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Functional analysis of alternatively spliced tyrosinase gene transcripts

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Three different cDNA clones (pmcTyr1, pmcTyr2 and pmcTyr3) representing mRNAs originating by alternative splicing of the primary transcript of the mouse tyrosinase gene, were identified and characterized by sequence analysis and by a functional assay. These cDNAs were subcloned into the newly contructed expression vector pHD. After electroporation of these hybrid clones into tyrosinase negative cells, protein extracts were prepared and tested for tyrosinase enzyme activity. Only the cDNA insert of pmcTyr1 was able to confer tyrosinase enzyme activity. This cDNA encodes a protein 533 amino acid residues in length containing a putative leader peptide of 18 amino acids and six putative glycosylation sites. Comparisons of the deduced amino acid sequence of the cDNA clone pmcTyr1 with the protein sequence of tyrosinases from man, *Streptomyces, Neurospora* and with haemocyanin subunits from a spider showed two regions of sequence conservation. One of these regions is known to be involved in copper binding. Since this gene with the coding capacity for tyrosinase is absent in all studied *c*-locus lethal deletion mutant mice, we have evidence that albinism in mice is caused by mutations of the tyrosinase gene.

Key words: tyrosinase/albinism/copper proteins/alternative splicing

Introduction

Tyrosinase (monophenol oxygenase, E.C. 1.14.18.1) is the key enzyme in melanin synthesis. Two reactions, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (Dopa) and the oxidation of Dopa to dopaquinone are catalysed by this enzyme, which contains a binuclear copper active site (Lerner *et al.*, 1949; Makino and Mason, 1973; Jolley *et al.*, 1974). The enzyme is found in melanoma cells in several different glycosylation states representing different isoforms T_1-T_4 (Hearing, 1987). The reported mol. wt of the unglycosylated isoform T_3 of mouse tyrosinase is 56–57 kd (Burnett, 1971; Hearing *et al.*, 1978).

Mutations in the gene encoded at the *c*-locus lead to albinism (Silvers, 1979). Albinism in mice was first documented in ancient Chinese literature dating back to 1100 BC. (reviewed by Morse, 1981). After the rediscovery of Mendel's rules the *c*-locus of the mouse was the first mammalian trait to be analysed (Cuénot, 1902) and shown to be recessive (Castle and Allen, 1903). It was also the first locus in a mammalian system for which linkage to another locus (pink-eyed dilution) could be demonstrated (Haldane *et al.*, 1915). Since the early 1920s several different *c*-locus alleles have been described: c^{ch} (Feldman, 1922), c^{e} (Detlefson, 1921), c^{h} (Green, 1961), c^{m} and c^{p} (reviewed by Green, 1981). In the last four decades histochemical (Russell and Russell, 1948) and biochemical evidence (Coleman, 1962) was obtained which argued that the *c*-locus encodes tyrosinase. This view was, however, challenged by observations supporting the hypothesis that the *c*-locus has to be regarded as a regulator of tyrosinase (Hearing 1973; Pomerantz and Li, 1974).

In 1986 the isolation of a putative mouse tyrosinase cDNA (pMT4) was reported (Shibahara *et al.*, 1986). It was subsequently realized by us that this cDNA hybridized to genomic DNA of mice carrying large *c*-locus deletions (Gluecksohn-Waelsch, 1979) indicating that this gene is not encoded at the *c*-locus (unpublished data). The evidence that pMT4 encodes a candidate for tyrosinase was based (i) on tissue specific gene expression, and (ii) the fact that pMT4 in an expression vector transfected into cells gave rise to a gene product which cross-reacted with a monoclonal antibody raised against purified tyrosinase T_4 isozyme (Shibahara *et al.*, 1986). Last year, another putative mouse tyrosinase cDNA clone (Tyrs33) (Yamamoto *et al.*, 1987) was described. This cDNA clone differed in its overall sequence and also in the encoded protein from pMT4. The evidence that Tyrs33 might be a candidate for mouse tyrosinase cDNA was based on cross-reaction of the gene product with tyrosinase antibodies and on limited protein sequence information of tyrosinase.

At the same time isolation of a candidate cDNA clone for human tyrosinase (pmel34) was reported (Kwon *et al.*, 1987). The evidence that this cDNA clone encodes human tyrosinase was based on reactivity of the gene product with antibodies raised against the purified enzyme and by showing that the corresponding genomic DNA is absent in *c*-locus deletion mice, but present in wild-type mice, indicating a close association with the *c*-locus. By sequence comparison of the human clone pmel34 with the mouse clone Tyrs33 we found, however, that the two putative proteins encoded differed markedly in sequence.

In this paper we resolve the historical discussion as to whether the *c*-locus encodes the gene for tyrosinase. We also resolve the recent confusion concerning the identity of functional, tyrosinase-encoding cDNAs. We have isolated several candidate clones for mouse tyrosinase which differed in sequence composition by using a probe homologous to the isolated human cDNA clone pmel34. These different cDNA clones correspond to transcripts originating by alternative splicing of a single gene (Ruppert *et al.*, 1988). In order to identify the clone which encodes tyrosinase activity we analysed with a functional assay the coding capacity of three different cDNA clones representing

different splicing products as well as of the clone pMT4 isolated by Shibahara *et al.* (1986). With this functional assay we characterized a cDNA encoding enzymatically active tyrosinase. In support of this conclusion we have identified two regions of amino acid sequence homology to characterized tyrosinases from prokaryotes and *Neurospora crassa*. One of these regions of homology is also shared with one copper-binding site of haemocyanin.

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 -20 CTTAGCCAAAACATGTGATAGTCACTCCAGGGGTTGCTGGAAAAGAAGTCTGTGACACTC

 +41 ATTAACCTATTGGTGCAGATTTTGTATGATCTAAAGGAGAAAATGTTCTTGGCTGTTTTG
 -18                                          MetPheLeuAlaValLeu

+101 TATTGCCTTCTGTGGAGTTTCCAGATCTCTGATGGCCATTTTCCTCGAGCCTGTGCCTCC
 -12 TyrCysLeuLeuTrpSerPheGlnIleSerAspGlyHisPheProArgAlaCysAlaSer

+161 TCTAAGAACTTGTTGGCAAAAGAATGCTGCCCACCATGGATGGGTGATGGGAGTCCCTGC
  +9 SerLysAsnLeuLeuAlaLysGluCysCysProProTrpMetGlyAspGlySerProCys

+221 GGCCAGCTTTCAGGCAGAGGTTCCTGCCAGGATATCCTTCTGTCCAGTGCACCATCTGGA
 +29 GlyGlnLeuSerGlyArgGlySerCysGlnAspIleLeuLeuSerSerAlaProSerGly

+281 CCTCAGTTCCCCTTCAAAGGGGTGGATGACCGTGAGTCCTGGCCCTCTGTGTTTTATAAT
 +49 ProGlnPheProPheLysGlyVAlaspAspArgGluSerTrpProSerValPheTyrAsn

+341 AGGACCTGCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGA
 +69 ArgThrCysGlnCysSerGlyAsnPheMetGlyPheAsnCysGlyAsnCysLysPheGly

+401 TTTGGGGGCCCAAATTGTACAGAGAAGCGAGTCTTGATTAGAAGAAACATTTTTGATTTG
 +89 PheGlyGlyProAsnCysThrGluLysArgValLeuIleArgArgAsnIlePheAspLeu

+461 AGTGTCTCCGAAAAGAATAAGTTCTTTTCTTACCTCACTTTAGCAAAACATACTATCAGC
+109 SerValSerGluLysAsnLysPhePheSerTyrLeuThrLeuAlaLysHisThrIleSer

+521 TCAGTCTATGTCATCCCCACAGGCACCTATGGCCAAATGAACAATGGGTCAACACCCATG
+129 SerValTyrValIleProThrGlyThrTyrGlyGlnMetAsnAsnGlySerThrProMet

+581 TTTAATGATATCAACATCTACGACCTCTTTGTATGGATGCATTACTATGTGTCAAGGGAC
+149 PheAsnAspIleAsnIleTyrAspLeuPheValTrpMetHisTyrTyrValSerArgAsp

+641 ACACTGCTTGGGGGCTCTGAAATATGGAGGGACATTGATTTTGCCCATGAAGCACCAGGG
+169 ThrLeuLeuGlyGlySerGluIleTrpArgAspIleAspPheAlaHisGluAlaProGly

+701 TTTCTGCCTTGGCACAGACTTTTCTTGTTATTGTGGGAACAAGAAATTCGAGAACTAACT
+189 PheLeuProTrpHisArgLeuPheLeuLeuLeuTrpGluGlnGluIleArgGluLeuThr

+761 GGGGATGAGAACTTCACTGTTCCATACTGGGATTGGAGAGATGCAGAAAACTGTGACATT
+209 GlyAspGluAsnPheThrValProTyrTrpAspTrpArgAspAlaGluAsnCysAspIle

+821 TGCACAGATGAGTACTTGGGAGGTCGTCACCCTGAAAATCCTAACTTACTCATCCCAGCA
+229 CysThrAspGluTyrLeuGlyGlyArgHisProGluAsnProAsnLeuLeuIleProAla

+881 TCCTTCTTCTCCTCCTGGCAGATCATTTGTAGCAGATCAGAAGAGTATAATAGCCATCAG
+249 SerPhePheSerSerTrpGlnIleIleCysSerArgSerGluGluTyrAsnSerHisGln

+941 GTTTTATGCGATGGAACACCTGAGGGACCACTATTACGTAATCCTGGAAACCATGACAAA
+269 ValLeuCysAspGlyThrProGluGlyProLeuLeuArgAsnProGlyAsnHisAspLys

+1001 GCCAAAACCCCCAGGCTCCCATCTTCAGCAGATGTGGAATTTTGTCTGAGTTTGACCCAG
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+1061 TATGAATCTGGATCAATGGATAGAACTGCCAATTTCAGCTTTAGAAACACACTGGAAGGA
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+1121 TTTGCCAGTCCACTCACAGGGATAGCAGATCCTTCTCAAAGTAGCATGCACAATGCCTTA
 +329 PheAlaSerProLeuThrGlyIleAlaAspProSerGlnSerSerMetHisAsnAlaLeu

+1181 CATATCTTTATGAATGGAACAATGTCCCAAGTACAGGGATCGGCCAACGATCCCATTTTT
 +349 HisIlePheMetAsnGlyThrMetSerGlnValGlnGlySerAlaAsnAspProIlePhe

+1241 CTTCTTCACCATGCTTTTGTGGACAGTATTTTTGAACAATGGCTGCGAAGGCACCGCCCT
 +369 LeuLeuHisHisAlaPheValAspSerIlePheGluGlnTrpLeuArgArgHisArgPro

+1301 CTTTTGGAAGTTTACCCAGAAGCCAATGCACCTATCGGCCATAACAGAGACTCTTACATG
 +389 LeuLeuGluValTyrProGluAlaAsnAlaProIleGlyHisAsnArgAspSerTyrMet

+1361 GTTCCTTTCATACCGCTCTATAGAAATGGTGATTTCTTCATAACATCCAAGGATCTGGGA
 +409 ValProPheIleProLeuTyrArgAsnGlyAspPhePheIleThrSerLysAspLeuGly

+1421 TATGACTACAGCTACCTCCAAGAGTCAGATCCAGGCTTTTACAGAAATTATATTGAGCCT
 +429 TyrAspTyrSerTyrLeuGlnGluSerAspProGlyPheTyrArgAsnTyrIleGluPro

+1481 TACTTGGAACAAGCCAGTCGTATCTGGCCATGGCTTCTTGGGGCAGCACTGGTGGGAGCT
 +449 TyrLeuGluGlnAlaSerArgIleTrpProTrpLeuLeuGlyAlaAlaLeuValGlyAla

+1541 GTTATTGCTGCAGCTCTCTCTGGGCTTAGCAGTAGGCTATGCCTTCAGAAGAAGAAGAAG
 +469 ValIleAlaAlaAlaLeuSerGlyLeuSerSerArgLeuCysLeuGlnLysLysLysLys

+1601 AAGAAGCAACCCCAGGAGGAAAGGCAGCCACTCCTCATGGACAAAGACGACTACCACAGC
 +489 LysLysGlnProGlnGluGluArgGlnProLeuLeuMetAspLysAspAspTyrHisSer

+1661 TTGCTGTATCAGAGCCATCTGTGAACATCCTAGGAAACAGAGTGGGACTGAAAGGTTTTA
 +509 LeuLeuTyrGlnSerHisLeu***

+1721 CCTCACTCGACTTATTTGTTGGTGTTTCTACAAATTTAAACTAGTATAAAACATAGACCA

+1781 TAGCTGTTTGGCTTTTTTTCAGACCCATGTTTTTTCCTAAGTCCTAGTTTCTAAGAAATG

+1841 ACTGGGATTTGCTAAAATATATATATATATAAATAATAACTTACTAATAGCTAAATAAAA

+1901 TTTCCTCTTACAACTAATTGGAAAAAAAAAAAAAAAAA
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Results

Sequence analysis of cDNAs originating by alternative splicing

Several independent putative mouse tyrosinase cDNA clones have been isolated from a mouse B16 melanoma cDNA library. Restriction analysis of these clones showed that four different types of cDNA clones existed, which originate from alternatively spliced transcripts (Ruppert *et al.*, 1988). The nucleic acid sequence and the deduced protein sequence of the most frequently isolated cDNA clone pmcTyr1 (containing all exons) is given in Figure 1. The cDNA insert of 1960 bp in length contains the entire coding region and the 3′ end of the gene including the polyadenylation signal AATAAA (Proudfoot and Brownlee, 1976) at position +1894 to +1899 and a poly(A) tail at position +1922 to +1939. The prominent proximal transcription start site mapped by primer extension analysis (Ruppert *et al.*, 1988) was designated as +1 and is indicated by a thick arrow in Figure 1. The transcript represented by pmcTyr1 was presumably initiated at the minor transcription start site at nucleotide −56 since the sequence extends to −20. The differences between cDNA clones pmcTyr1, pmcTyr2 and pmcTyr3 are also indicated in Figure 1. The clone pmcTyr2 representing 1.5% of the cDNA isolates has deleted that part of exon 1 corresponding to nucleotides +390 and +901 and pmcTyr3 representing ~10% of the isolated cDNAs has deleted exon 3 which corresponds to nucleotides +1119 to +1266. The cDNA clone pmcTyr4 which is identical to pmcTyr1, but extends beyond the poly(A) addition site is not included in this analysis (Ruppert *et al.*, 1988). Different exon usage leads to different reading frames downstream of the deleted exon regions in pmcTyr2 and pmcTyr3 in comparison to pmcTyr1. This results not only in different protein sequences, but also in earlier termination in pmcTyr2 and pmcTyr3. The clone pmcTyr2 has the coding capacity for a polypeptide of 105 amino acid residues in length when the same initiation codon is used as in pmcTyr1. The usage of an initiation codon (ATGII) 49 nucleotides downstream of ATGI would result in a coding capacity for a polypeptide 346 amino acid residues in length with an identical carboxy terminal part of the protein encoded by pmcTyr1 (+256 to +515; see Figure 2B). The open reading frame of the cDNA clone pmcTyr3 is 371 amino acid residues in length. This amino acid sequence is identical with the one encoded by pmcTyr1 from position −18 to +328 as indicated in Figure 2B.

The cDNA clone pmcTyr1 is able to encode a polypeptide

Fig. 1. DNA sequence of pmcTyr1 and deduced amino acid sequence. The nucleotide sequence of pmcTyr1 (top line) and the encoded protein sequence (bottom line) is shown. The transcription start site of the major promoter P1 (Ruppert *et al.*, 1988) is indicated by a big arrow at +1 of the nucleotide sequence. The cDNA clone pmcTyr1 represents a copy of a transcript which was presumably initiated at the transcription start site at nucleotide −56 of the minor promoter P2 (Ruppert *et al.*, 1988). The polyadenylation signal AATAAA (Proudfoot and Brownlee, 1976) at nucleotides +1894 to +1899 is underlined. The boxed regions represent the exons or parts of exons which have been spliced out in the mRNAs represented by pmcTyr2 (nucleotides +390 to +901) and pmcTyr3 (nucleotides +1119 to +1266). The deduced amino acid sequence has a putative signal peptide (von Heijne, 1983), the cleavage site is indicated by a bent arrow at amino acid +1. There are six potential glycosylation signals (Marshall, 1974) indicated by brackets; the putative glycosylation sites are at amino acids +71, +95, +145, +214, +321 and +356. The position of ATGII is at nucleotides +132 to +134.

533 amino acids in length with a putative leader peptide of 18 amino acid residues (Blobel and Dobberstein, 1975; von Heijne, 1983) and six potential glycosylation signals (Asn-X-Ser or Asn-X-Thr), (Marshall, 1974) as indicated in Figure 1. This predicts a polypeptide of 515 amino acid residues after cleavage of the leader peptide and with a mol. wt of 58 547 daltons, which is close to the estimated mol. wt of the unglycosylated T_3 isoform of mouse tyrosinase of 56–57 kd (Burnett, 1971; Hearing *et al.*, 1978). The size of the polypeptides potentially encoded by pmcTyr2 and pmcTyr3 is significantly smaller.

Only pmcTyr1 encodes tyrosinase

In order to test which of the putative mouse tyrosinase cDNA clones are able to encode tyrosinase we expressed these cDNAs in two different tyrosinase negative cells. We designed a new expression vector, named pHD, which contains a hybrid promoter: four 72-bp repeat units of simian virus 40 (SV40) are placed in front of the −105 to +19 region of the Herpes simplex virus thymidine kinase (HSV tk) gene, followed by a polylinker and an SV40 splice and polyadenylation signal (Figure 2A). The different cDNA inserts of pmcTyr1, pmcTyr2, pmcTyr3 and pMT4 (Shibahara *et al.*, 1986) and the CAT gene (Gorman *et al.*, 1982) were cloned into pHD in the orientation which allows transcription of the sense strand as indicated in Figure 2B. The clones were named pHDmcTyr1, pHDmcTyr2, pHDmcTyr3, pHDMT4 and pHDCAT respectively. Plasmid DNAs were transfected by electroporation into the melanoma cell line SKMel25 or into the human breast cancer cell line MCF-7. Protein extracts of transfected cells were assayed for tyrosine hydroxylase activity (Pomerantz, 1969). Hydroxylation of tyrosine to Dopa is the first reaction in melanin synthesis catalysed by tyrosinase. This assay measures the formation of ^3H_2O from 3,5-[^{3}H] L-tyrosine. Transfection of SKMel25 cells with pHDmcTyr1 yielded tyrosine hydroxylase activity of 431 (±59)c.p.m. ^3H_2O formation/min × mg protein. After transfection into MCF-7 cells 306 (±149) c.p.m. ^3H_2O formation/min × mg protein were obtained (Figure 3). In contrast, transfection of pHDmcTyr2, pHDmcTyr3 and pHDMT4 led to tyrosine hydroxylase activities within the range of background

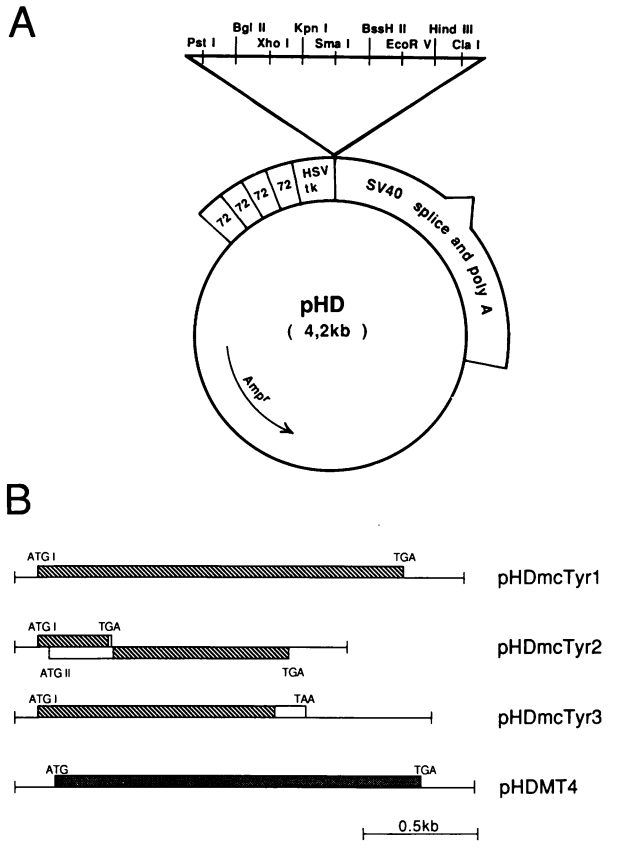

Fig. 2. Recombinant plasmids used for the expression of different putative tyrosinase cDNA clones. (**A**) Schematic representation of the expression vector pHD. Four 72-bp repeats of SV40 are located upstream of the HSV tk promoter region −105 to +19. This hybrid promoter is followed by a polylinker region with nine different restriction sites and processing signals of SV40. DNA fragments cloned into the polylinker region are placed under the control of the SV40/HSV tk hybrid promoter and are terminated by the SV40 signals downstream of the cloning sites. The restriction sites *PstI* and *Hin*dIII are not unique. (**B**) Schematic representation of the cDNAs inserted into the expression vector pHD to form the recombinants pHDmcTyr1, pHDmcTyr2, pHDmcTyr3 and pHDMT4. Each cDNA is inserted in the sense orientation downstream of the SV40/HSV tk promoter of pHD. The translational initiation (ATG) and termination signals (TAA and TGA) are also given and the resulting open reading frame is indicated by a bar. In the case of pHDmcTyr2 the usage of a second initiation codon ATG II 49 nucleotides downstream of ATG I would lead to a longer open reading frame, as indicated. Amino acid sequences of the open reading frame of the cDNA inserts of pHDmcTyr2 and pHDmcTyr3, identical with the one of pHDmcTyr1, are indicated by a striped bar; different sequences are indicated by an open bar. Lines indicate nontranslated nucleotide sequences.

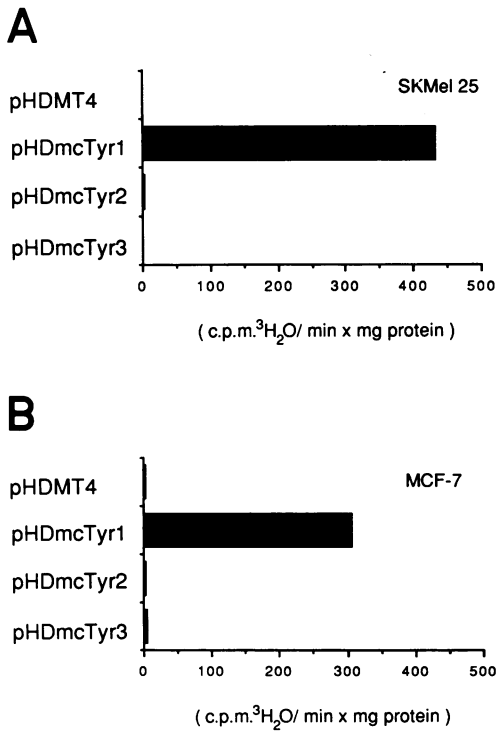

Fig. 3. Expression of tyrosinase activity in cells after electroporation with putative tyrosinase cDNA clones. The tyrosinase-negative cell lines SKMel25 (A) and MCF-7 (B) were electroporated with DNA of the expression clones pHDMT4, pHDmcTyr1, pHDmcTyr2, and pHDmcTyr3 (see Figure 2B). Extracts of the electroporated cells were analysed by an assay for tyrosinase-specific tyrosine hydroxylation (Pomerantz, 1969). The enzymatic activity is expressed as c.p.m. ^3H_2O formation/min × mg protein. All the experiments presented here were done in duplicate and the values were corrected for background levels by subtracting the value of the control transfection with pHDCAT (see Materials and methods).

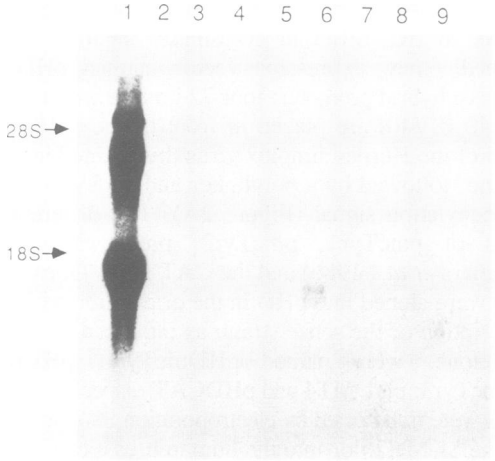

Fig. 4. Tissue-specific expression of tyrosinase mRNA. RNA analysis was performed with 10 μg total RNA extracted from B16 melanoma cells (**lane 1**), spleen (**lane 2**), liver (**lane 3**), muscle (**lane 4**), brain (**lane 5**), testes (**lane 6**), kidney (**lane 7**), heart (**lane 8**) and lung (**lane 9**). The RNAs were hybridized with antisense transcripts of the cDNA clone pmcTyr1.

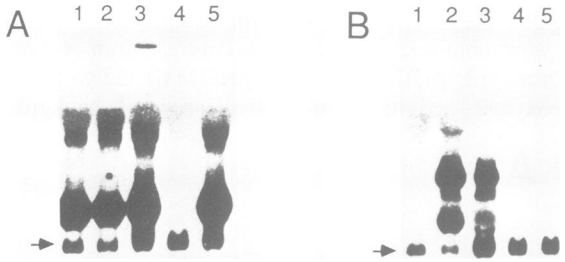

Fig. 5. Analysis of tyrosinase mRNA in melanoma cells. RNA blot analysis was performed with 10 μg total RNA of the amelanotic melanoma cell lines MML-I (**lane 1**), SKMel28 (**lane 3**), SKMel25 (**lane 4**) Colo38 (**lane 5**) and of the melanotic melanoma cell line A375 (**lane 2**). Northern blots were hybridized with the mouse tyrosinase clone pmcTyr1 (**A**) and with the cDNA clone pMT4 (Shibahara *et al.*, 1986) (**B**). The arrow indicates a signal obtained by hybridizing the Northern filters with a glyceraldehyde-3-phosphate-dehydrogenase cDNA probe (Fort *et al.*, 1985) as internal standard.

Fig. 6. Alignment of the deduced amino acid sequence of the mouse tyrosinase clone pmcTyr1 with the open reading frame of pMT4. The sequences are shown in single-letter code; the top line indicates the amino acid sequence of the mouse tyrosinase which is aligned with the sequence encoded by pMT4 (Shibahara *et al.*, 1986). Arrows indicate conserved cysteines. *Indicates that identical amino acids are found in both sequences; numbers 1–6 indicate that chemically similar amino acids are present (1 = long aliphatic hydrophobic group; 2 = short aliphatic hydrophobic group; 3 = single aromatic ring group; 4 = aliphatic hydroxy group; 5 = acidic group; 6 = basic group). The position of two regions which are highly conserved between tyrosinases of different species is indicated (I and II); see also Figure 7.

variability. These results prove that only pHDmcTyr1 has the coding capacity for tyrosinase.

The tyrosinase gene is expressed in a tissue-specific manner

Enzymatic activity of tyrosinases has only been found in melanocytes present in skin, hair bulbs and in the eye (reviewed by Hearing and Jimenez, 1987). In order to test whether expression of the tyrosinase gene is regulated in a tissue-specific fashion, we probed RNAs from different tissues with the cDNA clone pmcTyr1 as shown in Figure 4. RNA hybridizing with an antisense transcript of the clone pmcTyr1 is expressed in mouse B16 melanoma cells but not in lung, heart, kidney, brain, muscle and liver; only testes RNA shows a weak hybridization signal. RNA of B16 melanoma cells shows a broad signal of 1.8–2.0 kb and some minor signals larger in size (see Figure 4). Attempts to detect tyrosinase mRNA in mouse skin have failed, possibly due to the low representation of melanocytes in skin.

The tyrosinase gene is expressed in melanotic and in amelanotic cells

We analysed melanotic and amelanotic melanoma cells for the presence of transcripts hybridizing with the cDNA clones pmcTyr1 and pMT4 (Shibahara *et al.*, 1986). The results are shown in Figure 5. The clone pmcTyr1 showed a prominent hybridization signal at 1.8–2.0 kb and a signal larger in size to the RNA of the melanotic cell line A375 and three amelanotic melanoma cell lines MML-I, SKMel28 and Colo38; only the RNA of the amelanotic melanoma cell line SKMel25 showed no hybridization signal with antisense RNA of the clone pmcTyr1 (Figure 5A). Probes from the clone pMT4 hybridize to the RNA of the melanotic melanoma cell line A375 and to the RNA of the amelanotic melanoma cell line SKMel28; no hybridization signal was detected in the RNAs of the amelanotic melanoma cell lines Colo38, SKMel25 and MML-I (Figure 5B). In a further RNA analysis we found that the amelanotic melanoma cell line MeWo showed the same hybridization pattern as the

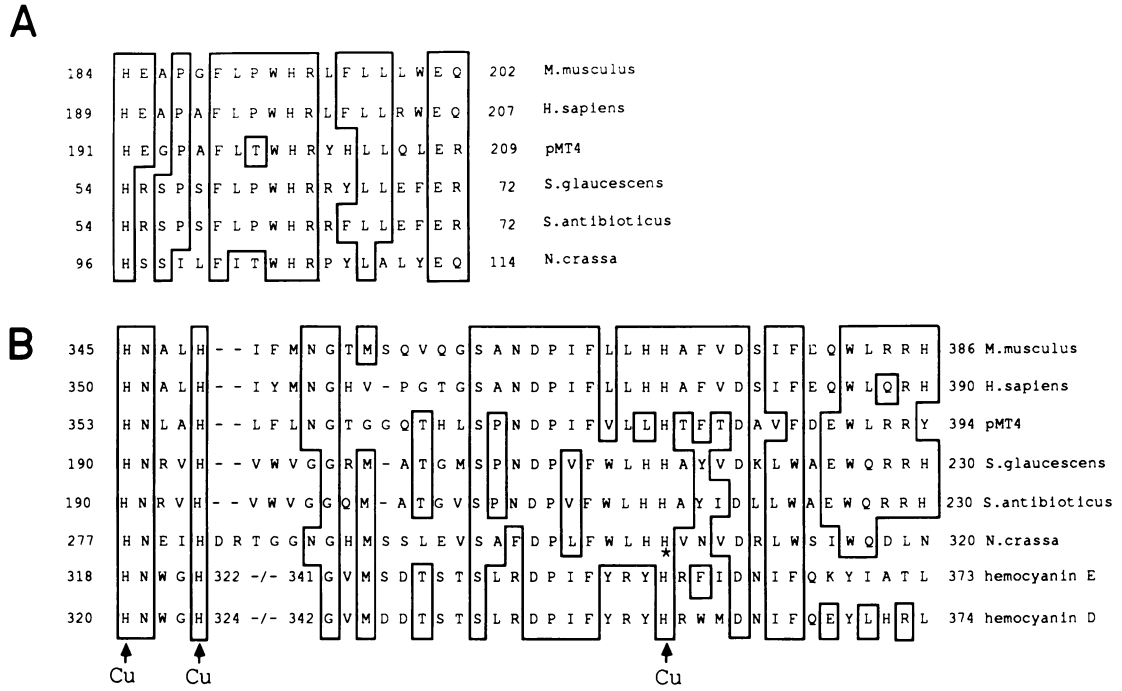

Fig. 7. Homologous regions between tyrosinases of different species, the tyrosinase-related protein encoded by pMT4 and haemocyanins. (**A**) Conservation of amino acid sequences in region I of tyrosinases is shown by sequence comparison of protein sequences of the tyrosinases of mouse, man (Kwon *et al.*, 1987), the protein encoded by pMT4 (Shibahara *et al.*, 1986), the tyrosinases of *S.glaucescens* (Huber *et al.*, 1985), *S.antibioticus* (Bernan *et al.*, 1985) and *N.crassa* (Lerch, 1982). The numbers indicate the position of the amino acids in the respective protein sequence. Conserved amino acids which were found in ≥50% of the cases are boxed. The amino acid sequence is given in single-letter code. (**B**) Conservation of amino acid sequences in region II is shown by comparison of the protein sequences of the tyrosinases of mouse, man (Kwon *et al.*, 1987), *S.glaucescens* (Huber *et al.*, 1985), *S.antibioticus* (Bernan *et al.*, 1985) *N.crassa* (Shibahara *et al.*, 1986) and with subunit E (Schneider *et al.*, 1983) and subunit D (Scharlau *et al.*, 1983) of haemocyanin of the spider *E.californicum*. The numbers indicate the positions of the amino acids in the protein sequence. Sequences which are conserved by ≥50% between all proteins listed or all tyrosinases are boxed. The histidine H at position 306 indicated by * of *N.crassa* tyrosinase is part of the catalytic centre (Lerch, 1983). Binding of one of the copper atoms is conferred by three histidyl groups in haemocyanins. The position of the copper-binding histidines which were described for homologous haemocyanins (Gaykema *et al.*, 1984) is indicated by Cu.

melanotic melanoma cell A375 (unpublished data). That is, tyrosinase gene transcripts are present in three out of four amelanotic cells. We determined tyrosinase activity of the amelanotic cell lines SKMel25, SKMel28 and Colo38 by the tyrosine hydroxylase assay (Pomerantz, 1969); no activity was found in any of the three cell lines (unpublished data). Thus, it can be concluded that the mechanism which leads to the absence of tyrosinase in the amelanotic melanoma cells Colo38 and SKMel28 is on the post-transcriptional level.

Mouse tyrosinase and the polypeptide encoded by pMT4 share sequence similarities

The cDNA clone pMT4 (Shibahara *et al.*, 1986) shows 45% DNA sequence homology to the cDNA clone pmcTyr1. In addition, the protein sequences which are predicted by these two cDNA clones demonstrate an overall homology of 39%. A comparison of these two protein sequences is shown in Figure 6. It is very striking that 15 out of 17 cysteine residues are conserved in equivalent positions between the two polypeptides. This suggests that these two proteins have similar secondary structures. There are two regions which are well conserved between tyrosinases of different species (see below). These two regions are also well conserved between the protein encoded by pMT4 and mouse tyrosinase.

Tyrosinases and haemocyanins show homologous protein sequences

A comparison of the deduced protein sequence of the mouse tyrosinase cDNA clone pmcTyr1 with the protein sequences of the tyrosinases from *N.crassa* (Lerch, 1982), *Streptomyces glaucescens* (Huber *et al.*, 1985), *Streptomyces antibioticus* (Bernan *et al.*, 1985) and man (Kwon *et al.*, 1987) showed two regions which were clearly conserved between all of these tyrosinases, as shown in Figure 7. The homology between mouse tyrosinase and the other tyrosinases in region I is 58% to *S.glaucescens*, 63% to *S.antibioticus*, 47% to *N.crassa*, 89% to human tyrosinase and 58% to the protein encoded by pMT4. In region II the homology between mouse tyrosinase and the other tyrosinases is 48% to *S.glaucescens*, 45% to *S.antibioticus*, 41% to *N.crassa*, 81% to human tyrosinase, 55% to the protein encoded by pMT4, 38% to subunit D and 38% to subunit E of haemocyanin from a spider.

Region II contains two interesting features. Firstly, the histidine at position 306 of *N.crassa* tyrosinase has been demonstrated to be part of the catalytic centre (Lerch, 1983). This histidine and flanking amino acid residues are conserved in tyrosinases, the protein encoded by pMT4 and haemocyanins (Figure 7B). Secondly, similarities of the binuclear copper complex between haemocyanins and *Neurospora* tyrosinase have been described by spectroscopic methods (for review, see Solomon, 1981) as well as by sequence comparisons (Huber *et al.*, 1985). It has been shown that a haemocyanin each of the two copper ions in the binuclear complex is bound by three histidyl groups (Gaykama *et al.*, 1984). One of the copper-binding sites of haemocyanins shows a

striking homology to conserved protein sequences of all sequenced tyrosinases as shown in Figure 7B.

Discussion

pmcTyr1 encodes tyrosinase

Several cDNA clones have been isolated as candidates for the mouse tyrosinase. By transfection of these clones into two different tyrosinase negative cells followed by the analysis of tyrosinase-specific enzymatic activity, we have shown that only the cDNA insert of pmcTyr1 was able to confer tyrosinase activity to cells. Consistent with the result that pmcTyr1 encodes mouse tyrosinase is the finding of a high degree of sequence homology to other known tyrosinases in two conserved regions. One of these regions, 42 amino acid residues in length, has been shown to be part of the catalytic (Lerch, 1983) as well as part of the copper-binding site (Huber *et al.*, 1985). Furthermore, pmcTyr1 encodes a protein of the expected size with a putative leader peptide and six putative glycosylation sites. These data are consistent with the fact that tyrosinase is a copper-containing glycoprotein which has to be transported to the melanosome of melanocytes.

We could also demonstrate by expression studies that the cDNA clones pMT4 (Shibahara *et al.*, 1986), pmcTyr2 and pmcTyr3 do not encode tyrosinase. The mouse cDNA clone Tyrs33 described previously (Yamamoto *et al.*, 1987) originates from a transcript which is putatively generated by alternative splicing removing exon 3 as has been shown to be the case in the cDNA clone pmcTyr3. The only differences we found in the coding region were one missing and five additional nucleotides in Tyrs33 in comparison to pmcTyr3. In order to verify that the sequence we obtained from pmcTyr3 was correct we sequenced this region of a genomic tyrosinase clone of a c^{ch}/c^{ch} mouse and found that the nucleotide sequence was in agreement with the sequence of pmcTyr3. Since we could also demonstrate that the presence of exon 3 is necessary for a cDNA clone to confer tyrosinase activity and since exon 3 encodes the most conserved protein sequence (region II) between tyrosinases of different species, Tyrs33 cannot encode tyrosinase.

The cDNA clone pmcTyr2 has two possible reading frames (see Figure 2B). Translation initiation from ATGI leads to a protein 12 kd in length. Translation initiation from ATGII would produce a protein 346 amino acid residues in length; in this protein 260 amino acid residues of the carboxy terminus would be identical with the deduced protein sequence of pmcTyr1 including the conserved region II, but not region I present in all studied tyrosinases (Figure 7). Nevertheless, we have shown that this clone confers no tyrosinase activity.

The human tyrosinase cDNA clone pmel34 (Kwon *et al.*, 1987) and the mouse tyrosinase clone pmcTyr1 show 78% DNA sequence homology and 80% protein sequence homology. It is clear from sequence comparisons between pmel34 and the mouse tyrosinase cDNA clones that pmel34 contains a full complement of exons. Since such a cDNA from the mouse (pmcTyr1) was able to mediate tyrosinase activity to cells by gene transfer, we assume that this clone encodes the human tyrosinase.

Expression analysis of the cDNA insert of pMT4 (Shibahara *et al.*, 1986) failed to yield any tyrosinase enzyme activity. A comparison of the protein encoded by pMT4 with other tyrosinases clearly showed a high degree of sequence conservation in regions I and II, as shown in Figure 7A and B. There is a large overall homology of the protein encoded by pMT4 and mouse tyrosinase (as shown in Figure 6). We assume therefore that pMT4 encodes a copper-containing enzyme with an enzymatic function related to the one of tyrosinase, and we also suspect that the corresponding genes have arisen from a common ancestor gene by duplication.

On the basis of the evidence presented below we wish to suggest that pMT4 encodes the 5,6-dihydroxyindole conversion factor which catalyses the conversion of 5,6-dihydroxyindole to melanin (Körner and Pawelek, 1982). Firstly, this enzymatic activity has only been found in preparations of the highly enriched tyrosinase isozyme T_4 and not in the enriched tyrosinase isozymes T_1 and T_3 (Hearing *et al.*, 1982; Körner and Pawelek, 1982). The tyrosinase isozyme T_4 is present in an aggregated form of several tyrosinase isozymes which can be complexed to other proteins (Hearing *et al.*, 1981). The bulk of tyrosinase activity is found in the T_4 form in melanosomal fractions (Hearing *et al.*, 1978) and this aggregated T_4 isozyme is the main source of purified tyrosinase. Mouse tyrosinase and the protein encoded by pMT4 (Shibahara *et al.*, 1986) are very similar in secondary structure (15 out of 17 cysteins are conserved), potentially in glycosylation (six and seven putative glycosylation sites are present) and in mol. wt (M_r of mouse tyrosinase is 58 547 daltons and M_r of the protein encoded by pMT4 is 57 872 daltons). Since both proteins are expressed in melanocytes it cannot be ruled out that the protein encoded by pMT4 is co-purified with mouse tyrosinase using the standard protocols for tyrosinase T_4 isozyme purification. Secondly, the monoclonal antibody TMH-1 (Tomita *et al.*, 1985) raised against purified tyrosinase T_4 isozymes reacts with the T_4 isozyme but not with the T_1 or T_2 isozymes of tyrosinase. Nevertheless TMH-1 reacts with the protein encoded by pMT4 (Shibahara *et al.*, 1986).

Evolutionary relationship of tyrosinases to other copper-binding proteins

Tyrosinase contains a binuclear copper complex and functions as a mono-oxygenase and as a two-electron oxidase. Both copper atoms in this protein are able to bind oxygen; the changes in spectral features before and after oxygen binding in tyrosinase are very similar to the observed changes in another oxygen-binding copper protein, haemocyanin (for review see Solomon, 1981). For these data it was concluded that there is a structural relationship between tyrosinases and haemocyanins. A comparison of the protein sequence of tyrosinases from *N.crassa* and *S.glaucescens* with the haemocyanin subunit E of *Euripelma californicum* showed some sequence conservation (five out of 15) (Huber *et al.*, 1985). We find a much larger region of sequence homology. Many of the 30 conserved amino acid residues in region II (see Figure 7B) are also present in haemocyanins: 16 are shared by haemocyanin subunit E and 18 by subunit D of *E.californicum*.

The tyrosinase gene is located in or close to the c-locus of the mouse

Detailed complementation mapping showed that radiation induced as well as neutral *c*-locus mutations cannot

complement each other for albinism (Green, 1981; Russell *et al.*, 1982). There is, therefore, only one complementation group affected by *c*-locus mutations indicating the presence of either a regulator for the tyrosinase gene (Hearing, 1973) or the tyrosinase structural gene in the *c*-locus region. We have demonstrated that the mouse tyrosinase cDNA clone pmcTyr1 confers tyrosinase activity to a breast cancer cell line which has no specialized functions for melanin synthesis. In addition, we have shown that the cDNA insert of pmcTyr1 encodes a protein homologous to tyrosinase of closely as well as distantly related species. Since in all studied *c*-locus lethal albino mice (Gluecksohn-Waelsch, 1979) the genomic DNA sequences hybridizing to pmcTyr1 and the homologous human clone are missing (Kwon *et al.*, 1987; Ruppert *et al.*, 1988) we conclude that the tyrosinase gene and not a regulatory gene involved in melanin synthesis is encoded at the *c*-locus. This hypothesis can now be tested in transgenic mice.

Materials and methods

DNA sequencing

The deletions 5′ and 3′ of the cDNA clones pmcTyr54 and pmcTyr63 (Ruppert *et al.*, 1988) were obtained by deleting insert sequences from both sides by using exonuclease III (New England Biolabs), S1 nuclease (Boehringer) and T4 DNA-polymerase (BRL) (Henikoff, 1984, with modification). The 5′ and 3′ deletion clones differing by ~150 bp, as judged by restriction analysis, were sequenced by using the dideoxy chain termination method (Sanger *et al.*, 1977) with modifications made for sequencing plasmid DNA (Chen and Seeburg, 1985). Either T3 or T7 promoter-specific primers were used for sequencing with sequenase (United States Biochemical Corporation).

Construction of plasmids

The expression vector pHD was constructed by modifying the plasmid pTK-CAT (Miksicek *et al.*, 1986). The modifications were the integration of four 72-bp repeat units of SV40, upstream of the HSV tk promoter (pTK-CAT-14C; also provided by R.Miksicek) and the substitution of the CAT gene and parts of the HSV tk fragment (+19 to +56) by the polylinker (as shown in Figure 2A).

The clones pHDmcTyr1 and pHDmcTyr3 were constructed by integrating the cDNA insert of pmcTyr1 or pmcTyr3 into the plasmid pHD. pHDmcTyr2 was constructed by first integrating a DNA fragment (−20 to +123) of pmcTyr1 to the 5′ region of the cDNA insert of pmcTyr2 in order to provide this cDNA clone with the initiation codon (ATGI); this hybrid fragment was subcloned into the plasmid pHD. The plasmids pHDMT4 and pHDCAT were constructed by integrating a subfragment of pMT4 (−174 to +1828) (Shibahara *et al.*, 1986) or the bacterial CAT-gene (Gorman *et al.*, 1982) into pHD; all constructs contained the whole coding region (see Figure 2B).

Cell culture

The human amelanotic melanoma cell lines SKMel28 (Houghton *et al.*, 1982) Colo38 (McCabe *et al.*, 1979), MML-I (Tilgen *et al.*, 1983), a subclone of SKMel25 (all provided by Dr S.Matzku, Heidelberg, FRG) and the human and mouse melanotic melanoma A375 (provided by Dr S.Matzku) and B16 (provided by Dr W.Birchmaier, Tübingen, FRG) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS) (Seromed). The human breast cancer cell line MCF-7 (Horwitz *et al.*, 1978) was cultured in DMEM containing 10% FCS and 1 μg/ml insulin.

Electroporation of cells

Cells were grown to confluency, trypsinized and centrifuged. The pellets were washed twice with phosphate-buffered saline (PBS). The resuspended cells were adjusted to a concentration of 1.25×10^7 cells/ml. Of this suspension 0.8 ml (1×10^7 cells) was mixed with 5 pmol plasmid DNA and used for electroporation (Fromm *et al.*, 1985; Chu *et al.*, 1987) with a Gene Pulser (BioRad). The clone pHDCAT (as a negative control) and the clones pHDmcTyr1, pHDmcTyr2, pHDmcTyr3 and pHDMT4 were transfected in parallel. One day after cell culture the medium was changed.

Tyrosine hydroxylase assay and protein determination

Electroporated cells were harvested after 2 days. Cells were washed three times in PBS before scraping. The cell suspension was pelleted (2 min, 5000 *g*) and taken up in a 5-fold volume of 1 × PBS, 0.5% Triton X-100. After resuspension by vortexing, the cells were sonicated by short pulses until no intact cells remained (40−50 s) (Fuller *et al.*, 1987). After sonication 50 μl cell extract was mixed with 10 μl of a solution containing [^{3}H]tyrosine (100 μCi/ml, 0.3 mM) 0.5 mM Dopa and 100 mM $NaPO_4$, pH 6.8. This mixture was incubated for 40 min at 37°C. Unreacted [^{3}H]tyrosine was removed by adding 1 ml of a suspension of 100 mg/ml Norit A (Serva) in 0.1 M citric acid followed by vortexing and centrifuging for 2 min at 8000 *g*. The supernatant fraction was poured over a Dowex 50 column (Serva) equilibrated with 0.1 M citric acid in order to bind residual tyrosine. ^3H_2O was eluted with 0.1 M citric acid (Pomerantz, 1969; Körner and Pawelek, 1977). The eluate was neutralized with 1 M Tris pH 9 before adding scintillation cocktail Unisolve I (Zinsser Analytic). Protein extracts of B16 melanoma cells were used as a positive control. Extracts of SKMel25 or MCF-7 cells electroporated with pHDCAT DNA were used as a negative control. The value of the negative control which was done in parallel was subtracted from the values of the other protein extracts. All experiments were done in duplicate. Tyrosine hydroxylase activity could be detected in 5 μg total protein of B16 melanoma cells 15-fold over background (unpublished data). The abundance of tyrosinase is estimated to be <0.01% of the total protein in melanoma cells (Hearing, 1987); therefore one can detect <0.1 ng tyrosinase by this assay.

Protein determinations were carried out using a protein assay kit, obtained from Bio-Rad, based on a protein assay described by Bradford (1976). As a reference standard bovine serum albumin (fraction V) was used.

RNA analysis

RNA from cells in culture was isolated by the method of Auffray and Rougeon (1980). This RNA was electrophoretically separated on formaldehyde agarose gels (Dobner *et al.*, 1981). The RNA was blotted on Gene Screen membrane (Du Pont), baked and UV-cross-linked. The filters were hybridized with ^{32}P-labelled antisense RNA (Melton *et al.*, 1984) in 50% formamide, 5 × SSC, 50 mM sodium phosphate, pH 6.5, 8 × Denhardt's solution, 0.5 mg/ml yeast RNA and 1% SDS at 55°C and 3 × washed in 2 × SSC, 1% SDS at 45°C and exposed on X-ray film (Kodak).

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