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(54) Title: A cDNA CLONE FOR HUMAN TYROSINASE

(57) Abstract

The gene for human tyrosinase was cloned as a cDNA sequence prepared as a part of a lambda gt library of normal human melanocytes. The cloned gene encodes a polypeptide of 548 amino acids with a molecular weight of 62, 160 excluding a hydrophobic signal peptide.

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A cDNA CLONE FOR HUMAN TYROSINASE

BACKGROUND OF THE INVENTION

Human tyrosinase is an essential enzyme which regulates the production of melanin, a group of brown or black pigments in the skin and eyes of humans. The lack of a human tyrosinase gene in usable form has greatly limited medical research in the field of albinism, and other medical and nonmedical applications relating to the control of pigment production in human melanocyte cells. This invention relates to the discovery of the cDNA gene which expresses human tyrosinase. When the cDNA is fused to an expression vector, the cDNA is useful to produce pure tyrosinase. When used as a cDNA probe the DNA is useful in the production or the control of production of human melanin. As a cDNA probe the cDNA gene is useful for genetic analysis of human albinism and melanotic and amelanotic melanoma, and also prenatal diagnosis of albinism.

There has been a minimum of research on the genetic control of melanin formation because of the lack of availability of suitable nucleic acid probe (gene sequence). cDNA for human tyrosinase is a key material for the study. The material will serve to understand why some melanoma cells lose the expression of tyrosinase gene and become more invasive. Differences in tyrosinase expression in cancer cells make it possible that the gene probe can be used in understanding and as a marker for malignancy. Understanding of the regulation of melanin biosynthesis will lead to the development of a rational chemotherapy of human melanoma because the intermediate substances of melanin are known to be toxic to pigment cells. Deletion of genes around albino locus (tyrosinase structural gene) becomes lethal in mice. The cDNA probe will open up a new research area to identify genes causing lethal effect in mouse embryo.

SUMMARY OF THE PRESENT INVENTION

The present invention resulted when a λ gt11 cDNA library of normal human melanocytes were screened with antibodies directed against purified hamster tyrosinase. Sixteen independent clones which gave a positive signal were isolated from 5 x 10^5 independent plaques. cDNA inserts of 13 clones among the 16 candidates cross-hybridized with each other, indicating that they were from related mRNA species. mRNA homologous to a representative cDNA λ mel 34 was expressed specifically in melanocytes, detecting an approximately 2.4 kb mRNA species of human melanocytes. The nucleotide sequence of the three overlapping cDNA inserts spanning 1.88 kb was determined and an amino acid sequence was deduced. The human tyrosinase is composed of 548 amino acids with a molecular weight of 62,160 excluding a hydrophobic signal peptide. Mouse genomic DNA blot analysis revealed that the gene for λ mel 34 was deleted in albino mouse homozygous for the deletion at and around the albino locus on chromosome 7. It is concluded that λ mel 34 contained cDNA encoding human tyrosinase. Moreover, the cDNA gene for human tyrosinase was recovered from the λ mel 34 and can be used for many purposes including the production of pure human tyrosinase.

In addition, the three clones of the 11 not represented by λ mel 34 are represented by λ mel 17-1. The gene for λ mel 17-1 cDNA was not mapped at the albino locus, detected single hybridizing restriction fragment in human and mouse DNA, and was highly conserved from mouse to human. The abundance of λ mel 17-1 cRNA paralleled the melanin content in human and mouse melanocytes. The expression of λ mel 17-1 cRNA was elevated after stimulation of mouse and human melanoma cells with MSH or/and IBMX, and U-V light (such as suntan). This was also closely correlated with the elevation of melanin content. The fact that the λ mel 17-1 gene is conserved evolutionarily indicates that the molecule encoded by the λ mel 17-1 has biologiacally important functions. The expression of that gene is controlled by hormones (MSH) or U-V light and positively correlated with the melanin content. These data indicate that the gene is involved in melanin biosynthesis in addition to tyrosinase. Current studies suggest that λ mel 17-1 gene product act on melanin

biosynthesis's pathway distal to tyrosinase.

It is a primary object of the present invention to provide a new and improved cDNA gene which expresses human tyrosinase.

It is another object of the present invention to identify a new and improved cDNA gene which expresses human tyrosinase by its nucleotide sequence.

It is still another object of the present invention to teach that the cDNA from bacteriophage λ mel 34 may be used as a probe for the production or control of production of human melanin.

It is another object of the present invention to teach that the cDNA gene contained in the λ mel.34 is useful as a probe for genetic analysis of human albinism and melanotic and amelanotic melanoma, and also for prenatal diagnostics for albinism.

It is another object of the present invention to teach that the cDNA gene contained in λ mel 17-1 is useful as a probe for detecting the change in the degree of melanization of normal human melanocytes and melanoma cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 in three panels depicts the isolation of human tyrosinase cDNA by antibody screening.

Figure 2 is a Northern blot analysis of poly(A)$^+$ RNA derived from normal melanocyte, human melanotic melanoma (LC), HePG-2, HL-60, human and mouse neuroblastoma.

Figure 3 is a SDS PAGE presentation of the detection of λ mel 16 beta-galactosidase tyrosinase fusion protein.

Figure 4 is a Southern blot analysis of genomic DNA of deletion homozygous (c^{3H}/c^{3H}) in newborn mice.

Figure 5 is a Partial Restriction map and sequencing strategy for human tyrosinase cDNA.

Figures 6A, 6B, 6C and 6D together are a table showing the nucleotide sequence of cDNA encoding human tyrosinase and its deduced amino acid sequence.

DETAILED DESCRIPTION OF THE INVENTION

As used herein the following abbreviations are used: Dopa, 3,4-dihydroxyphenylalanine; MSH, melanocyte stimulating hormone; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; IBMX, isobutylmethylxanthine; TBS, 50 m M Tris HCL, pH 8.0, 0.15 M NaCl; IPTG, isopropyl-beta-D-thiogalactopyranoside; PMSF, phenylmethylsolforyl fluoride; PBS, 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SSC, 0.3 M sodium chloride, 0.03 M sodium citrate; Denhardt, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin.

Described herein is the isolation of a cDNA clone for human tyrosinase. Since human tyrosinase has not been sequenced, the following properties of the cDNA lead us to conclude that the mRNA homologous to λ mel 34 cDNA encodes authentic human tyrosinase: 1) the protein encoded by the cDNA binds to antityrosinase antibodies; 2) the mRNA corresponding to the cDNA (cRNA) is expressed in melanocytes but not in human neuroblastoma, lymphocytes, fibroblasts or hepatoma cells; 3) the molecular weight of protein encoded by λ mel 34 and λ mel 34-related cDNA clones is in good agreement with the reported size of de novo form of human tyrosinase (Hermann, W.P. and Uhlenbruck, G. (1975) Arch. Dermatol. Res. 254 , 275-280.); and 4) the mouse gene corresponding to 34 cDNA is mapped at the albino locus of the mouse chromosome 7.

The availability of tyrosinase cDNA will open up a way to investigate the cis-acting regulatory sequence responsible for melanocyte-specific gene expression and the molecular genetic basis of human albinism and the related genetic disorders such as herein discussed (Witkop, C.J., Jr. (1984) In Clinics in Dermatology Vol. 2 (1), pp 70-98, J.B. Lippincott Co., U.S.A.). There are several varieties of human albinism (Witkop, C.J. Jr., Quevodo, W., Jr. and Fitzpatrick, T.B. (1983) In the Metabolic Basis of Inherited Disease. pp 301-346 (Stanbury, J.B., Wyngarden, J.B., Fredrickson, D.S., Goldstein, J.L., Brown, M.S., eds)

McGraw-Hill, New York; King, R.A. and Witkop, C.J., Jr. (1976) Nature 263 , 69-71; King, R.A. and Witkop, C.J., Jr. (1977) Am. J. Human Genet. 29 , 164-168; King, R.A., Olds, D.P., and Witkop, C.J., Jr. (1978) J. Invest, Dermatol. 71 , 136-139; King, R.A. and Olds, D.P. (1981) J. Invest. Dermatol. 77 , 201-204), and in some of them a normal level of hair bulb tyrosinase can be measured. It may be possible, using the cDNA probe, to determine which of the differences are related to mutations in the tyrosinase structural gene and which may be due to mutations which affect synthesis of melanosomes, packaging of enzyme into melanosomes, or degradation of the enzyme. In conjunction with the accumulated genetic information on various mouse mutants affecting coat and eye color, the cDNA probe may be used to classify the molecular genetic nature of the mutations. There are strong indications for the existence of regulatory factors encoded near the mouse albino locus which appear to control various genes that map on other chromosomes, such as liver-specific enzymes and serum proteins (Gluecksohn- Waelsch, S. (1979) Cell 16 , 225-237; Schmid, N. Muller, G., Schutz, G. and Gluecksohn-Waelsch, S. (1985) Proc. Natl. Acad. Sci. USA 82 , 2866-2869; Loose, D.S., Schaw, P.A., Krauter, K.S., Robinson, C., Englard, S. Hanson, R.W. and Gluecksohn-Waelsch, S. (1986) Pro. Catl. Acad. Sci. USA, 83 , 5184-5188). Since mel 34 detects the albino locus in mice this can be a starting material to identify such transacting regulatory genes in mice.

Posession of the cDNA probe for tyrosinase will allow further study of the regulation of tyrosinase synthesis by cAMP, MSH, and other hormones. In those cases where mRNA for tyrosinase is increased or decreased, a direct quantitation of mRNA can be made.

Tyrosinase's (EC 1.14.18.1) are copper containing enzymes that catalyze the conversion of tyrosine to dopa to dopaquinone, and thence to melanin (Mason, H.S. (1948) J. Biol. Chem. 172 , 83-99). Several of the mammalian enzymes have been shown to be glycoproteins and to contain sialic acid residues (Miyazaki, K. and Ohtaki, N. (1975) Arch. Derm, Forsch. 252 , 211-216; Hermann, W.P. and Uhlenbruck, G. (1975) Arch.

Dermatol. Res. 254 , 275-280; Nishioka, K. (1978) Eur. J. Biochem. 85 , 137-146). There are multiple sizes of tyrosinases in all mammalian species which have been studied. These may be caused by differing contents of carbohydrate and as a result of the actions of difference structural genes.

. Tyrosinase is an important factor in the development of pigmentation. Oculocutaneous albinism, a group of autosomal recessive diseases in humans (Witkop, C.J., Jr. Quevedo, W., Jr. and Fitzpatrick, T.B. (1983) In the Metabolic Basis of Inherited Disease. pp 301-346 (Stanbury, J.B., Wyngarden, J.B., Fredrickson, D.S., Goldstein, J.L., Brown, M.S., eds) McGraw-Hill, New York) and animals, is characterized by reduced or no melanin in skin and eye. However, some people with the condition have as much tyrosinase activity as fully pigmented individuals (King, R.A. and Witkop, C.J., Jr. (1976) Nature 263 , 69-71; King, R.A. and Witkop, C.J., Jr. (1977) Am. J. Human Genet. 29 , 164-168; King, R.A., Olds, D.P., and Witkop, C.J., Jr. (1978) J. Invest. Dermatol. 71 , 136-139). The albino (C) locus in mice has been mapped to chromosome 7 (Coleman, D.E. (1962) Arch. Biochem. Biophys. 69 , 562-568; Gluecksohn-Waelsch, S. (1979) Cell 16 , 225-237).

Studies on the regulation of tyrosinase have focused on the role of MSH in humans and in whole animals (Lerner, A.B. and McGuire, J.S. (1961) Nature 189 , 176-179; Pomerantz, S.H. and Chuang, L. (1971) Endocrinol. 87 301-310; Lee, T.H., Lee, M.S., and Lu, M.X. (1972) Endocrinol. 91 . 1180-1188) and in normal and malignant melanocytes in culture (Halaban, R., Pomerantz, S.H., Marshall, S., Lambert, D.T. and Lerner, A.B. (1983) J. Cell Biol. 97 , 480-488; Halaban, R., Pomerantz, S. H., Marshall, S. and Lerner, A.B. (1984) Arch. Biochem. Biophys. 230 , 383-387). The later studies showed that MSH increased the rate of synthesis of tyrosinase.

A nucleic acid probe for tyrosinase would be valuable for a thorough study of the regulation of tyrosinase, the molecular genetic basis of human albinism and of various mouse mutants affecting coat and eye color,

and the relationship between melanocyte differentiation and tyrosinase gene expression. Described here is the isolation of a cDNA clone for human tyrosinase which maps at or near the mouse C locus.

EXPERIMENTAL PROCEDURES

Cell culture Normal human melanocytes were cultured from newborn foreskin by a method modified by us (Halaban, R. and Alfano, F.D. (1984) In Vitro 20 , 447-450) from that of Eisinger and Marko (Eisinger, M. and Marko, O. (1982) Proc. Natl. Acad. Sci. USA 79 , 2018-2022). The cells were grown in Ham's F-10 medium (American Biorganics) containing 8% fetal calf serum (Gibco), 8% Nu-serum (Collaborative Research), 100 ug/ml penicillin, 200 units/mil streptomycin (Gibco), 85 nM TPA (Consolidated Midland Corp.), 0.1 mM IBMX (Sigma) and 2.5 nM cholera toxin (List Biological Laboratories). In the most recent experiments cholera toxin was replaced by human placental extract (20 ug/ml) (Halaban, F. Ghosh, S., Duray, P., Kirkwood, J.M. and Lerner, A.B. (1986) J. Invest. Dermatol. 87 , 95-101).

Melanoma and neuroblastoma cells were grown in Ham's F-10 medium supplemented with penicillin, streptomycin, 8% fetal calf serum and 8% Nu-serum. The melanoma cell line used was human metastatic LG (melanotic) cultured by us from the brain (Halaban, et al. supra). The human neuroblastoma SK-N-SH (Ross, R.A. and Biedler, J.L. (1985) Adv. Neuroblast. Res. pp. 249-259. (Evans, A.E., D'Ansio. G.J., Seeser, R.C., eds). Raven Press, New York) was received from Dr. J.L. Biedler, Sloan Kettering Institute, New York, NY. The murine neuroblastoma cell line NIE115 was obtained from Dr. X.O. Breakefield, Harvard University, Cambridge, Mass.

To radiolabel proteins of normal melanocytes the cultures were first incubated for 24 hours in methionine-free medium (American Biorganics) supplemented with TPA, cholera toxin, 3% dialyzed calf serum and [^{35}S] methionine (Amersham) (100 uCi/ml, 1390 Ci/mole). The cells

were lysed with 0.5 ml PBS containing 1% NP-40 (Sigma) and 0.1 mM PMSF (Sigma) and agitated on a vortex mixer. The lysate was centrifuged at 15,000g for 15 minutes, the specific radioactivity in protein of the supernatant was determined in 5 ul aliquots, and the supernatant was used as a crude radiolabeled tyrosinase preparation.

Preparation of cDNA libraries of normal human melanocytes Total cellular RNA of normal human melanocytes was prepared by guanidine isothiocyanate-cesiumchloride gradient centrifugation essentially as described by Chirgwin et al. (Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1978) Biochemistry 18 , 5194-5299). Poly(A)$^+$ RNA was selected by chromatography on a column of oligo d(T) cellulose (Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69 . 1408-1412) (Collaborative Research Type 2). Double stranded cDNAs were prepared from poly (A)$^+$ mRNA as previously described (Schwarzbauer, J.E., Tamkun, J.W., Lemischka, I.R. and Hynes, R.O. (1983) Cell 35 , 421-431; Land, H., Grez, J., Hauser, H., Lindenmaier, W. and Schutz, G. (1981) Nucleic Acids Res. 9 , 2251-2261). The cDNA was EcoRI-methylated at internal EcoRI restriction sites with EcoRI methylase (New England Biolabs) (Maniatis, T., Hardison, R.C., Lacy, E., Lauer, J., O'Connel, C., Quon, D., Sim, G.K. and Efstratiadis, A. (1978) Cell, 687-701) to prevent degradation at the step of EcoRI-linker addition. EcoRI linkers were ligated to both ends of cDNA molecules which were blunt-ended by treating with S1 nuclease followed by E. coli DNA polymerase 1 large fragments. The cDNAs were fractionated on 1 ml columns of Bio-gel A-150m (Bio Rad) and enriched for those composed of over 400 base pairs. λ gt11 DNA (Young, R.A., and Davis, R.W. (1983) Proc. Natl. Acad. Sci. USA 80 , 1194-1198) was digested with EcoRI and treated with bacterial alkaline phosphatase (Worthington, 0.2u/ug DNA) at 65° for 1 hour to decrease self-ligation. The cDNAs were inserted into the unique EcoRI site of λ gt11 cloning vector. The recombinant phage DNA was packaged in vitro as described by Grosveld et al (Grosveld, F.G., Dahl, H.H.M., deBoer, E. and Flavell, R.A. (1981) Gene 13 , 227-237). The λ gt11 library contained 1.7 x 10^6 independent plaques. A pilot experiment showed that 88% of the plaques

contained cDNA inserts based on beta-galactosidase activity. The λgt11 cDNA library was amplified in E. coli strain Y1088 (Huynh, T.V., Young, R.A. and Davis, R.W. (1984) In DNA CLoning: A Practical Approach, vol. 1, pp. 49078 (D. Glover, ed.) IRL Press, Arlington, VA) as a host and stored at 4°C.

Screening for the gene products of the λ gt11 melanocyte cDNA library with antityrosinase antibodies The immunobiological screening was carried out as described by Young and Davis (Young, R.A. et al. supra). Recombinant phages were absorbed to bacterial strain Y1090 and plated on 150 mm L-agar plates. The plates were incubated at 42°C for 3 hours. Nitrocellulose filters, soaked in 50 mM IPTG were placed on the plates and incubated for 3 hours at 37°C. The filters were removed and washed in TBS twice and treated with 20% fetal calf serum in TBS for one hour. The filters were washed with TBS twice and TBS + 0.1% NP-40 once. The filters were incubated with antityrosinase antibodies (available from Dr. Seymour H. Pomerantz, Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201) in TBS plus 20% fetal calf serum in a sealed plastic bag overnight at 4°C. At the same time another set of IPTG-treated filters were overlayed on plaques and incubated overnight at 37°C to obtain duplicate filters. The second set of filters were processed the same way as the first set of filters except that the binding period to antityrosinase antibody was two hours at room temperature. Both sets of filters were washed extensively with TBS and TBS plus 0.1% NP-40 at room temperature. [^{125}I] protein A (New England Nuclear) was diluted to 1 x 10^5 cpm/ml in TBS and incubated with the filters for 1 hour at room terperature with shaking. Filters were washed three times with TBS and blotted dry and autoradiographed with intensifying screen at -70°C. The duplicate filters were compared to search for positive signals in both filters.

Detection of fusion protein The lysogens of the recombinant and nonrecombinant λ gt11 were prepared employing E. coli strain Y1089 (Huynh, T.V., Young, R.A. and Davis, R.W. (1984) In DNA Cloning: A Practical Approach, vol. 1, pp. 49-78 (D. Glover, ed.) IRL Press,

Arlington, VA). The lysogens were grown at 32°C until they reached logarithmic phase (OD_{600} = 0.5). Lytic replication was then induced by a temperature shift (42°C, 20 min) and cultures were incubated at 37° for 3 hours in the presence of IPTG at a final concentration of 1 mM. The bacterial cells were suspended in a buffer containing 10 mM Tris HCL pH 7.4, 0.15 M NaCl, 1 mM PMSF, 50 ug/ml DNase 1 (Sigma) and 50 ug/ml of RHase (Boehringer Mannheim). The soluble fractions of the sonicated bacterial cell suspension were run on a 6% polyacrylamide gel (Laemmli, U.K. (1970) Nature 227 , 680-685).

Immunoprecipitation and competition with antityrosinase antibodies For competition experiments, antityrosinase antibodies were incubated on ice overnight with bacterial lysates prepared from E. coli Y1089 lysogens of λ gt11 or λ gt11 plus cDNA. [^{35}S] methionine labeled extract from black foreskin melanocytes (6 x 10^6 cpm in protein/assay) was added to each assay tube, followed 15 minutes later by 10 ul of IgGSorb (The Enzyme Center, Inc., 250 mg/ml). At the end of 15 minutes incubation with IgGSorb, the cell lysates were centrifuged at 13,000g for 0.5 minutes, the pellet of IgGSorb with bound immune complexes washed 3 times with PBS plus 0.1% NP-40 and once with PBS. The immune complexes were eluted from the IgGSorb with sample buffer (Laemmli supra) and subjected to polyacrylamide gel electrophoresis followed by fluorography of the gels as described before (Halaban et al. supra). For quantitation of labeled protein, gel slices were taken from relevant radioactive bands, rehydrated with 25 ul water and digested overnight with 5% Protosol (National Diagnostics) in Econofluor (NEN) at 37°C, and the radioactivity counted.

Northern blot analysis Poly(A)$^+$ RNA from normal human melanocytes, melanoma cells, neuroblastoma cell lines, HL-60 (human promyelocytic leukemia cell line) and HepG-2 (human hepatocarcinoma cell line) was fractionated on a 1.2% formaldehyde denaturing gel (Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77 , 5201-52005) and transferred to a nitrocellulose filter or Gene Screen plus membrane (NEN). [^{32}P] labeled cDNA probes were hybridized overnight to the filter at 42°C in 5

times concentrated SSC, 50% formaldehyde, 50 mM sodium phosphate buffer pH 6.8, 10% dextran sulfate, Denhardt, 0.1% SDS and 250 ug/ml denatured salmon sperm DNA. Filters were then washed three times for 5 minutes each in 2 times concentrated SSC and 0.1% SDS at room temperature and three times at 42°C in 5 times concentrated SSC, 5% formaldehyde, 50 mM sodium phosphate buffer pH 6.8, 10% dextran sulfate, Denhardt, 0.1% SDS and 250 ug/ml denatured salmon sperm DNA. Filters were then washed three times for 5 minutes each in 2 times concentrated SSC and 0.1% SDS at room temperature and three times at 42°C in 0.1 concentrated SSC and 0.1% SDS. The filters were autoradiographed at -70°C. When a Northern blot of Gene Screen plus was used multiple times for hybridization, the previous probe was removed by treating the membrane in 10 mM Tris-HCl pH 7.0 and 0.2% SDS at 85°C for 1 hour.

Genomic Southern blot analysis High molecular weight DNAs of murine embryos homozygous for the lethal deletion c^{3H}/c^{3H}, heterozygous for the deletion, (c^{3H}/c^{ch}) and of normal homozygote chinchilla (c^{ch}/c^{ch}), were prepared as described previously (Grass-Bellard, M., Oudet, P. and Chambon, P. (1973) Eur. J. Biochem. 36 , 32-38). c^{3H}/c^{3H} and c^{3H}/c^{ch} mice were obtained from Dr. Gluecksohn-Waelsch at the Albert Einstein College of Medicine and may be purchased from Johnson Laboratories, Inc., Bar Harbor, Maine. Restriction endonuclease digests of DNA were electrophoresed in a 0.8% agarose gel at 4°C. The gel was denatured with 0.5 M NaCH/1 M NaCl and neutralized with 1 M Tris HCl pH 8.0/1 M NaCl. The DNA in the gel was transferred to Gene Screen plus as described by Southern (Southern, E. (1975) J. Mol. Biol. 98 , 503-517). For a low stringent hybridization and washing the blot was hybridized with the [^{32}P] labeled cDNA in 6 times concentrated SSC, 5 times concentrated Denhardt solution, 0.5% SDS and 100 ug/ml denatured salmon sperm DNA for 24 hours at 58°C. The filters were then washed two times at room temperature for 10 minutes each in 2 times concentrated SSC, 0.1% SDS and two times at 55°C for 30 minutes each in 2 times concentrated SSC and 0.1% SDS, and two times at room temperature for 30 minutes in SSC.

Isolation of λ phage DNA and preparation of inserted DNA Recombinant phage DNA was prepared as described by Davis et al (Davis, R.W., Botstein, D. and Roth, J.R. (1980) Advanced Bacterial Genetics, pp. 106-107, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The recombinant DNA was cut with EcoRI, and the cDNA inserts were separated from λ phage arms by 1% agarose gel. The cDNA bands were cut out of the agarose gels, run on 5% polyacrylamide gels, eluted from the gels in 0.1 SSC, and precipitated with ethanol.

DNA sequencing DNA restriction fragments, subcloned in M13 vectors (Messing, J., Crea, R. and Seeburg, P.H. (1981) Nucleic Acids Res. 9 . 309-322), were sequenced by the dideoxy chain termination technique (Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74 , 5463-5467), with modifications made to accommodate 2'-deoxy-adenosine 5'-[alpha-[^{35}S]thio] triphosphate (Biggin, M., Gibson, T. and Hung, G. (1983) Proc. Natl. Acad. Sci. USA 80 , 3963-3965).

RESULTS

Isolation of cDNA clones from human melanocyte λ gt11 library which react with antityrosinase antibodies A λ gt11 cDNA library of normal human melanocytes was screened with rabbit antityrosinase antibodies raised against purified hamster tyrosinase. The antityrosinase antibodies have been shown to cross react with human, murine and avian tyrosinase (Halaban, R. et al. (1983) supra; Halaban, R. et al. (1984) supra). The antibodies immunoprecipitated newly-synthesized as well as processed tyrosinase and were abosrbed completely by extracts from melanocytes but not from fibroblasts.

Initial screening of approximately 500,000 recombinant phages with rabbit antityrosinase antibodies identified sixteen independent clones which gave positive signals. These clones were consistently reactive with antityrosinase antibodies but not with nonimmune rabbit serum during

several rounds of screening for plaque-purification. Figure 1 shows an example of duplicate primary (a and b) and the third round (c) of screening. Figure 1 depicts the isolation of human tyrosinase cDNA by antibody screening. A λ gt11 cDNA library of human melanocytes was screened with antityrosinase antibody directed against hamster tyrosinase and ^{125}I-labeled protein A (NEN). Filter panels a and b represent duplicate filters prepared from the same plate of λ gt11 cDNA library. Signals in filter a (1, 2 and 3) correspond to those in filter b. It was found that the duplicate screening method was helpful in discriminating false-positive signals. Filter panel c shows the third round of screening during the plaque purification of λ mel 34.

Recombinant phage DNAs from each of the 16 clones were prepared, digested with EcoRI to excise the cDNA insert and fractionated on a 1% agarose gel. Sizes of the cDNA inserts varied from 0.2 to 1.6 kb. The longest cDNA insert (§1.6) kb) from λ mel 34 was hybridized to 12 other cDNA inserts and shared an overlapping restriction pattern. The other three cDNA inserts were not related to the thirteen cDNA clones. The thirteen cDNA inserts were partially overlapping but none of them were the same clone. The fact that overlapping sequences were found repeatedly indicates that the antibody reacted to the peptides encoded by specific sequences present in the cDNA species. The 1.6 kb cDNA insert of λ mel 34 was subcloned into PBR322 to yield pmel 34 and used to amplify the cDNA insert.

<u>mRNA homologous to λ mel 34 is expressed preferentially in melanocytes</u> Tyrosinase is presumed to be expressed only in pigment cells. To examine whether λ mel 34 homologous mRNA is expressed only in melanocytes, Northern blot analysis of poly(A)$^+$ mRNA from normal human melanocytes, human melanotic melanoma cells (LG), human neuroblastoma, murine neuroblastoma, HePG-2 (human hepatoma cell line) and HL-60 (human promyelocytic leukemia cell line) was performed using [^{32}P] labeled λ mel 34 cDNA as a probe. Figure 2 is a Northern blot analysis of poly (A)$^+$ RNA (a) of normal human melanocyte (lane 1), LG (lane 2), HepG-2 (lane 3) and HL-60 (lane 4) cells, and 10 ug of

poly(A)$^+$ RNA (b) of normal human melanocyte (lane 5), LG (lane 6), human neuroblastoma (lane 7) and mouse neuroblastoma cells (lane 8) were fractionated on 1.2% formaldehyde denaturing agarose gel, blotted and hybridized with [^{32}P]- labeled λ mel 34 cDNA (upper arrow). The same filter was used to hybridize to [^{32}]-labeled mel 14-2 (bottom arrow) to show that each lane contained RNA as indicated above and the RNA was relatively intact. λ mel 14-2 is a cDNA clone which was isolated from human melanocyte cDNA library and has been used as a control probe because the corresponding RNA was detectable in similar amounts from all human and mouse cells tested. As shown in Figures 2a and b, λ mel 34 hybridized to 21S (§2.4 kb) mRNA species of normal human melanocytes and human melanotic melanoma cells but not to HePG-2, HL-60, human or murine neuroblastoma mRNA. A cDNA fragment (λ mel 14-2) is isolated from melanocyte cDNA library was used as a control probe to show that a similar amount of mRNA was loaded in each lane (Fig. 2a and b). In addition, λ mel 34 cRNA was not expressed in other human and mouse fibroblasts and lymphocytes (data not shown). Further studies showed that λ mel 34 cRNA expression was correlated with tyrosinase activity and melanin content in human melanoma cells. Amelanotic melanoma cells (no or little melanin-containing cells) expressed far less λ mel 34 cRNA than normal or melanotic melanoma cells.

Fusion protein of λ mel 34-related clone, λ mel 16 To characterize the fusion protein further, lysogens of λ gt11 and λ mel 16 (§C.7 kb), which were isolated at an earlier stage of this work and which cross hybridized to λ mel 34, were prepared and analyzed by 6% SDS PAGE and competitive immunoprecipitation assay. Figure 3 depicts the detection of λ mel 16 beta-galactosidase-tyrosinase fusion protein. Panel a: Bacterial lysates were prepared from the lysogens Y1089/ λ gt11 (lanes 1 and 2), Y1089/ λ mel 19 (lane 3) and Y1089/ λ mel 16 (lanes 4 and 5) following inactivation of the temperature-sensitive repressor at 42°C and subsequent incubation in the absence (lanes 1 and 5) or presence (lanes 2, 3, and 4) of IPTG. λ mel 16 and λ mel 19 were λ mel 34-related clones which were isolated in the early phase of this work. Reduced samples were run on a 6% SDS-polyacrylamide gel, and stained with

Coomassi-blue. The IPTG-dependent production of beta-galactosidase (lane 2) and ~140 kd λ mel 16 fusion protein (lane 5, arrow) was noted. Protein sizes in kilodaltons are marked left. Panel b: The lysate of mel 16 or gt11 lysogen was used to compete with metabolically labeled human melanocyte cell extract for antityrosinase antibodies. Constant amounts of lysates of gt11 or mel 16 lysogens (~2 x 10^7 cells in 100 ul volume) were incubated with 5 ul of 1:100 dilute of antityrosinase antibodies. The absorbed antibodies were used to immunoprecipitate the [^{35}S] labeled human melanocyte extract (6 x 10^6 cpm in protein/tube). The eluted immune complexes were analyzed on an 8.5% polyacrylamide gel. There were 3,780 cpm in control lane and 1,186 cpm in λ mel 16 lane, in the gel slices taken from the tyrosinase bands (arrow). As shown in Figure 3a, a fusion protein was produced in Y1089 (λ mel 16) which had a relative size of approximately 140,000 daltons. This was approximately 25,000 daltons larger than that of E. coli beta-galactosidase. This indicates that the cDNA in λ mel 16 is fused to beta-galactosidase gene in frame, producing an almost entire protein encoded by the cDNA. Synthesis of both beta-galactosidase and the fusion protein was dependent on induction with IPTG. The lysate of λ mel 16 lysogen was used to compete with metabolically labeled melanocyte cell extract for antityrosinase antibodies. A typical experiment is shown in Figure 3B. When bacterial lysates of λ gt11 or λ mel 16 lysogens (approximately 2 x 10^7 cells in 100 ul volume) were incubated with 1 ul of 1:100 dilution of antityrosinase antibodies, approximately 70% of antityrosinase activity was absorbed by λ mel 16 lysate compared with λ gt11 control lysates based upon the intensity of tyrosinase bands and radioactivity count of the gel slices taken from tyrosinase bands. Since the antibody preparation is poly-clonal the λ mel 16 encoding protein, containing only a partial sequence of tyrosinase, may not bind all of the antityrosinase antibodies.

Gene for λ mel 34 is deleted in chromosome of albino locus-deleted mouse, c^{3H}/c^{3H} Earlier studies showed that the skin of mice carrying the albino locus deleted mutant c^{3H}/c^{3H} has no tyrosinase activity (Gluecksohn-Waelsch, S. (1979) supra). However, the

tyrosinase activity levels in the skin of mice heterozygous to lethal albino deletions and chinchilla (c^{3H}/c^{ch}) were shown to be intermediate between normal (c^{ch}/c^{ch}) and mutant (c^{3H}/c^{3H}) homozygotes, confirming that the albino locus of mice encodes the structural gene of tyrosinase.

Because the deletion around the albino locus in c^{3H}/c^{3H} mice is large enough to cover the tyrosinase gene (Gluecksolh-Waelsch, S. (1979) supra), authentic tyrosinase cDNA should not detect any band (c^{3H}/c^{3H}) mice DNA but should detect hybridizing bands of half normal intensity of the homozygote (c^{ch}/c^{ch}) in the heterozygous litter mate (c^{3H}/c^{ch}). To test this prediction, genomic DNAs from (c^{3H}/c^{3H}), (c^{3H}/c^{ch}) and (c^{ch}/c^{ch}) were digested with EcoRI and the fragments were separated on 0.8% agarose gel, transferred to Gene Screen Plus, and probed with the [^{32}]P-labeled λ mel 34 cDNA. After autoradiograph, the λ mel 34 cDNA probe was stripped and the same filter was probed with [^{32}]P-labeled λ mel 17-1 cDNA. λ mel 17-1 is one of the three cDNA clones among the initial 16 clones which is unrelated to λ mel 34.

As shown in Figure 4a, λ mel 34 detected three hybridizing fragments whose sizes were approximately 4.5 kb, 12.0 kb and 14.5 kb in (c^{ch}/c^{ch}) DNA. According to the hybridization conditions the mouse tyrosinase gene. Those three bands were also detected in (c^{3H}/c^{ch}) at approximately half the intensity of that with (c^{ch}/c^{ch}) DNA. No hybridizing fragment was detected in (c^{3H}/c^{3H}) DNA even after longer exposure. Therefore, the mouse genes whose sequences are homologous to cDNA contained in λ mel 34 are located at or near the albino locus. Figure 4 is a Southern blot analysis of genomic DNA of deletion homozygous (c^{3H}/c^{3H}) (lane 1), (c^{3H}/c^{ch}) (lane 2) and (c^{ch}/c^{ch}) (lane 3) mice was digested with EcoRI, run on a 0.8% agarose gel, transferred to Gene Screen Plus and hybridized to λ mel 34 cDNA (a). The same filter was probed with mel 17-1 after stripping λmel 34 cDNA probe (b). λ mel 17-1 is one of the sixteen clones which we initially isolated and proved

not to be related to λ mel 34. Size markers are Hind III fragments of λ phage DNA. As shown in Figure 4b, λ mel 17-1, one of the three other clones we initially isolated, detected a 13.0 kb band in all three mouse DNAs at relatively similar intensities. Therefore λ mel 17-1 clone is unrelated to the albino locus.

Nucleotide sequence analysis The entire nucleotide sequence of three overlapping cDNA clones (λ mel 34, λ mel 16 and mel 40) was determined according to the strategy shown in Figure 5. Figure 5 shows partial restriction map and sequencing strategy for human tyrosinase cDNA. The protein-coding region is indicated by an open box and a putative signal peptide is represented by a shaded box. Horizontal arrows under the 3 inserts show the direction and extent of sequencing used to generate the sequence presented in Figure 6. Restriction sites used for sequencing are indicated. The scale at the top indicates the nucleotide number. The 5' sides of the other 10 clones were also sequenced. The nucleotide sequence of tyrosinase cDNA revealed a single long open reading frame, beginning with the first nucleotide after the EcoRI linker. This open reading frame is in frame with the lacZ gene of the λ gt11 vector. We found that the other 10 clones are also fused in frame with lacZ gene. This property was helpful in assigning the open reading frame even though the cDNAs did not start the first ATG codon. This reading frame codes for a polypeptide of 560 amino acids with a molecular weight of 63,549 (Figure 6). Figure 6 lists nucleotide sequence of cDNA encoding human tyrosinase and its deduced amino acid sequence. The nucleotide sequence of message strand is numbered in the 5' to 3' direction. Numbers above each line refer to nucleotide position. Nucleotide residue 1 is the T of the first codon TTC of putative mature protein, and the nucleotides of a portion of putative signal peptide are indicated by negative numbers. The predicted amino acid sequence is shown below the nucleotide sequence. Numbers below the amino acid sequence refer to amino acid position, beginning with the amino-terminal residue of the mature tyrosinase. The preceding residues of a portion of putative signal peptide are indicated by negative numbers. Potential glycosylation signals and potential polyadenylation signals are

underlined. - - - indicates stop codon. The codon specifying carboxy-terminal leucine is followed by the translation termination codon TAA (Nucleotide residue, 1645-1647). No nucleotide differences were observed among the three cDNA clones except that they differ in length. The 3'-untranslated sequence determined from λ mel 34, λ mel 16 and λ mel 40 does not extend as far as the poly(A)$^+$ tail. However λ mel 40 contains a potential polyadenylation signal of ATTAAA (Goeddel, D.V., Leung, D.W., Dull, T.J., Gross, M., Lawn, R.M., McCandliss, R., Seeburg, P.H., Ullrich, A., Yelverton, E. and Gray, P.W. (1981) Nature 290 , 20-26) (nucleotide residues, 1822-1827).

Amino acid sequence The sequence of the first eleven amino acid residues exhibits a feature characteristic of the signal peptide of secretary and membrane-associated proteins (Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67 , 852-862), which mainly contains hydrophobic amino acide (9 out of 11) and terminate with serine having a small side chain (Steiner, D.F., Quinn, P.S., Chan, S.J., Marsh, J. and Tager, H.S. (1980) Ann. N.Y. Acad. Sci. 343 , 1-16) (Figure 6). Therefore, a possible site for cleavage of the signal peptide of putative tyrosinase precursor is after the serine residue at position 1 of phenylalanine (Figure 6). This prepeptide is probably involved in the transfer of tyrosinase into the melanosome. Thus, mature tyrosinase is composed of 548 amino acids with a molecular weight of 62,160. As tyrosinase is a glycoprotein, we exammined the possible N-glycosylation sites. There are five potential aspargine-linked glycosylation signals, Asn-X-Ser-S or Thr, where X is any amino acid except proline (Marshall, R.D. (1974) Biochem. Soc. Symp. 40 , 17-26; Bause, E. (1983) Biochem. J. 209 , 331-336). The possible glycosylation sites are the asparagine residues at positions 73, 98, 148, 217 and 324 as underlined in Figure 6. Tyrosinases contain two copper atoms per enzyme molecule (Nishioka, K. (1978) Eur. J. Biochem. 85 , 136-146; Lerch, K. (1976) FEBS Letters 69 , 157-160). Therefore, we examined the possible copper-binding sites by comparison with human (Jabusch, J.R., Farb, D.L., Kerschensteiner, D.A. and Deutsch, H.F. (1980) Biochemistry 19 , 2310-2316) and bovine (Richardson, J.S., Thomas, K.A., Rubin, B.H. and Richardson, D.C.

(1975) Proc. Natl. Acad. Sci. USA 72 , 1349-1353) superoxide dismutase. Crystallographic study of bovine superoxide dismutase (Richardson, J.S. et al. (1975) supra) has shown that His-44, His-46, and His-61 are ligands to copper. There are 15 histidine residues in the deduced amino acid sequence of human tyrosinase. Histidine residues at positions 350, 354, 360 and 377 showed a similar arrangement to bovine superoxide dismutase. By analyzing albino and normal human DNA using λ mel 34 as a probe the genetic defect of tyrosinase gene in human albinism will be established. The cDNA contained in λ mel 34 will be further used to analyze amniotic cell DNA of suspected individuals for prenatal diagnosis of albinism. The cDNA (λ mel 34) will be used to produce a pure human tyrosinase by recombinant DNA technology in bacteria. This will facilitate obtaining a large quantity of pure human tyrosinase. Obtaining a large quantity of pure human tyrosinase is essential to study of the three dimensional structure of the enzyme. Clarification of the tyrosinase structure will lead to establishment of the rationale for chemotherapy against malignant melanoma because presursors to melanin is toxic to melanotic melanoma cells.

To produce the tyrosinase in E. coli , λ mel 34 cDNA will be fused to an expression vector (Tac) which has T rp and l ac promoter together. Tac, the expression vector is available communally through suppliers such as U.S. Pharmacia, Inc. The construct will be expressed in E. coli strain MM294. Subsequently the tyrosinase will be purified by affinity column chromatography.

Subsequently it was found that the cDNA inserts of the three clones which were not related to λ mel 34 were cross-hybridized to each other and expressed specifically in melanocytes. This indicates that they were not isolated spuriously, rather that the protein encoded by the cDNAs were reactive to the antityrosinase antibody and had melanocyto-specific functions.

The representative clone λ mel 17-1 detected approximately 2.5 kb mRNA species in only melanocytes. The gene for λ mel 17-1 cDNA was not mapped at the albino locus, detected single hybridizing restriction fragment in human and mouse DNA, and was highly conserved from mouse to human. The abundance of λ mel 17-1 cRNA paralleled the melanin content in human and mouse melanocytes. The expression of λ mel 17-1 cRNA was elevated after stimulation of mouse and human melanoma cells with MSH or/and IBMX, and U-V light (such as suntan). This was also closely correlated with the elevation of melanin content. The fact that the mel 17-1 gene is conserved evolutionarily indicates that the molecule encoded by the λ mel 17-1 has biologically important functions. The expression of the gene is controlled by hormones (MSH) or U-V light and positively correlated with the melanin content. These data indicate that the gene is involved in melanin biosynthesis in addition to tyrosinase. Current studies suggest that λ mel 17-1 gene product act on melanin biosynthesis's pathway distal to tyrosinase.

The foregoing description has been directed to particular embodiments of the invention in accordance with the requirements of the Patent Statutes for the purposes of illustration and explanation. It will be apparant, however, to those skilled in this art that many modifications and changes will be possible without department from the scope and spirit of the invention. It is intended that the following claims be interpreted to embrace all such modifications.

Bacteriophage λ mel 34 cDNA has been deposited at the American Type Culture Collection under ATC No. 40265 and will be available after this Patent Application issues.

Bacteriophage λ mel 17-1 cDNA has been deposited at the American Type Culture Collection under ATC No. 40264 and will be available after this Patent Application issues.

I claim:

1. A cDNA gene for human tyrosinase.

2. A cDNA gene for human tyrosinase comprising a nucleotide sequence as shown in Figures 6A, 6B, 6C and 6D.

3. A bacteriophage vector for use in cloning human tyrosinase cDNA comprising a replicon identified as λ mel 34 cDNA deposited at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATC No. 40265.

4. A method of making human tyrosinase comprising the steps of:

a. fusing λ mel 34 cDNA to an expression vector (Tac);
b. express tyrosinase in E. coli ;
c. purify the resulting tyrosinase.

5. A cDNA gene for human tyrosinase comprising the cDNA recovered from the λ mel 34 cDNA as deposited at the A.T.C. Collection under No. 40265.

6. The method of using the cDNA gene recovered from the λ mel 34 cDNA as deposited at the A.T.C. Collection under No. 40265 as a probe for genetic analysis of human albinism.

7. The method of using the cDNA gene recovered from the λ mel 34 cDNA as deposited at the A.T.C. Collection under No. 40265 as a probe for melanotic and/or amelanotic melanoma.

8. The method of using the cDNA gene recovered from the λ mel 34 cDNA as deposited at the A.T.C. Collection under No. 40265 as a probe for the diagnostics of prenatal albinism.

9. The method of using the cDNA gene recovered from the λ mel 34 cDNA as deposited at the A.T.C. Collection under No. 40265 for the production or regulation of the production of human melanin.

10. A bacteriophage vector for use as a probe for melanin biosynthesis comprising a replicon identified as λ mel 17-1 cDNA and on deposit at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATC No. 40264.

11. The cDNA gene comprising the cDNA recovered from the λ 17-1 cDNA as deposited at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATC No. 40264.

12. The method of using the cDNA gene recovered from the λ mel 17-1 cDNA as deposited at the American Type Culture Collection under ATC No. 40264 to detect the change in the degree of melanization of human melanocytes.


a
b
c
λ mel 34

FIG. 1

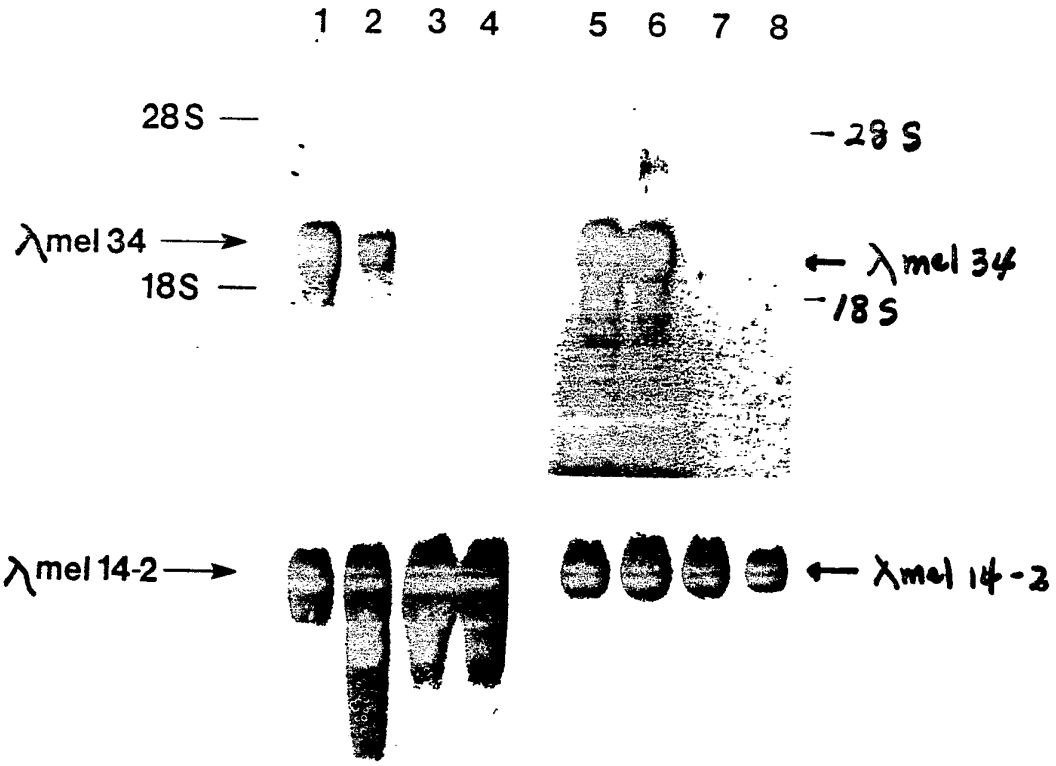
1 2 3 4 5 6 7 8
28S —
λmel 34 —
18S —
λmel 14-2 —
-28S
← λmel 34
-18S
← λmel 14-2

FIG. 2

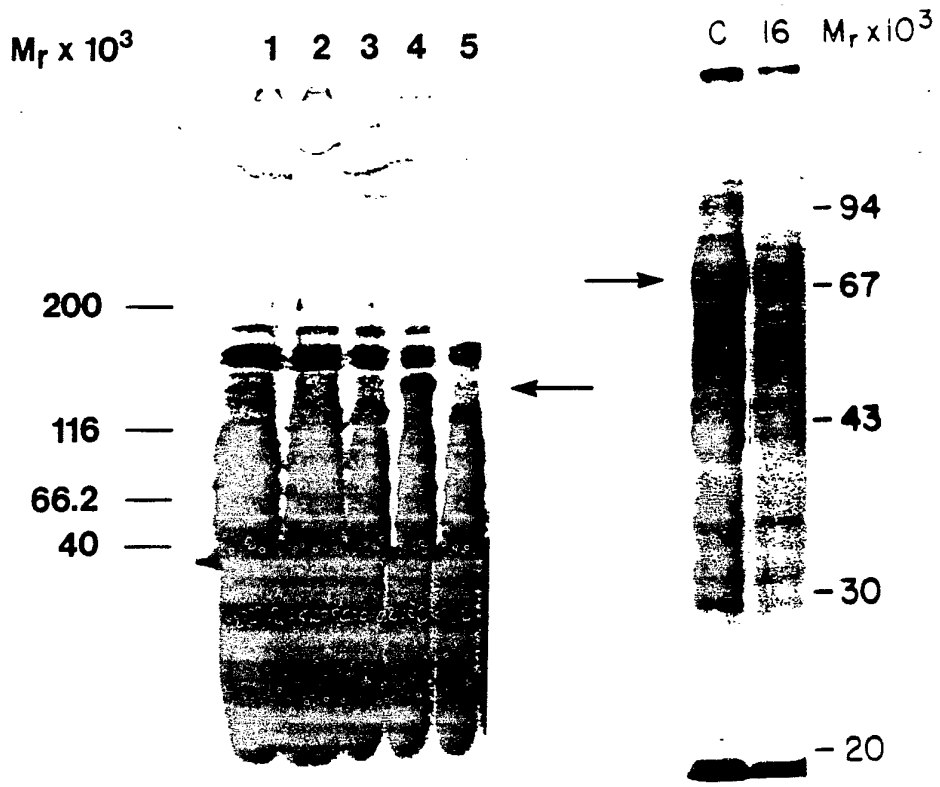
Mr x 10³
1 2 3 4 5
C 16 Mr x10³
200
116
66.2
40
94
67
43
30
20

FIG. 3

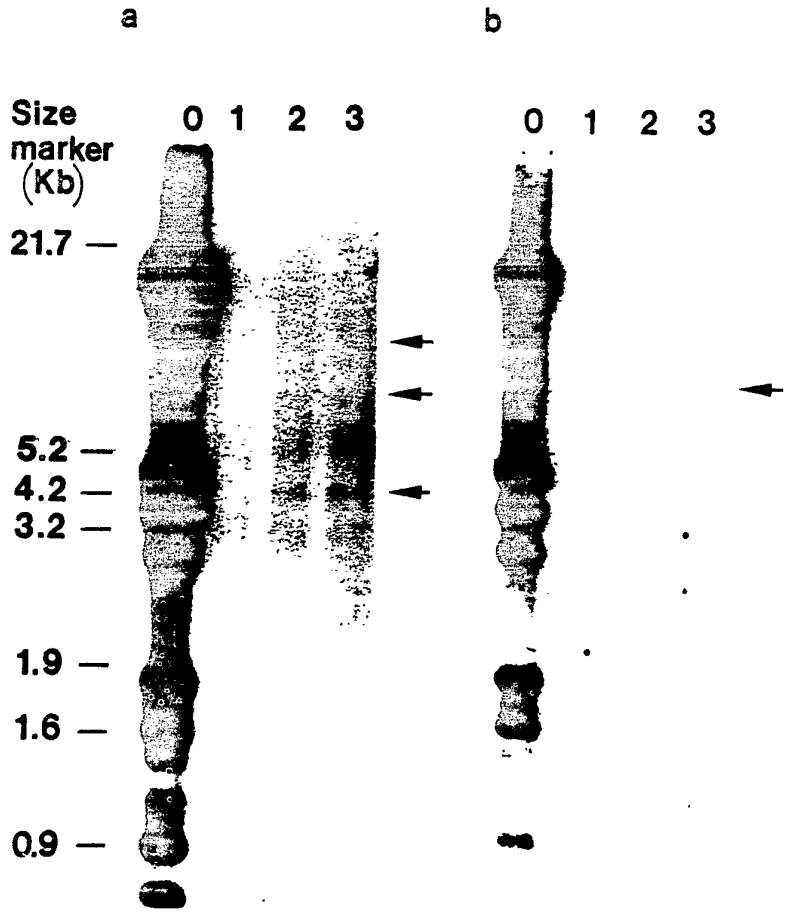
a
b
Size marker (Kb)
0 1 2 3
0 1 2 3
21.7
5.2
4.2
3.2
1.9
1.6
0.9

FIG. 4

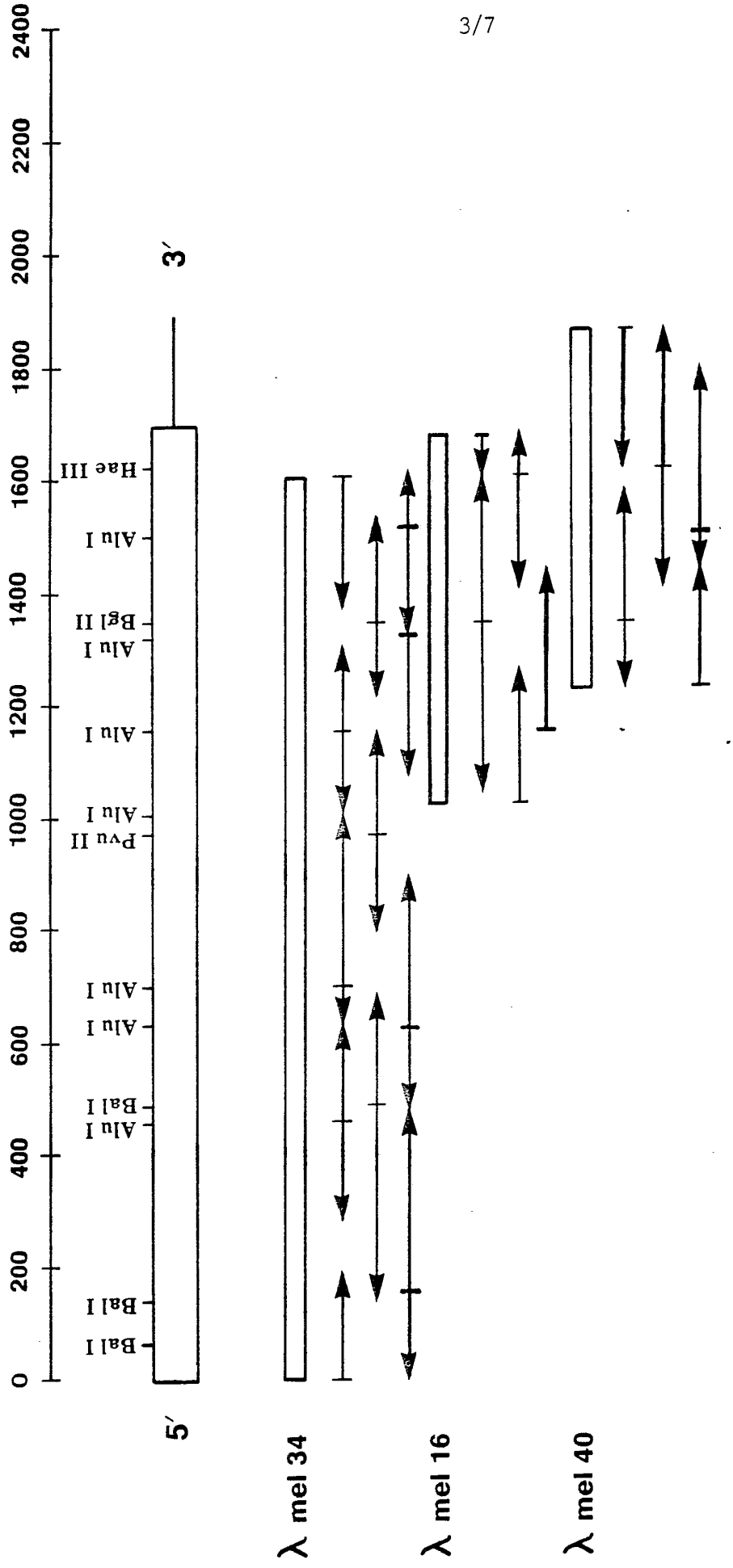
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
5′
3′
Bal I
Bal I
Alu I
Bal I
Alu I
Alu I
Pvu II
Alu I
Alu I
Alu I
Bgl II
Alu I
Hae III
λ mel 34
λ mel 16
λ mel 40

FIG. 5

```
-40          -30          -20          -10          -1 1          10

GAA TTC CTG CTC CTG GCT GTT TTG TAC TGC CTG CTG TGG AGT TTC CAG ACC TCC GCT GGC
------- Leu Leu Leu Ala Val Leu Tyr Cys Leu Leu Trp Ser Phe Gln Thr Ser Ala Gly
EcoRI          -10                                    -1    1

 20          30          40          50          60          70

CAT TTC CCT AGA GCC TGT GTC TCC TCT AAG AAC CTG ATG GAG AAG GAA TGC TGT CCA CCG
His Phe Pro Arg Ala Cys Val Ser Ser Lys Asn Leu Met Glu Lys Glu Cys Cys Pro Pro
          10                                    20

 80          90          100          110          120          130

TGG AGC GGG ACA GGA GTC TGT GGC CAG CTT TCA GGC AGA GGT TCC TGT CAG AAT ATC CTT
Trp Ser Gly Thr Gly Val Cys Gly Gln Leu Ser Gly Arg Gly Ser Cys Gln Asn Ile Leu
          30                                    40

140          150          160          170          180          190

CTG TCC AAT GCA CCA CTT GGG CCT CAA TTT CCC TTC ACA GGG GTG GAT GAC CGC GAG TCG
Leu Ser Asn Ala Pro Leu Gly Pro Gln Phe Pro Phe Thr Gly Val Asp Asp Arg Glu Ser
          50                                    60

200          210          220          230          240          250

TGG CCT TCC GTC TTT TAT AAT AGG ACC TGC CAG TGC TCT GGC AAC TTC ATG GGA TTC AAC
Trp Pro Ser Val Phe Tyr Asn Arg Thr Cys Gln Cys Ser Gly Asn Phe Met Gly Phe Asn
          70                                    80

260          270          280          290          300          310

TGT GGA AAC TGC AAG TTT GGC TTT TGG GGA CCA AAC TGC ACA GAG AGA CGA CTC TTG GTG
Cys Gly Asn Cys Lys Phe Gly Phe Trp Gly Pro Asn Cys Thr Glu Arg Arg Leu Leu Val
          90                                    100

320          330          340          350          360          370

AGA AGA AAC ATC TTC GAT TTG AGT GCC CCA GAG AAG GAC AAA TTT TTT GCC TAC CTC ACT
Arg Arg Asn Ile Phe Asp Leu Ser Ala Pro Glu Lys Asp Lys Phe Phe Ala Tyr Leu Thr
          110                                    120

380          390          400          410          420          430

TTA GCA AAG CAT ACC ATC AGC TCA GAC TAT GTC ATC CCC ATA GGG ACC TAT GGC CAA ATG
Leu Ala Lys His Thr Ile Ser Ser Asp Tyr Val Ile Pro Ile Gly Thr Tyr Gly Gln Met
          130                                    140

440          450          460          470          480          490

AAA AAT GGA TCA ACA CCC ATG TTT AAC GAC ATC AAT ATT TAT GAC CTC TTT GTC TGG ATG
Lys Asn Gly Ser Thr Pro Met Phe Asn Asp Ile Asn Ile Tyr Asp Leu Phe Val Trp Met
          150                                    160
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FIG. 6A

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500          510          520          530          540          550

CAT TAT TAT GTG TCA ATG GAT GCA CTG CTT GGG GGA TAT GAA ATC TGG AGA GAC ATT GAT
His Tyr Tyr Val Ser Met Asp Ala Leu Leu Gly Gly Tyr Glu Ile Trp Arg Asp Ile Asp
            170                                                 180

560          570          580          590          600          610

TTT GCC CAT GAA GCA CCA GCT TTT CTG CCT TGG CAT AGA CTC TTC TTG TTG CGG TGG GAA
Phe Ala His Glu Ala Pro Ala Phe Leu Pro Trp His Arg Leu Phe Leu Leu Arg Trp Glu
            190                                                 200

620          630          640          650          660          670

CAA GAA ATC CAG AAG CTG ACA GGA GAT GAA AAC TTC ACT ATT CCA TAT TGG GAC TGG CGG
Gln Glu Ile Gln Lys Leu Thr Gly Asp Glu Asn Phe Thr Ile Pro Tyr Trp Asp Trp Arg
            210                                                 220

680          690          700          710          720          730

GAT GCA GAA AAG TGT GAC ATT TGC ACA GAT GAG TAC ATG GGA GGT CAG CAC CCC ACA AAT
Asp Ala Glu Lys Cys Asp Ile Cys Thr Asp Glu Tyr Met Gly Gly Gln His Pro Thr Asn
            230                                                 240

740          750          760          770          780          790

CCT AAC TTA CTC AGC CCA GCA TCA TTC TTC TCC TCT TGG CAG ATT GTC TGT AGC CGA TTG
Pro Asn Leu Leu Ser Pro Ala Ser Phe Phe Ser Ser Trp Gln Ile Val Cys Ser Arg Leu
            250                                                 260

800          810          820          830          840          850

GAG GAG TAC AAC AGC CAT CAG TCT TTA TGC AAT GGA ACG CCC GAG GGA CCT TTA CGG CGT
Glu Glu Tyr Asn Ser His Gln Ser Leu Cys Asn Gly Thr Pro Glu Gly Pro Leu Arg Arg
            270                                                 280

860          870          880          890          900          910

AAT CCT GGA AAC CAT GAC AAA TCC ACA ACC CCA AGG CTC CCC TCT TCA GCT GAT GTA GAA
Asn Pro Gly Asn His Asp Lys Ser Thr Thr Pro Arg Leu Pro Ser Ser Ala Asp Val Glu
            290                                                 300

920          930          940          950          960          970

TTT TGC CTG AGT TTG ACC CAA TAT GAA TCT GGT TCC ATG GAT AAA GCT GCC AAT TTC AGC
Phe Cys Leu Ser Leu Thr Gln Tyr Glu Ser Gly Ser Met Asp Lys Ala Ala Asn Phe Ser
            310                                                 320

980          990          1000         1010         1020         1030

TTT AGA AAT ACA CTG GAA GGA TTT GCT AGT CCA CTT ACT GGG ATA GCG GAT GCC TCT CAA
Phe Arg Asn Thr Leu Glu Gly Phe Ala Ser Pro Leu Thr Gly Ile Ala Asp Ala Ser Gln
            330                                                 340
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FIG 6B

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1040        1050        1060        1070        1080        1090

AGC AGC ATG CAC AAT GCC TTG CAC ATC TAT ATG AAT GGA CAT GTC CCA GGT ACA GGA TCT
Ser Ser Met His Asn Ala Leu His Ile Tyr Met Asn Gly His Val Pro Gly Thr Gly Ser
            350                                 360

1100        1110        1120        1130        1140        1150

GCC AAC GAT CGT ATC TTC CTT CTC ACC ATG CAT TTG TTG ACA GTA TTT TTG AGG CAG TGG
Ala Asn Asp Arg Ile Phe Leu Leu Thr Met His Leu Leu Thr Val Phe Leu Arg Gln Trp
            370                                 380

1160        1170        1180        1190        1200        1210

CTC CAA AGG CAC CGT CCT CTT CAA GAA GTT TAT CCA GAA GCC AAT GCA CCC ATT GGA CAT
Leu Gln Arg His Arg Pro Leu Gln Glu Val Tyr Pro Glu Ala Asn Ala Pro Ile Gly His
            390                                 400

1220        1230        1240        1250        1260        1270

AAC CGG GAA TCC TAC ATG GTT CCT TTT ATA CCA CTG TAC AGA AAT GGT GAT TTC TTT ATT
Asn Arg Glu Ser Tyr Met Val Pro Phe Ile Pro Leu Tyr Arg Asn Gly Asp Phe Phe Ile
            410                                 420

1280        1290        1300        1310        1320        1330

TCA TCC AAA GAT CTG GGC TAT GAC TAT AGC TAT CTA CAA GAT TCA GAC CCA GAC TCT TTT
Ser Ser Lys Asp Leu Gly Tyr Asp Tyr Ser Tyr Leu Gln Asp Ser Asp Pro Asp Ser Phe
            430                                 440

1340        1350        1360        1370        1380        1390

CAA GAC TAC ATT AAG TCC TAT TTG GAA CAA GCG AGT CGG ATC TGG TCA TGG CTC CTT GGG
Gln Asp Tyr Ile Lys Ser Tyr Leu Glu Gln Ala Ser Arg Ile Trp Ser Trp Leu Leu Gly
            450                                 460

1400        1410        1420        1430        1440        1450

GCG GCG ATG GTA GGG GCC GTC CTC ACT GCC CTG CTG GCA GGG CCT GTG AGC TTG CTG TGT
Ala Ala Met Val Gly Ala Val Leu Thr Ala Leu Leu Ala Gly Pro Val Ser Leu Leu Cys
            470                                 480

1460        1470        1480        1490        1500        1510

CGT CAC AAG AGA AAG CAG CTT CCT GAA GAA AAG CAG CCA CTC CTC ATG GAG AAA GAA GGA
Arg His Lys Arg Lys Gln Leu Pro Glu Glu Lys Gln Pro Leu Leu Met Glu Lys Glu Gly
            490                                 500

1520        1530        1540        1550        1560        1570

TTA CCA CAG CTT GTA TCA GAG CCA TTT ATA AAA GGC TTA GGC AAT AGA GTA GGG CCA AAA
Leu Pro Gln Leu Val Ser Glu Pro Phe Ile Lys Gly Leu Gly Asn Arg Val Gly Pro Lys
            510                                 520
```

FIG 6c

1580 1590 1600 1610 1620 1630

AGC CCT GAC CTC ACT CTA ACT CAA AGT AAT GTC CAG GTT CCA GAG AAT ATC TGC TGG TAT
Ser Pro Asp Leu Thr Leu Thr Gln Ser Asn Val Gln Val Pro Glu Asn Ile Cys Trp Try
530 540

1640 1650 1660 1670 1680 1690

TTT CTG TAA AGA CCA TTT GCA AAA TTG TAA CCT AAT ACA AAG TGT AGC CTT CTT CCA ACT
Phe Leu ---

1700 1710 1720 1730 1740 1750

CAG GTA GAA CAC ACC TGT CTT TGT CTT GCT GTT TTC ACT CAG CCC TTT TAA CAT TTT CCC

1760 1770 1780 1790 1800 1810

CTA AGC CCA TAT GTC TAA GGA AAG GAT GCT ATT TGG TAA TGA GGA ACT GTT ATT TGT ATG

1820 1830 1840

TGA ATT AAA AGT GCT CTT AGG AAT TC

FIG 6D

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/02561

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07H 15/12;C12Q 1/68;C12P 21/02; See Attachment

US CL : 536/27;435/6,70,108,172.3,189,320

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System :	Classification Symbols
U.S.	536/27; 435/6,70,108,172.3,189,320

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	NUCLEIC ACIDS RESEARCH (Oxford), Volume 14, issued 25 March 1986 (SHIBAHARA ET AL), "Cloning and expression of cDNA mouse tyrosinase". See pages 2413-2427.	1-9
Y	CHEMICAL ABSTRACTS (Columbus, Ohio, USA), Volume 100, No. 5, issued 30 January 1984 (SEKIGUCHI) "Pleiotrophic cytoplasmic control of gene expression in mammalian reconstituted cells (nuclear transplanted cells).", see page 295, column 2, the abstract No. 32667x, JINRUI IDENGAKU ZASSHI, 1983, 28(2), 82-92 (Japan).	1-7,9-12
Y	JOURNAL OF BIOLOGICAL CHEMISTRY (Baltimore, Maryland, USA), Volume 258, issued 25 December 1983 (GAULTON ET AL), "Control of tryosinase gene expression and its relationship to neural crest induction in Rana pipiens". See pages 14845-14849.	1-12

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
31 December 1987	25 JAN 1988
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Thomas D. Mays

Form PCT/ISA/210 (second sheet) (May 1986)

Attachment To Form PCT/ISA/210, Part I.

IPC(4): C12Q 1/26; C12P 13/22; C12N 15/00;
C12N 7/00

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	CHEMICAL ABSTRACTS (Columbus, Ohio, USA), Volume 81, No. 5, issued 5 August 1974 (PAWELEK ET AL) "Genetic control of melanization. Isolation and analysis of amelanotic variants from cultured melanoma cells", see page 271, column 2, the abstract No. 23599p, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, 1974, 71(4), 1073-1077 (Eng).	1-12
Y	CHEMICAL ABSTRACTS (Columbus, Ohio, Volume 72, No. 13, issued 30 March 1970 (OTTOLENGHI-NIGHTINGALE) "Induction of melanin synthesis in albino mouse skin by DNA from pigmented mice", see page 162, column 1, the abstract No. 64375v, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, 1969, 64(1), 184-189 (Eng).	7-12

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ **OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE** [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** [11]

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-6

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment To Form PCT/ISA/210, Part VI.

I. Claims 1-6 drawn to a cDNA gene from lambda mel 34 clone, bacteriophage vector, method of making tyrosinase and method of using cDNA gene as a probe for albinism; class 536 subclass 27 and class 435 subclasses 6, 108, 172.3, 189 and 320.

II. Claim 7 drawn to a second use of said cDNA gene as a probe for melanoma; class 435 subclass 6.

III. Claim 8 drawn to a third use of said cDNA gene as a probe for diagnosing prenatal albinism; class 435 subclass 6.

IV. Claim 9 drawn to a fourth method of using said cDNA gene for regulation of production of human melanin; class 435 subclass 70.

V. Claims 10-12 drawn to a second cDNA gene from lambda mel 17-1 clone, bacteriophage vector and use of said second cDNA gene as a probe for melanin biosynthesis; class 536 subclass 27 and class 435 subclass 6 and 320.