Number and Evolutionary Conservation of α- and β-Tubulin and Cytoplasmic β- and γ-Actin Genes Using Specific Cloned cDNA Probes

Don W. Cleveland, Margaret A. Lopata, Raymond J. MacDonald, Nicholas J. Cowan,* William J. Rutter and Marc W. Kirschner
Department of Biochemistry and Biophysics
School of Medicine
University of California
San Francisco, California 94143
*Department of Biochemical Sciences
Princeton University
Princeton, New Jersey 08540

Summary

Bacterial clones containing inserted DNA sequences specific for α-α tubulin, β-tubulin, β-actin and γ-actin have been constructed from mRNA of embryonic chick brain. Plasmids containing approximately 75, 90 and >90%, respectively, of the sequences present in α-α tubulin, β-tubulin and β-actin mRNAs have been isolated as well as clones containing parts of the extensive 3' untranslated regions of the β- and γ-actin mRNAs. The sequences for the two tubulins do not cross hybridize. Hybridization of labeled, cloned probes for each of the tubulins with chicken DNA digested with several restriction endonucleases reveals about four fragments for (β- and four for β-tubulin. This seems to be the number of genes, since both the 5' and 3' ends of either cloned tubulin cDNAs hybridize to at least four common fragments in genomic DNA which has been digested with restriction endonucleases. The tubulin probes are able to hybridize under stringent conditions to DNA of all vertebrate genomes tested, as well as to sea urchin DNA, but not to yeast DNA. In digested sea urchin sperm DNA there are more than 20 different fragments which hybridize to both the 5' and 3' ends of the tubulin cDNAs. A full-length β-actin cDNA clone hybridizes to 4-7 bands in restricted chicken DNA and cross hybridizes to DNA from every other species tested, including sea urchin and yeast. Hybridization to chicken DNA of cloned probes specific for the 3' untranslated regions of β- and γ-actin mRNA indicates that the β sequence is present only once in the genome and the γ is present in at most three copies. Neither 3' untranslated sequence is conserved evolutionarily.

Introduction

Although α- and β-tubulin, the principal components of microtubules, are major structural proteins in all eucaryotic cells, little is known about the number and organization of the genes coding for these proteins. Reports of multiple α- or β-tubulins appear regularly (Feit, Sulsarek and Shelanski, 1971; Piperno and Luck, 1976; Bibring et al., 1976; Lu and Elzinga, 1977a; Feit, Neudeck and Gaskin, 1977; Stephens, 1978; Gozes and Littauer, 1978; Marotta, Harris and Gilbert, 1978; Sheir-Neiss, Lai and Morris, 1978; Gozes, Saya and Littauer, 1979). With the exception of work performed by Kemphues et al. (1979), however, in which evidence for an apparent genetically altered, testis-specific β-tubulin has been described, the evidence for multiple tubulins is confined to the appearance of multiple bands or spots on one- or two-dimensional protein gels. Since such heterogeneity may be post-translational or even artifactual, these electrophoretic studies cannot address the question of the genetic complexity of the tubulins. Protein sequencing has provided little information to date. Unlike actin, for which six distinct gene products have been identified by protein sequencing (Lu and Elzinga, 1977b; Vandekerckhove and Weber, 1978), no tubulin from any source has been fully sequenced.

To elucidate the genetic structure of the tubulin genes, we have constructed bacterial clones harboring plasmid-containing nucleic acid sequences complementary to α- or β-tubulin mRNA from embryonic chick brain. We have also identified a full-length cDNA clone for β-actin and two additional clones containing cDNAs specific for the 3' untranslated regions of β- or γ-actin mRNA, respectively. We have used these five cDNA clones to investigate the apparent complexity of the tubulins and actins within the chicken genome and the conservation of these sequences within other eucaryotic DNAs.

Results

Identification and Characterization of Clones

Bacterial clones containing inserted sequences complementary to α-tubulin, β-tubulin, β-actin and γ-actin mRNAs from embryonic chick brain were constructed as detailed in Experimental Procedures. Figure 1 shows plasmid DNA from five characterized clones which contain sequences of interest. In each case, the inserted cDNA sequences have been excised from the plasmid vector with the appropriate restriction endonuclease. Slot 4 contains a single 470 bp inserted sequence called A3 (excised from hybrid plasmid pA3) which codes for part of the 3' untranslated region of α-tubulin. Slot 5 contains a 590 bp inserted sequence called A2, which is complementary to the 3' untranslated region of γ-actin mRNA. Slot 6 shows a 2000 base inserted cDNA, A1, which codes for most if not all of the mRNA of β-actin. Slot 7 shows a 1400 base cDNA insert, T1, which codes for an α-tubulin and contains a single internal Pst I restriction site, and hence gives two insert fragments of 1125 and 280 bases. Finally, slot 8 displays the Pst I fragments of a clone, pT2, which contains 1700 bp of sequence complementary to β-tubulin mRNA. This β-tubulin
Figure 1. Excision of Cloned Tubulin and Actin cDNA Sequences

About 300 ng of supercoiled plasmid DNA were digested to completion with either Hind III or Pst I to excise the cloned cDNA sequence. The digest products were electrophoresed on a 1.5% agarose gel which was stained with 1 μg/ml of ethidium bromide to visualize the DNA fragments. (Slot 1) Hind III-digested λ DNA run as a molecular weight standard (bands are 23,000, 9800, 6600, 4500, 2200 and 490 bp, respectively); (slot 2) Hae III-digested αX174RF DNA, molecular weight standard (the four highest molecular weight bands visible are 1342, 1078, 872 and 606 bp); (slot 3) Hind III-digested linear pBR322; (slot 4) Hind III-digested plasmid pA3 DNA showing the linearized plasmid and a 470 base sequence complementary to the 3' untranslated region of α-actin mRNA; (slot 5) Hind III-digested DNA from plasmid pA2 containing a 590 bp sequence complementary to the 3' untranslated region of β-actin; (slot 6) Pst l-digested DNA from plasmid pA1 containing a 2000 bp sequence complementary to nearly the full length (if not all) of the β-actin mRNA; (slot 7) Pst l-digested DNA from plasmid pT1 containing a total insert length of 1400 bp coding for an α-tubulin mRNA—note that the 1400 base inserted sequence contains an internal Pst l site, yielding fragments of 1125 and 280 bp; (slot 8) Pst l-digested DNA from plasmid pT2 containing a total insert length of 1700 bp coding for β-tubulin mRNA—this inserted sequence has three internal Pst l sites, yielding fragments of 1250, 250, 170 and 35 bp. The 35 bp fragment is not visible on this gel.

cDNA sequence contains three internal Pst l sites, yielding restriction fragments of 1250, 250, 170 and 35 bp. The 35 bp fragment is not visible in this figure.

The clones shown in Figure 1 were screened from the initial population of transformants for the ability to hybridize 32P-labeled cDNAs reverse-transcribed from RNAs enriched in β-actin, β-tubulin or α-tubulin. A modification of the colony hybridization technique of Grunstein and Hogness (1975) (see Experimental Procedures) was used. Many clones were examined. Final identification of cloned sequences was made using a positive hybridization-translation assay. Linearized plasmid DNA was denatured, bound to nitrocellulose filters and used to select the complementary mRNA by hybridization to unfractionated poly(A)-containing mRNA from chick brain. The specifically hybridized mRNA was eluted and translated in vitro in the presence of 35S-methionine (see Experimental Procedures for details). The translation products were run on an 8.5% SDS-polycrylamide gel (Laemmli, 1970), the resolving gel for which had been adjusted to pH 9.1. A fluorograph of the gel is shown. (Slot 1) Translation of the initial mRNA; (slot 2) background translation of the carrier tRNA in the reticulocyte system; (slot 3) the in vitro translation products of mRNA specifically hybridized to DNA from plasmid pT2; (slot 4) the in vitro translation products of mRNA specifically hybridized to DNA from plasmid pT1; (slots 5–7) the translation products of mRNA specifically hybridized to DNA from (slot 5) plasmid pA1; (slot 6) plasmid pA3; (slot 7) plasmid pA2. Slots 1–4 are 1 hr exposures; slots 6 and 7 are 15 hr exposures.
cDNA Clones for α- and β-Tubulin and Actin

ized to plasmids pT2 and pT1: pT2 and pT1 clearly hybridize mRNAs, giving translation products that co-migrate with authentic β- and α-tubulin markers, respectively. Two-dimensional gel electrophoresis of these translation products shows that they co-electrophorese with the appropriate chick brain tubulin markers (data not shown). In addition, in collaboration with P. Valenzuela, M. Quiroga and J. Zaldivar, we have obtained confirmation of the assignments of pT2 and pT1 as β- and α-tubulin clones by partial DNA sequencing. For pT2, we have sequenced almost all the cDNA and have identified sequences corresponding to the known N terminal (Luduena and Woodward, 1973) and C terminal (Lu and Elzinga, 1978; H. Pone-stingl, personal communication) protein sequences for β-tubulin. pT2 thus contains the entire coding sequence of β-tubulin mRNA. For pT1, we have identified DNA sequences which code for two of the unordered cyanogen bromide fragments (a4CB and a4J) from beef brain α-tubulin (Lu and Elzinga, 1978).

Slots 5–7 of Figure 2 show the translation products from the actin clones pA1, pA2 and pA3. All three selectively hybridize to mRNAs which are translated to give polypeptides with mobilities corresponding to that of actin. To determine which actin polypeptide was represented in the translation products of the three actin clones, the translation products were examined on two-dimensional gels according to the method of O’Farrell (1975). The possibility that the translation products might be different was initially suggested by the absence of cross hybridization of inserts A2 and A3. The fluorographs of the regions containing actin on two-dimensional gels are shown in Figure 3. Figure 3A shows the translation products of unfractionated chick brain mRNA. β-Actin and γ-actin are detected in an apparent ratio of approximately 3:2. Other proteins, such as tubulin, are outside this region. Translation of mRNA selectively hybridized to plasmid pA1 yields a pattern of two actin spots (Figure 3B) indistinguishable from that obtained with the unfractionated mRNA. In contrast, the translation product of mRNA hybridized to plasmid pA3 (Figure 3C) yields only γ-actin. Similarly (Figure 3D), the translation of mRNA hybridized to the clone pA2 yields only β-actin. Figure 3E shows that both β- and γ-actins are present in a mixture of the translation products from clones pA2 and pA3, clearly demonstrating that the single spots of Figures 3C and 3D represent different actins. Clone pA1, which hybridizes to both β- and γ-actin mRNAs, hybridizes only to pA2 and not to pA3. The failure to detect cross hybridization between pA2 and pA3 suggests that neither of the short actin inserts contains appreciable (if any) sequences coding for translated portions of either actin mRNA. We have used partial DNA sequencing to confirm that both A2 and A3 are copies of the 3′ ends of the respective mRNAs [that is, A2 and A3 both contain a 3′ poly(A) tail] and that neither A2 nor A3 contains any sequences which, when translated, correspond to actin protein sequences. The strong cross hybridization of the clones pA1 and pA2 along with the estimate of the size of the β-actin mRNA (see below) suggests that the 2000 base cDNA sequence of pA1 is a full- (or nearly full) length copy of β-actin mRNA.

Size Estimate of the Tubulin and Actin mRNAs

The size of the tubulin and actin mRNAs was determined from gel electrophoresis of unfractionated
mRNA followed by covalent attachment to paper and localization by hybridization with specific probes. Slot 1 of Figure 4 displays an RNA band of approximately 2000 bases which hybridizes to a probe prepared from the clone pA2, the 3' untranslated region of β-actin mRNA. Similarly, slot 2 also shows a single band at 2000 base, although a very faint band at 2700 bases can also be observed in longer exposures. In slot 3 a band of 2000 bases hybridizes to the probe copied from the clone pA3, the 3' untranslated region of γ-actin mRNA. A mixture of β-actin and γ-actin probes displays only one band, suggesting that β- and γ-actin mRNAs are virtually identical in molecular weight (not shown). In slot 5 a band of 1900 bases is detected with the α-tubulin-specific probe from plasmid pT1.

Two higher molecular weight bands of 3000 and 4300 bases can also be seen in slot 4; these RNAs may be precursors to the mature β-tubulin mRNA.

Hybridization of Cloned Tubulin and Actin Probes to Genomic Chicken DNA Digested with Restriction Endonucleases

High molecular weight chicken DNA was prepared (Hughes et al., 1979); digested with Hind III, Eco RI, Pst I or Bam HI; electrophoresed on an agarose gel and transferred to nitrocellulose (Southern, 1975). Figure 5 shows hybridization of labeled probes transcribed from the 2000 base β-actin, the 590 base 3' untranslated region of β-actin mRNA; (slot 2) the insert from pA1, a 2000 bp full-length copy of β-actin mRNA; (slot 3) the insert from pA3, a 470 base 3' untranslated region of γ-actin mRNA; (slot 4) the 1250 bp largest inserted sequence in plasmid pT2, the β-tubulin-specific probe, whereas in slot 5 a band of 1900 bases is detected with the α-tubulin-specific probe from plasmid pT1. Two higher molecular weight bands of 3000 and 4300 bases can also be seen in slot 4; these RNAs may be precursors to the mature β-tubulin mRNA.

Figure 4. Estimate of the Size of the Tubulin and Actin mRNA from Chick Brain

A 1.2% agarose gel containing 10 mM methylmercury hydroxide was loaded with five aliquots (5 µg each) of total poly(A)-containing RNA from embryonic chick brain. This RNA was transferred and covalently bound to a cellulose filter by the method of Alwine et al. (1977). The filter was cut into strips and hybridized against cloned 32P-labeled probes made from (slot 1) the insert from plasmid pA2, a 590 bp 3' untranslated region of β-actin mRNA; (slot 2) the insert from pA1, a 2000 bp full-length copy of β-actin mRNA; (slot 3) the insert from pA3, a 470 bp 3' untranslated region of γ-actin mRNA; (slot 4) the 1250 bp largest inserted sequence in plasmid pT2, the β-tubulin clone; (slot 5) the 1125 bp largest Pst I fragment from plasmid pT1, the α- tubulin clone. The strips were taped back together into a single filter after hybridization and exposed to Kodak RP2 X-omat film with an intensifying screen.

Figure 6 shows multiple chicken genomic fragments which hybridized to the α- and β-tubulin cDNA clones. Neither of these probes contains a restriction site for any of the four endonucleases used to digest the chicken DNA (although Bam HI cleaves the β-tubulin probe once). The autoradiograms of the hybridization of these probes to the filters containing digested chicken DNA are shown in Figures 6A and 6B for α- and β-tubulin. Three to five strongly hybridizing restriction fragments can be seen in each digestion with either of the probes. A number of very weakly hybridizing fragments are also visible.

Estimation of the Number of α- and β-Tubulin Genes by Hybridization to Probes Constructed from the Cloned cDNAs Corresponding to the 5' and 3' Ends of the Tubulin mRNAs

Figure 6 shows multiple chicken genomic fragments which hybridized to the α- and β-tubulin probes and were generated by digestion with restriction endonucleases that did not cut either probe. This result suggested that within the chicken genome there were multiple α- and β-tubulin genes, or that within unique genes for α- and β- there were multiple intervening
sequences which contained sequences recognized by the endonucleases, or both. To test whether individual restriction fragments which hybridized to the α- or β-tubulin probes contained sequences complementary to both 5' and 3' ends of the cloned cDNA probes, four additional filters of digested chicken DNA were prepared as in Figure 6. The 5' and 3' ends of the cDNA complementary to α-tubulin mRNA were prepared by digesting pT1 with Sal I, which cleaves once within the vector pBR322 and once within the cloned cDNA. The two resultant fragments, one containing 750 bp of sequence distal to the 3' end of the α-tubulin mRNA and the second containing 650 bp of sequence from the 3' end of the mRNA, were separated by electrophoresis and labeled by reverse transcription. Labeled probes for the 5' and 3' ends of the cloned β-tubulin cDNA were similarly prepared by digestion of pT2 with Bam HI, which cleaves once within pBR322 and once nearly midway in the 1250 bp Pst I fragment of T2. (Note that DNA sequencing and restriction site mapping of T2 place the portion of the β-tubulin cDNA complementary to the 3' end of the β-tubulin mRNA on this 1250 bp Pst I fragment, and that the three internal Pst I sites within the cloned β-tubulin cDNA are 5' to this 1250 bp fragment.)

The fragments of digested chicken DNA which contain sequences complementary to the 5' and 3' ends of the α-tubulin cDNA are shown in Figures 7A and 7B. As shown in slot 1, three bands are common to both 5' and 3' probes. In slots 2 and 3 all four strongly hybridizing fragments are detected by both 5' and 3' probes. In slot 4 the Bam HI digest shows three bands and a complex band which hybridize to both 5' and 3' probes. These data argue that there must be at least three or four genes with sequences complementary to α-tubulin in the chicken genome; the presence of intervening sequences within a single gene would allow only a single fragment to contain sequences complementary to both 5' and 3' probes. However, not all of the fragments which hybridize to the 5' probe also hybridize to the 3' probe, specifically two such fragments in slot 3 and four in slot 4 of Figures 7A and 7B. In addition, there are two fragments in slot 3 and one in slot 4 which hybridize to the 3' probe but not to the 5' probe. The appearance of such non-cross hybridizing fragments is consistent with

— the presence within a gene of intervening DNA sequences which contain restriction sites not present in the cDNA,

— the absence of restriction sites in the cDNA as a result of an error by the reverse transcriptase in the initial construction of the cDNA.

The data presented here do not distinguish among
Figure 7. cDNA Probes Complementary to the 5' and 3' Ends of α-Tubulin mRNA Hybridized against Restriction Endonuclease-Digested Genomic Chicken DNA

Chicken DNA was digested with a 5 fold excess of each of four restriction enzymes, displayed (5 μg per slot) on a 0.8% agarose gel and transferred to nitrocellulose. The filters were then hybridized against 32P-labeled probes copied from the end of the cloned α-tubulin cDNA complementary to (A) the 5' portion of the mRNA or (B) the 3' portion of the mRNA. Slots 1-4 in (A) and (B) contain DNA digested with Eco RI, Hind III, Pst I and Bam HI, respectively.

These possible explanations for the differences in the 5' and 3' patterns. However, although the differences between the patterns are difficult to interpret, the existence of fragments which hybridize to both the 3' and 5' ends of α-tubulin mRNA constitute strong evidence for the presence of at least three or four genes for α-tubulin.

The analogous experiment with the 5' and 3' ends of the β-tubulin cDNA is shown in Figures 8A and 8B. For example, note slots 1, 2 and 3, in which all four prominent fragments hybridize to both 5' and 3' probes. Three common hybridizing fragments are found in slot 4. These data indicate the presence of at least three or four β-tubulin genes in chicken. The interpretation of the results in Figures 7 and 8 to indicate the presence of multiple α- or β-tubulin genes assumes that the 5' probes do not hybridize to the corresponding 3' probes. We have been unable to detect any such cross hybridization for either α or β probes (data not shown).

Evolutionary Conservation of Tubulin and Actin Detected by Hybridization of cDNA Probes to DNAs from Other Species

We examined the ability of the actin and tubulin probes to hybridize to DNA from sea urchin, angilar fish, chicken, mouse, rat and human. Aliquots of each DNA were digested with Hind III, electrophoresed on agarose and transferred to nitrocellulose. Autoradiograms of the resultant filters after hybridization to 32P-labeled α- or β-tubulin cloned probes are shown in Figures 9A and 9B for the yeast, sea urchin, chicken and human DNAs. Slot 3 of both figures shows the characteristic Hind III pattern for chicken DNA with α- and β-tubulin probes (compare with Figure 6). Strong cross hybridization is observed with both the α and β probes for all DNAs tested, although at this level of stringency no signal was detected with yeast DNA (Figure 9, slots 1). Human DNA showed on the order of ten bands for α-tubulin and ten bands for β-tubulin. These values are comparable to those obtained with rat, mouse or fish DNA (data not shown). The sea urchin DNA shows more than 20 strongly hybridizing bands with both α- and β- probes. Furthermore, more than 20 bands have been observed in sea urchin DNA digested with three other restriction endonucleases, and more than three fourths of these bands hybridize to both the 5' and 3' probes of either the α- or β-tubulin cDNAs (data not shown).

A parallel experiment using the three actin probes is shown in Figure 10. Slot 3 of Figures 10A, 10B and 10C again shows the characteristic bands for the 3' untranslated β-actin probe, the full-length β-actin...
cDNA Clones for α- and β-Tubulin and Actin

9.8 - 6.6 - 4.5 - 2.2 -
1234 1234 1234

Figure 9. Tubulin Probes Hybridized against Restricted DNAs from Yeast, Sea Urchin, Chicken and Human
5 μg aliquots of DNA (only 2.5 μg from yeast) were restricted with a 5 fold excess of Hind III, electrophoresed on an agarose gel and blotted onto nitrocellulose. Slots 1-4 in (A) and (B) represent restricted yeast, sea urchin, chicken and human DNAs, respectively. Hybridization of 32P-labeled probe constructed from (A) the 1125 bp fragment of plasmid pT1, the α-tubulin clone, and (B) the 1250 bp fragment of plasmid pT2, the β-tubulin clone. Molecular weight markers (in kb) are shown at the left.

The construction and identification of cDNA clones containing sequences complementary to α-tubulin, β-tubulin, β-actin and γ-actin mRNAs allows new approaches to a number of significant questions concerning the genetic organization and expression of these structural proteins. In the present paper we report initial experiments designed to answer some of these general questions. Hybrid plasmids containing cDNA sequences complementary to α-tubulin, β-tubulin, β-actin and γ-actin mRNAs were identified by specific hybridization of mRNA followed by translation and analysis by gel electrophoresis. Confirmation of the identities of both the α- and β-tubulin clones has been obtained by DNA sequencing. The apparent size of the mRNAs identified with labeled, cloned probes (Figure 4) indicates that the α- and β-tubulin clones must contain 75 and >90%, respectively, of the sequences present in the mature mRNAs. Two presumptive precursor RNAs of 3000 and 4300 bases have also been identified for β-tubulin. Similarly, β- and γ- actin mRNAs from chick brain have each been estimated to be about 2000 bases long. This is a slightly larger estimate (especially for the γ-actin mRNA) than that previously made for the chick brain actin mRNAs using elution from agarose gels containing methylmercury and translation as an assay (Cleveland, Kirschner and Cowan, 1978). Since clone pA1 carries a cDNA sequence which is 2000 bases long and complementary to β-actin, this clone must contain virtually the entire β-actin mRNA sequence.

The tubulins are widely assumed to be evolutionarily conserved proteins. This assumption is based chiefly on the observations that tubulins from a wide variety of eucaryotic sources migrate with comparable mobilities on one- or two-dimensional gels and that the tubulins from these diverse sources will form co-polymers (for example see Snyder and McIntosh, 1976; Kirschner, 1978 for reviews). In addition, N terminal protein sequence data for the first 25 amino acids have indicated nearly 100% conservation between chicken and sea urchin tubulins (Luduena and Woodward, 1973). We have found strong cross hybridization of both α- and β-tubulin mRNA sequences from chicken to DNAs of all higher eucaryotic organisms tested. Only in more primitive species such as yeast and Aspergillus (D. Kirsch and R. Morris, unpublished data) have such hybridization attempts failed under stringent hybridization conditions. The absence of strong cross hybridization to yeast with these tubulin probes is consistent with reports of phenotypic differences between yeast and higher eucaryotic tubulin, such as the failure of yeast tubulin to bind colchicine...
(Haber et al., 1972). Under less stringent conditions, however, N. Neff and D. Botstein have recently detected hybridization to single bands in yeast DNA, and N. Agabian has demonstrated hybridization to DNA from trypanosomes (T. gambiense) using either of these α- or β-tubulin probes. This high degree of conservation of the nucleic acid sequence is not mandated by protein sequence conservation, since considerable mismatch can occur due to third base substitution. This result might suggest that for these proteins evolutionary pressures have affected the entire nucleic acid sequence.

The appearance of 3–4 fragments in restriction endonuclease-digested chicken DNA containing sequences complementary to both the 5' and 3' ends of either α- or β-tubulin cDNAs is consistent with the presence of about four α- and four β-tubulin genes in the chicken genome. At this time we cannot rule out the possibility that multiple genes may be present on some or all of the larger restriction fragments, that duplicated genes which yield identical restriction fragments may also be present, or that some of the more weakly hybridizing fragments may represent pseudogenes similar to X and Y in the chick ovalbumin system (Royal et al., 1979). The simplest interpretation, however, would suggest that a large number of different tubulins are not functionally required even for higher eucaryotic species. On the other hand, the more than 15 fragments in sea urchin DNA which hybridize to both ends of either α- or β-tubulin cDNA argue for a much larger number of genes in this species. Indeed, the multitude of hybridizing fragments in sea urchin DNA is puzzling from a functional point of view, but may reflect a special property of the organization of the sea urchin tubulin genes. In any case, for all DNAs tested, the fragments from restriction endonuclease digests that hybridize to α-tubulin probes appear to be distinct from those selected by β-tubulin probes. Hence, although limited protein sequence data for the N termini of α- or β-tubulin suggest that α- and β-tubulin arise from a common precursor, we have no evidence of a strongly conserved sequence homology between the tubulins at the genomic nucleic acid level. In addition, we observed no hybridization of labeled probe copied from the 1.2 kb α-tubulin Pst I fragment to the β-tubulin clone (linearized plasmid plus insert), or of labeled probe produced from the 1.2 kb β-tubulin Pst I fragment to the α-tubulin clone (D.W. Cleveland, unpublished data).

The full-length β-actin probe hybridizes to 4–7 restriction fragments in chicken DNAs and 6–7 fragments in human and sea urchin DNAs. This is not surprising since Vandeckerckhove and Weber (1978) have found (by protein sequencing) at least six actins in mammals. Therefore, in higher organisms such as chickens and humans, the number of total actin genes appears to be at most 5–7. Of these there appears to be only a single β-actin gene and at most three γ-actin genes. We have independent evidence from chromosome fractionation studies that at least two of the three apparent γ-actin genes are on separate chromosomes (D. W. Cleveland et al., unpublished results). These extensive 3' untranslated sequences of β- and γ-actin, unlike the coding sequences, do not appear to be evolutionarily conserved. The total number of six or seven actin genes in chicken contrasts with the larger estimate of actin genes in Dictyostelium (Kindie and Firtel, 1978), where 15–20 bands were detected on Southern blots. Thus we have two examples of highly conserved genes apparently present in larger numbers in primitive species than in more advanced species: tubulin genes in sea urchin and actin genes in Dictyostelium. The significantly smaller number of genes detected in the chicken must suggest caution in interpreting functional heterogeneity on the basis of apparent genetic complexity.

Experimental Procedures

Isolation of mRNA from Embryonic Chick Brain

Free polysomes were prepared from 14–15 day embryonic chicken brains essentially by the method of Harrison, Brownline and Mitewin (1974), as modified by Cleveland et al. (1978). For the selection of poly(A)-containing RNA, polysome pellets were dissolved and passed over oligo(dT)-cellulose as described (Cleveland et al., 1976). This poly(A)-containing RNA was then enriched in tubulin and actin sequences by collection of the 18S region of a sucrose gradient to which total poly(A)-containing RNA had been applied (Cleveland et al., 1978). Confirmation of the presence of tubulin and actin mRNAs was obtained by in vitro translation using the cell-free rabbit reticulocyte system described by Pelham and Jackson (1976).

For preparation of RNA to be used to estimate the sizes of the tubulin and actin mRNAs, total RNA from embryonic chick brain was prepared by the guanidine thiocyanate-cesium chloride method of Chirgwin et al. (1979), and poly(A)-containing RNA was selected on an oligo(dT)-cellulose column.

Preparation of cDNA Used in Cloning the Untranslated Regions of β-Actin and γ-Actin mRNA

Starting with 25 μg of embryonic chicken brain mRNA, first and second strand cDNA reactions were performed using avian myeloblastosis virus reverse transcriptase (Beard, Life Sciences) as described by Ullrich et al. (1977), with the exception that [3H]-dCTP was used in place of a-32P-dCTP to label the cDNA. The double-stranded cDNA was then treated with S1 nuclease to create blunt ends and ligated to 32P-labeled Hind III decanucleotide linkers (Collaborative Research) as described by Ullrich et al. (1977) and Seeburg et al. (1977). The ligation mixture was treated with Hind III endonuclease.

High molecular weight RNA (>400 bp) was purified on a 5% polyacrylamide gel (using the buffer system of Peacock and Dingman, 1964) and ligated to plasmid pDI032 which had been linearized with Hind III and (repeated with alkaline phosphatase (a gift from W. Swain, UCSF, who prepared it according to the method of Ullrich et al., 1977). This ligated preparation was used to transform E. coli J1778 (see below).

Preparation of cDNA for the α-Tubulin, β-Tubulin and Full-Length β-Actin Clone

Double-stranded cDNA was constructed from 25 μg of embryonic chick brain mRNA essentially by the protocol of Wickens, Buell and Schimke (1977). The first strand reaction was performed at 42°C for 60 min in the presence of 140 mm KCl. The second strand synthesis used DNA polymerase I (New England Biolabs) at a final concentration of 50 U/μg of initial mRNA. Double-stranded cDNA was phenol-extracted and chromatographed on Sephadex G-75, and excluded...
cDNA Clones for α- and β-Tubulin and Actin

Transferase (BRL) according to the protocol of Chang et al. (1978). The fraction of the total cDNA (approximately 7 µl) between 1000 and 2500 bp was identified by sizing the cDNA on an agarose gel. The appropriate region was excised and the cDNA was eluted and precipitated with 50 µg of yeast tRNA (Miles). This sized, tag-cut, double-stranded cDNA was mixed at a weight ratio of 1:3 with plasmid pBR322 which had been linearized at the PstI restriction site and tagged with approximately 15 guanosine residues (a gift from J. Edman, UCSF). Total DNA concentration was 1 µg/ml. The mixture was incubated at 65°C for 2 min and then incubated for 2 hr at 4°C, followed by 2 hr at 30°C and finally 1 hr at 14°C. IRNA was added to 20 µg/ml and the sample was ethanol-precipitated, pelleted, dried and resuspended in 20 µl DH2O and stored for transformation.

Transformation of χ1776

Hybrid DNA, ligated using either the Hind III linkers or the GC tailing method, was used to transform χ1776 (provided by R. Curtis) according to the protocol of Goodman and MacDonald (1979). All manipulations were performed in a P3 facility as described in the NIH Guidelines for research involving recombinant DNA molecules.

Screening of Recombinant Clones

Recombinants cloned in the Hind III site were initially selected for ampicillin resistance (50 µg/ml in agar plates) and cloned in the Pst I site for tetracycline resistance (20 µg/ml in agar plates). Surviving colonies were then screened for sensitivity to tetracycline (for the Hind III clones) and to ampicillin (for the Pst I clones). Colonies possessing the appropriate drug responses were then screened according to the Craig, McCarthy and Wadswoth (1979) modification of the colony hybridization protocol of Grunstein and Hogness (1975). Colonies were grown on Whittam's 541 filter plates for 2 days and the filters were processed (Craig et al., 1979). χP-labeled probes enriched in β-actin, α-tubulin or β-tubulin sequences were constructed (see below) by reverse transcription using an oligo(dT)10 primer of mRNA enriched in the appropriate sequences. Such enriched mRNAs were prepared as previously described (see Figure 7, slots 4, 7 and 13, of Cleveland et al. (1978) for the mRNA enriched in β-actin, β-tubulin and α-tubulin, respectively). The χP-labeled probes were hybridized to the filters according to the hybridization procedure (see below) for nitrocellulose filters. Strongly hybridizing colonies were identified by autoradiography on Kodak RP-2 X-omat film and DuPont Lightning Plus intensifying screens. Supercoiled plasmids were purified (Bolivar et al., 1977) from chloramphenicol-amplified, lysed, and cleared cells by propidium iodide-enriched in β-actin, P-tubulin and α-tubulin, respectively. 32P-labeled mRNA was incorporated by boiling each filter in 300 µl of distilled water for 90 sec. Carrier mRNA (5 µg) was added and eluted mRNA and carrier were precipitated with ethanol. The precipitate was collected, dried, resuspended in 2 µl of distilled water and added to a 20 µl aliquot of 32S-methionine-containing rabbit reticulocyte lysate (Pelham and Jackson, 1976) for in vitro translation. Translation products were identified by analysis on 6.6% polyacrylamide gels (Laemmli, 1970) followed by fluorography (Bonner and Laskey, 1974).

For clones which hybridized actin mRNA, the translation products were analyzed further on two-dimensional gels according to the method of O Farrell (1977).

Gel Electrophoresis

Gel electrophoresis of DNA was carried out on vertical slab gels cast from 0.8–1.2% agarose (Sigma) containing 10 mM sodium acetate, 2 mM EDTA (pH 8.1). Gels were stained with 1 µg/ml of ethidium bromide for 30 min prior to photographing on Polaroid film using ultraviolet illumination.

For electrophoresis of RNA, 1.5% agarose gels containing 10 mM methyleneglycine hydroxide were run according to the procedure of Bailey and Davidson (1976). These gels were stained with 10 µg/ml of ethidium bromide for visualization.

Filter Hybridization

DNA in agarose gels was transferred to nitrocellulose filters according to the method of Southern (1975). After transfer, the filters were heated to 30°C for 2 hr and "prehybridized" overnight at 41°C in 50% formamide, 3 x SSC, 20 mM HEPES (pH 7.4), 1 mM EDTA, 100 µg/ml yeast RNA, 0.01% bovine serum albumin, 0.01% Ficoll 400, 0.01% polyvinylpyrrolidone 360, and 10 µg/ml sonicated, denatured E.coli DNA. This prehybridization solution was removed and replaced with a fresh aliquot of the same solution containing 1–5 x 105 dpm of χP-labeled probe (see below), and hybridization was allowed to occur at 41°C for 24–48 hr. After incubation, the filters were washed three times in 0.1% SDS, 0.1 x SSC at 50°C for a total time of about 1 hr. The filters were then exposed using Kodak RP-2 X-omat film and DuPont Lightning Plus intensifying screens.

For transfer of RNA from agarose gels containing methyleneglycine hydroxide to diazobenzyloxymethyl-paper. Whatman 541 paper was deribavilated and activated, and RNA was transferred from the agarose gel according to the method of Abnormally, Kemp and Kark (1977). After completion of the transfer (24 hr), the paper was dried at room temperature and prehybridized as above for nitrocellulose with the exception that 10 mg/ml of glycine were included in the prehybridization solution. Hybridization of labeled probes was as detailed above for nitrocellulose.

In Vitro Labeling of RNA and DNA

To prepare χP-labeled cDNA for use in colony hybridization screening, mRNA was reverse-transcribed using reverse transcriptase. Typically, a 20 µl reaction contained 0.1 µg of mRNA; 50 mM Tris-Cl (pH 8.3); 0.5 mM each of dCTP, dATP and dTTP; 10 mM MgCl2; 20 mM dithiothreitol; 90 µg/ml oligo(dT)12–18; 8 units of reverse transcriptase; and 50 µCi of χP-dCTP (400 Ci/mmole). The reaction was incubated for 1 hr at 42°C. After hydrolysis of the RNA with 0.1 N NaOH at 39°C overnight, the labeled cDNA was freed of unincorporated χP by chromatography on Sephadex G-75.

Cloned cDNA sequences were labeled in vitro with χP essentially as described by Shank et al. (1978). Briefly, high specific activity (400 Ci/mmole) χP-dCTP was incorporated into short DNA segments by reverse transcriptase using cloned DNAs as templates. DNA synthesis was primed with a 1000 fold weight excess of oligomers derived from calf thymus DNA. Template is limiting in these reactions and all segments of the DNA are copied efficiently.

Determination of Genomic DNA with Restriction Endonucleases

DNA samples to be digested with Eco RI, Hind III, Bam HI or Pst I were incubated at 37°C in (the appropriate buffers as given by New...
England and Rhode Island, with a 5% loss of seeds from one mould. They were then collected in Rhode Island and stored in a tube containing 800 ng of supercoiled pBR322. The test samples were incubated in parallel with the digest with only genomic DNA for 4 hr at 37°C. The test samples were analyzed by electrophoresis on a 0.8% agarose gel; digests were judged to be complete if the test sample DNA was completely converted from the supercoiled to the linear form.

Preparation of Genomic DNAs
Chicken genomic DNA was prepared from 11 day chick embryos by the method of Hughes et al. (1979). DNA from Strongylocentrotus purpuratus was prepared from the sperm of a single sea urchin (provided by B. Nagle and D. Mazia) in an analogous fashion. Yeast genomic DNA (Saccharomyces cerevisiae) was a gift from J. Edman (UCSF); human, mouse and rat DNAs were each prepared from tissues of single individuals and were provided by R. Pictet and G. Bell (UCSF).

Acknowledgments
We thank William Swain, Jeff Edman and Graeme Bell for helpful suggestions and for making several valuable DNA samples available to us.

D.W.C. is the recipient of a Chaim Weizmann postdoctoral fellowship. This work has been supported by grants from M.W.K., N.J.C. and W.J.R. from the NIH, the American Cancer Society and the Whitehead Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 26, 1979; revised February 11, 1980

References


D.W.C. is the recipient of a Chaim Weizmann postdoctoral fellowship. This work has been supported by grants from M.W.K., N.J.C. and W.J.R. from the NIH, the American Cancer Society and the Whitehead Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 26, 1979; revised February 11, 1980

References


cDNA Clones for α- and β-Tubulin and Actin

2167


