell types (37,38).

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FOOTNOTE

The probability was calculated as follows. It was assumed that the phage pool represents an infinite number of copies of each clone of which 11 different examples were selected at random and analysed. If in the pool there are n unique actin-coding EcoRI fragments the probability (P) of observing r single repeats in m isolates is given by the formula

$$P(r \text{ repeats in } m/n) = \frac{n!}{(n-m+r)!} \frac{m!}{(m-2r)!2^r} \cdot \left(\frac{1}{n}\right)^m$$

This formula allows for the observed repeats to be obtained in any order of the m draws. For the values $r = 2$, $m = 11$ this formula is convergent and can be solved for different values of n . Between $n = 20$ and $n = 30$ the probability is a maximum, indicating that the isolates were picked from a pool of between 20-30 different genes. The probability that $n > 20$ is calculated to be >90% using the formula

$$P(N > 20) = 1 - \frac{\sum_{N=9}^{20} P(\text{data}/N)}{\sum_{N=9}^{\infty} P(\text{data}/N)}$$

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