Table 3 Hepatic concentrations of $MeSO_2$-PCB metabolites after the administration of KC500, PentaCB or HexaCB to mice, hamsters, rats and guinea pigs

Treatment	Animals	Total $MeSO_2$-PCBs (ng/g liver)
KC500	Mice	267
	Hamsters	46
	Rats	102
	Guinea pigs	138
PentaCB	Mice	265
	Hamsters	13
	Rats	62
	Guinea pigs	31
HexaCB	Mice	577
	Hamsters	31
	Rats	190
	Guinea pigs	270

Animals were killed at 4 days after the administration of KC500 (37.5 mg/kg, ip), PentaCB (11 mg/kg, ip) or HexaCB (19 mg/kg, ip). Results are expressed as the mean for 5-6 animals.

(Table 2)．一方，total T_3濃度の低下はモルモットのみであった．

また，PentaCB投与では，マウス，ハムスターおよびラットで，HexaCB投与では，マウスのみに血中total T_4濃度の低下が見られた．なお，マウスにPentaCBを投与したときのみtotal T_3濃度の低下も見られた．

血中甲状腺刺激ホルモン（TSH）濃度は4種の動物にいずれのPCBを投与した場合にも変化しなかった(Table 2)．

これらの結果から，PCB投与による血中甲状腺ホルモン濃度への影響には，少なくとも一部，動物種差が見られることが明らかになった（Kato et al., 2003b; 2003c)．

血中total T_4濃度の低下（あるいはその動物種差）を生む要因として，① 甲状腺への直接作用（および各動物の甲状腺のPCBあるいはその代謝物に対する感受性の相違）や，② PCBやその代謝物によるT_4-UDP-GTの誘導（およびその動物種差），また，③ PCBやその代謝物の血中T_4輸送タンパク（トランスサイレチン：TTR）との結合などが考えられる．

以下，血中T_4濃度の低下機構について，動物種差発現の解明を目指し，検討した結果を記す．

2．甲状腺への直接的作用

PCBによる血中T_4濃度の低下の要因として，甲状腺濾胞上皮細胞におけるT_4生合成の抑制や，甲状腺からのT_4の放出抑制が考えられる（Collins and Capen, 1980; Saeed and Hansen, 1997)．そこで，マウス，ハムスター，ラットあるいはモルモットに，KC500，PentaCBあるいはHexaCBを投与し，甲状腺の空胞変性，濾胞上皮細胞の肥大および過形成について検討した．

各PCBを投与したいずれの動物にも甲状腺への上記悪影響は見られず，各PCB投与時に起こる血中T_4濃度の低下は，PCBあるいはその代謝物による甲状腺への直接作用によるものではないことが示唆された．

3．PCB類の代謝とその動物種差

PCBのメチルスルフォン代謝物の3-メチルスルフォニル-（3-$MeSO_2$-）および4-$MeSO_2$-PentaCB，および3-$MeSO_2$-HexaCBなどは，UGT1A1/6誘導活性を有し，この酵素誘導がT_4の代謝を亢進させ，血中T_4濃度の低下を引き起こすと考えられている（Kato et al., 2000)．したがって，$MeSO_2$代謝物の生成量の違いが，各動物種における血中T_4濃度の低下に差を生む要因になっている可能性が考えられる．

そこで，マウス，ハムスター，ラットあるいはモルモットにKC500，PentaCBあるいはHexaCBを投与し，血中T_4濃度の低下と，$MeSO_2$代謝物の生成量との関連性を検討した．KC500を投与後の肝臓中の総$MeSO_2$代謝物量は，マウスで最も高く，モルモット，ラット，ハムスターではそれぞれマウスの約1/2，1/3，および1/6量であった（Table 3)．また，PentaCB投与後の$MeSO_2$代謝物生成量もマウスで最も高く，続いてラット，モルモット，ハムスターの順であった．さらに，HexaCB投与でも，$MeSO_2$代謝物はマウスにおいて最も多く生成され，その生成量は，以下，モルモット＞ラット＞ハムスターの順であった（Table 3)．

以上，各PCB投与時の，各動物での血中T_4濃度の低下と肝臓中各$MeSO_2$代謝物量との間には必ずしもはっきりとした相関性は認められず，血清中total T_4濃度の低下が，単に$MeSO_2$代謝物の生成量に依存して起こるのではないことが示唆された．

4．肝UDP-GTへの影響

一般に，PCBによるラット血中T_4濃度の低下の主因として，T_4のグルクロン酸抱合をはじめとするT_4の代謝系の促進が考えられている（Barter and Klaassen, 1994; Schuur et al., 1997; van Birgelen et al., 1995)．そこで，PCB投与時の各動物における血中T_4濃度の低下と，肝T_4-UDP-GT活性ならびに胆汁中へのT_4のグルクロン酸抱合体排泄量との関連性を調べた．

モルモットにKC500を，また，マウスにHexaCBを投与すると，いずれの場合にも肝T_4-UDP-GTが誘導された（Table 4)．しかし，T_4のグルクロン酸抱合体の胆汁排泄量には有意な変化は見られなかった（Table 4)．KC500をマウス，ハムスター，ラットに投与した場合にも，モルモットの場合と同様に，血中T_4濃度は低下するものの，T_4-UDP-GTの誘導は，モルモットの場合とは異なり，起こらなかった（Tables 2 and 4)．このように，KC500やHexaCBによる血中T_4濃度の低下を，単に

Table 4 Effects of KC500, PentaCB and HexaCB on hepatic microsomal T_4-UDP-GT activity and the biliary excretion of $[^{125}I]T_4$-glucuronide in mice, hamsters, rats and guinea pigs

Treatment	Animals	T_4-UDP-GT	$[^{125}I]T_4$-glucuronide
KC500	Mice	→	→
	Hamsters	→	→
	Rats	→	↑
	Guinea pigs	↑	→
PentaCB	Mice	→	n.t.
	Hamsters	→	n.t.
	Rats	→	n.t.
	Guinea pigs	→	n.t.
HexaCB	Mice	↑	n.t.
	Hamsters	→	n.t.
	Rats	→	n.t.
	Guinea pigs	→	n.t.

Animals were killed at 4 days after the administration of KC500 (37.5 mg/kg, ip), PentaCB (11 mg/kg, ip) or HexaCB (19 mg/kg, ip).
Results are expressed as the mean for 4-6 animals. n.t.： not tested.

UDP-GTの誘導やT_4の胆汁排泄量で説明することは難しい.

また，PentaCB投与の場合には，マウス，ハムスターおよびラットのいずれでもUDP-GTは誘導されず，各動物で見られる血中T_4濃度の低下を，単にUDP-GT誘導で説明することはできなかった．なお，PentaCBを投与したモルモットでは，血中T_4濃度の低下やUDP-GTの誘導は認められなかった（Tables 2 and 4）.

そこで，PCB投与時のラットにおける血中T_4濃度の低下に，肝UDP-GTの誘導（活性上昇）が関与しているか否かをより明確にするために，Wistar系ラットおよびGunnラット（遺伝的にUGT1Aサブファミリーを欠損したWistar系ラットの突然変異体）を用い，PentaCB（112 mg/kg）あるいはKC500（100 mg/kg）投与後の血中T_4濃度の変動と，肝T_4-UDP-GTの発現量およびT_4グルクロン酸抱合活性との関連性を追究した.

血中total T_4，free T_4濃度は各PCB投与により両ラットで著しく低下した（Table 5）．一方，T_4-UDP-GT（UGT1A，UGT1A1，UGT1A6）の発現量およびT_4-UDP-GT活性はWistar系ラットで著しく増加したが，Gunnラットではこれら発現量や活性には有意な変化は認められなかった（Table 6）.

したがって，KC500 あるいはPentaCB投与時のGunnラットに見られる血中T_4濃度の低下は，肝T_4-UDP-GTの活性には依存していないことが示された．このことは，Wistar系ラットでのKC500あるいはPentaCB投与による血中T_4濃度の低下もまた，少なくとも一部T_4-UDP-GT非依存的な機序で起こっている可能性を示唆している（Kato et al., 2004）.

Table 5 Effects of KC500 and PentaCB on the levels of serum total T_4 and free T_4 in Wistar and Gunn rats

Treatment	Total T_4 (% of control)		Free T_4 (% of control)	
	Wistar	Gunn	Wistar	Gunn
Control	100	100	100	100
KC500	17	19	15	18
PentaCB	23	17	39	15

Animals were killed at 4 days after the administration of KC500 (100 mg/kg, ip) or PentaCB (112 mg/kg, ip).
Results are expressed as the mean for 3-8 animals.

5．トランスサイレチンとの結合

PCBの水酸化代謝物は血中T_4の輸送タンパクであるTTRと親和性をもち，T_4と競合的に結合することが報告されている（Lans et al., 1993）．この競合的結合が血中T_4の標的器官への輸送を撹乱し，血中T_4濃度を低下させる可能性が考えられる（Brouwer et al., 1998; Meerts et al., 2002）．そこで，マウス，ハムスター，ラット，モルモットにKC500（37.5 mg/kg および100 mg/kg）および$[^{125}I]$ T_4を投与し，血中$[^{125}I]$ T_4とTTRあるいはアルブミンとの結合率の変動を測定した.

その結果，ラットおよびモルモットでは，KC500投与によりT_4とTTRとの結合阻害が起こり，T_4とアルブミンとの結合率が増加することが明らかになった．一方，マウスおよびハムスターではPCBを投与しても，T_4と血中タンパク（TTR，アルブミン）との結合率にはほとんど変化は認められなかった.

したがって，ラットとモルモットでのKC500投与による血中T_4濃度の低下には，KC500（PCBs）あるいはそれらの水酸化代謝物によるTTRとの結合が関与している可能性が考えられるが，マウスやハムスターにおける血中T_4濃度の低下には，このような機構は考え難い.

6．血中T_4の肝臓への移行

前述までの研究結果から，PCBによる血中T_4濃度の低下機序やその動物種差を十分に説明することは、困難であった．そこで，T_4の体内動態に注目して，マウス，ハムスター，ラットあるいはモルモットにKC500を投与した場合の，$[^{125}I]T_4$のクリアランスをさらに調べた．KC500（100 mg/kg）投与により，いずれの動物においても，血中からの$[^{125}I]T_4$の血清クリアランスは増し，分布容積（組織移行性）も増加した．また，この組織移行性増加の度合いには，種差が見られ，ラットで4.2倍，ハムスターで3.7倍，モルモットで1.8倍，マウスで1.4倍であった.

これらの結果から，KC500投与による血中T_4濃度の低下には，血中から組織へのT_4の急速な移行が関与し

Table 6 Effects of KC500 and PentaCB on the levels of the hepatic microsomal UGT isoforms and T_4-UDP-GT activity in Wistar and Gunn rats

Treatment	UGT1A (% of control)		UGT1A1 (% of control)		UGT1A6 (% of control)		T_4-UDP-GT (% of control)	
	Wistar	Gunn	Wistar	Gunn	Wistar	Gunn	Wistar	Gunn
Control	100	n.d.	100	n.d.	100	n.d.	100	100
KC500	370	n.d.	138	n.d.	642	n.d.	392	111
PentaCB	163	n.d.	167	n.d.	106	n.d.	202	141

Animals were killed at 4 days after the administration of KC500 (100 mg/kg, ip) or PentaCB (112 mg/kg, ip). Results are expressed as the mean for 4-10 animals. n.d.: not detected.

ていることが示唆された．そこで，血中から消失した$[^{125}I]T_4$がどのような組織に移行したかを明らかにするために，KC500（100 mg/kg）投与後の，$[^{125}I]T_4$の組織分布を調べた．まずはじめに，各対照（KC500未処理）動物に$[^{125}I]T_4$を投与し，その組織分布を測定した．その結果，分布量は肝臓で特に高いことが判明した．また，KC500投与により，いずれの動物においても，特に肝の分布量が顕著に増加することが明らかになった．

したがって，KC500による血中T_4濃度の低下には，血中T_4の速やかな肝臓への移行が関わっているものと考えられる．

7．甲状腺ホルモンのトランスポーター

最近，有機アニオン輸送ポリペプチド（Oatp1，Oatp2，Oatp3，Oatp4）（Abe et al., 1998; Cattori et al., 2000; Friesema et al., 1999），Na^+/タウロコール酸共輸送ペプチド（Ntcp）（Friesema et al., 1999），L型アミノ酸トランスポーター（LAT1，LAT2）（Friesema et al., 2001），モノカルボン酸トランスポーター（MCT8）（Friesema et al., 2003）などが甲状腺ホルモンをも基質とすることが次々と明らかにされている．また，T_4の輸送に関わるとされるOatp1，Oatp2，Oatp3，Oatp4，NtcpおよびMCT8がラットの肝臓に発現していることも報告されている（Friesema et al., 2003; Li et al., 2002; Meier, 1995）．

そこで，ラットにKC500を投与し，各トランスポーター遺伝子発現への影響をRT-PCR法を用いて測定した．KC500をラットに投与すると，肝臓のOatp2 mRNAおよびLAT1 mRNAの発現量が有意に増加した．これらの結果は，これらトランスポーターの発現増加が肝臓のT_4蓄積量の増加，そして，血中T_4濃度の低下をもたらす要因になっている可能性を示している．

結　　語

マウス，ハムスター，ラットあるいはモルモットに，KC500を投与した場合，いずれの動物でも血中total T_4濃度の低下が見られた．また，マウス，ハムスター，ラットにPentaCBを投与した場合や，マウスにHexaCBを投与した場合にも，血中total T_4濃度の低下が見られたが，これら投与時には，一部，種差が認められた．

本研究では，各PCB投与による各動物の血中T_4濃度の低下機序やその動物種差を明らかにするため，PCB投与時の甲状腺の病理組織学的検討をはじめ，肝臓T_4-UDP-GTの誘導（活性増加），PCBの代謝，血中T_4-TTR結合量の変化，および肝臓でのT_4トランスポーター発現量への影響などを検討した．しかしながら，PCB投与による血中T_4濃度の低下機序やその動物種差を，単一の要因で説明することは難しく，これら検討した要因，あるいは未知の要因が複雑に絡み合って各動物の感受性が決定されているように考えられる．これまで，PCBによる血中T_4濃度の低下は，肝臓のT_4-UDP-GTの誘導（活性増加）が主因とされてきたが，本研究結果は，本酵素誘導は，単に一つの要因であるに過ぎないことを示唆し，PCBによる毒性発現は極めて複雑な機序を通して起こっていることを示している．

以上，ヒトを含む多くの動物種のPCBによる血中甲状腺ホルモン濃度の低下機構の解明はいまだ不十分であり，今後のさらなる研究が必要である．

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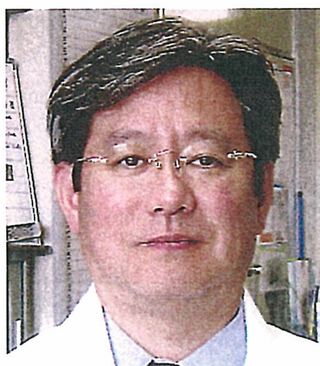
第3号

巻頭言

「油症」を診て感じたこと

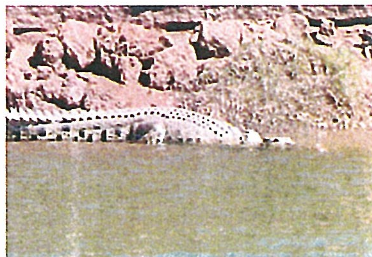


（九州大学大学院皮膚科学教授）
古江増隆

油症の患者さんをはじめて診察したのは、九州大学に着任した翌年の1998年のことです。多くの患者さんは昔に比べれば症状はかなりよくなったとおっしゃっていましたが、耳周囲、顔、うなじ、腋窩、陰部などに多発した面庖や嚢腫を診たときは驚きました。皮膚科医ですので、皮膚症状そのものにびっくりしたわけではありません。油症が発生して30年も経っているのに、強い症状に悩んでおられる患者さんがいまだにいらっしゃるのがショックだったのです。「今でも時々膿むのが悲しい」という老婦人の言葉にも心が痛みました。

2000年に九州大学油症治療研究班長を、2001年から全国油症治療研究班長を拝命しました。正直に申し上げますと、いったい何をすればいいのか不安でたまりませんでした。でも患者さんたちのことを考えると愚痴などこぼせないと気を引き締め、油症の文献をはじめから読むことにしました。油症研究班の業績は発生当時から克明に記載され発表されており、また倉恒匡徳先生他監修による英文単行本「Yusho」も発刊されていました。油症はPCBだけでなくPCDFなどのダイオキシン類によって汚染されたカネミ油の摂取による食中毒です。患者さんの血中PCB濃度やパターンは測定されていましたが、ダイオキシン類の血中濃度測定は測定感度や再現性の問題から検診では行なわれておりませんでした。一方、研究班内ではダイオキシン類をかなり低コストで高精度に測定することが可能になりつつありました。そこでダイオキシン類の血中濃度測定を検診に組み込むことは、医学的根拠に基づく診断基準の改訂につながるだけでなく、認定者の病態をより詳細に把握することが可能となり、ひいてはダイオキシン類の濃度を下げる薬剤の開発や症状軽減の治療薬の開発につながるという共通の考えが班内に自然と広がりました。厚労省の担当課の方々も即座に賛同いただき全面的にご援助くださり、感度・再現性・妥当性をクリアし、2004年9月29日にPCDF濃度を加えた新診断基準が作成されました。PCB/ダイオキシン類濃度と様々な症状や検査値がどのような関係にあるのか、なんらかの治療薬を開発できないかなどを今後のフォローの中心課題にすえたいと考えています。油症に関する情報は以下のサイトをご覧ください。「油症の検診と治療の手引き 2004」；http://www.kyudai-derm.org/ yusho/index.html、「油症研究30年の歩み・小栗一太他監修」；http://www.kyudai-derm.org/yusho_kenkyu/ index.html

Freshwater crocodile



Saltwater crocodile

ワニにも影響か？

Polychlorinated biphenylsによる血中サイロキシン濃度低下作用機構

静岡県立大学薬学部　加藤善久、山田静雄、出川雅邦

Polychlorinated biphenyl (PCB)は、多くの野生生物の組織のみならず、ヒトの血液、母乳、肝臓、脂肪組織などにも見出され、生体に対する影響が懸念されている。ラットでは、すでに、PCB投与により血中サイロキシン(T_4)濃度が低下することが報告され、ヒトにおいてもPCB曝露により甲状腺ホルモン（T_4、3、5、3'-トリヨードサイロニン(T_3)）の撹乱が引き起こされる可能性が指摘されている。一般に、PCBによるラットでの血中T_4濃度の低下は、肝臓のUDP-グルクロン酸転移酵素(UDP-GT)が誘導され、T_4のグルクロン酸抱合化や胆汁排泄が促進されることによると考えられている。しかし、PCBによる血中T_4濃度の低下と肝臓でのT_4-UDP-GT活性の増加には、必ずしも定量的関連性は見られないなど、このT_4の低下メカニズムについては、不明な点が多く残されている。

そこで、PCB投与時のラットにおける血中T_4濃度の低下に、肝UDP-GTの誘導が関与しているか否かをより明確にするために、Wistar系ラットおよびGunnラット（遺伝的にUGT1Aサブファミリーを欠損したWistar系ラットの突然変異体）を用い、2、2'、4'、5、5'-pentachlorobiphenyl (PentaCB)あるいはKanechlor-500 (KC500、PCB製品であるPCB混合物)投与後の血中T_4濃度と、肝T_4-UGT-GT (UGT1A、UGT1A1、UGT1A6)の発現量およびT_4のグルクロン酸抱合活性との関連性を検討した。

その結果、血中total T_4、free T_4濃度は、各PCB投与によりWistarとGunnの両ラットともに著しく低下することが明らかになった。一方、Wistar系ラットにおいては、UGT1A、UGT1A1、UGT1A6の発現量およびT_4-UDP-GT活性が著しく増加したが、Gunnラットではこれら発現量や活性には有意な変化は認められなかった。したがって、KC500あるいはPentaCB投与時のGunnラットに見られる血中T_4濃度の低下は、肝T_4-UDP-GTの活性には依存していないことが示された。このことは、Wistar系ラットでのKC500あるいはPentaCB投与による血中T_4濃度の低下もまた、少なくとも一部T_4-UDP-GT非依存的な機序で起こっている可能性を示唆している。

また、T_4のグルクロン酸抱合のほかに、T_4の代謝を亢進させる生体内反応として、脱ヨード化反応が知られている。そこで、KC500およびPentaCBを投与したWistarおよびGunnラットの肝のI型ヨードサイロニン脱ヨード化酵素活性を測定した。しかし、いずれのラットでも、本酵素活性の増加は認められなかった。なお、Aroclor1254を用いた場合にも、同様の結果が報告されている。したがって、WistarおよびGunnラットにPCBを投与した場合の血中T_4濃度の低下には、I型ヨードサイロニン脱ヨード化酵素はほとんど関与していないと考えられる。

PCBの水酸化代謝物は、血中T_4の輸送タンパクであるトランスサイレチン（TTR）と親和性をもち、T_4と競合的に結合すること、さらに、TTRとの親和性はモノヒドロキシ体よりジヒドロキシ体の方が高いことが報告されている。したがって、これらの競合的結合が、血中T_4の標的器官への輸送を撹乱し、血中T_4濃度を低下させる可能性も考えられる。

今回、KC500を投与したWistarとGunnラットにおける主水酸化代謝物は4-OH-2、3、3'、4'、5-pentachlorobiphenyl（T_4の3.3倍の結合親和性を持つ）であり、それらの割合は、それぞれ血中全水酸化体の89％、56％であった。なお、Gunnラットでは、37％がジヒドロキシ体であった。また、PentaCBを投与したWistarやGunnラットの血中全水酸化体濃度の80％以上は3'、4'-$(OH)_2$-PentaCBであった。

このことは、ラットでのKC500あるいはPentaCB投与による血中T_4濃度の低下の一部は、PCBの水酸化代謝によって引き起こされる可能性を示唆している。

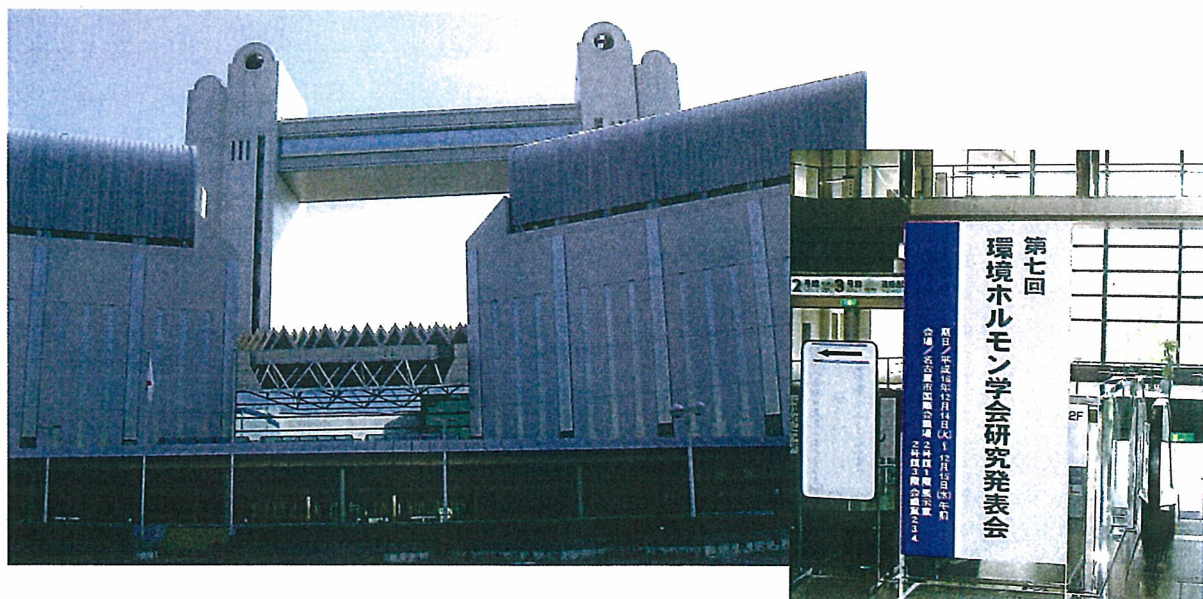
以上、PCBを投与した動物における血中T_4濃度の低下機序やその動物種差を明らかにするため、著者らは、すでに、PCB投与時の甲状腺の病理組織学的解析をはじめ、肝臓でのT_4-UDP-GTの誘導、PCBの代謝および肝臓でのT_4トランスポーター発現量などへの影響を検討してきた。しかしながら、PCB投与による血中T_4濃度の低下やその動物種差を、単一の要因で説明することは難しく、これまでに検討してきた要因、あるいは未知の要因が複雑に絡み合って、血中T_4濃度の低下が惹起されるものと考えられる。

これまで、PCBによる血中T_4濃度の低下は、主に肝臓のT_4-UDP-GTの誘導が主因とされてきたが、本研究結果は、本酵素誘導は、単にひとつの要因であるに過ぎないことを明らかにし、PCBによる生体影響が極めて複雑な機序で現れてくることを改めて示している。このように、PCBによる血中甲状腺ホルモン濃度の低下機構の解明はいまだ不十分であり、今後のさらなる研究が必要である。

第７回環境ホルモン学会研究発表会報告

（独）国立環境研究所　**米元純三**

本年は12月14，15日の両日にわたって名古屋国際会議場で開催されました。研究発表として55件の口頭発表および279件のポスター発表がなされ、また、参加者は会員364名、非会員59名、学生参加159名の合計582名であり、盛会裏に終了しました。口頭発表は、2会場に分かれて行われ、A会場では、生物検定法、分析法・環境動態、動物実験によるメカニズム解明に関する研究発表が行われました。B会場では、ヒトへの影響、生態系への影響に関する研究発表が行われました。SPEED98の改訂や、環境ホルモン関係の研究費の減少など環境ホルモン研究をとりまく状況は厳しくなりつつありますが、研究レベルでは、着実に広がりと深まりを見せているとの印象を受けました。環境ホルモン学会と連携して、第7回内分泌撹乱化学物質に関する国際シンポジウムが、同じ場所で15日から17日まで開催されました。初日の一般向け講演として養老孟司教授による特別講演「ホルモンのはたらき」が行われました。パネルディスカッションとして「環境ホルモン問題をどう伝えていきますか」が行われ、また、専門家プログラムとしても「リスクコミュニケーション」というセッションが設けられ、今回は、リスクコミュニケーションを意識したプログラム構成となっています。専門家プログラムでは、このほかに、基礎科学、野生生物への影響、曝露、ヒトへの影響、今後の研究の方向性、などのセッションが行われ、最先端の研究が紹介されました。なお、来年の環境ホルモン学会は、10月に東京で通例の研究発表会を、沖縄で環境ホルモン国際会議をやることを考えています。環境省の国際シンポジウムは、12月初旬に沖縄那覇で開催される予定です。



第14回環境ホルモン学会講演会のお知らせ

テーマ：内分泌撹乱化学物質の汚染と毒性

日　時：2005年1月28日（木）

会　場：江戸東京博物館ホール

墨田区横綱1-4-1　総武線両国駅下車

会　費：会員3,000円　非会員5,000円

定　員：300名

演　者：

＊「生体試料中の有機フッ素系化合物の分析と残留について」
中澤裕之　先生　星薬科大学

＊「臭素化ジフェニルエーテルの環境汚染」
酒井伸一　先生　（独）国立環境研究所

＊「水酸化ＰＣＢの人体残留と毒性」
鯉淵典之　先生　群馬大学大学院

＊「フタル酸エステルの昨今－環境残留を中心に」
片瀬隆雄　先生　日本大学大学院

＊「茨城県神栖町のジフェニルアルシン酸等による健康被害」
石井　一宏　先生　筑波大学

平成17年度総会と第15回講演会日時のお知らせ

日　時：2005年6月2日（木）

会　場：江戸東京博物館ホール

墨田区横綱1-4-1　総武線両国駅下車

講演会内容に付きましては次号のニュースレターに掲載を予定しております。

＜その他＞

「使いやすいPRTR情報」公開について

平成14年度データを公開しています。

http://env.safetyeng.bsk.ynu.ac.jp/ecochemi/PRTR.html

連絡先：エコケミストリー研究会

tel:fax:045-336-4036 e-mail:ecochemi@ynu.ac.jp

＜研究発表会忘れ物＞

研究発表会ポスター会場にて印鑑（認印・訂正印）の届出がありました。事務局にて保管しておりますので心当たりのある方はご連絡下さい。

第７回環境ホルモン学会研究発表会

2004年12月14・15日　名古屋国際会議場

＜懇親会＞

編集後記

PCBは、30年前に製造・使用が禁止されたにもかかわらず、未だに環境中に存在し、魚やヒトの母乳から検出され、その影響が懸念されている。また、我が国ではカネミ油症という不幸な食中毒事件も経験している。今年、９月にカネミ油症の診断基準が23年ぶりに改訂された。本号では、全国油症治療研究班班長の古江増隆教授に巻頭言をお願いし、PCB、ダイオキシン類の生体影響に関する最前線の研究ならびにダイオキシンの規制の動向についての原稿をお願いした。PCBは、古くて新しい問題だという想いを強くした次第である。

（（独）国立環境研究所　米元純三）

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A novel induction mechanism of the rat *CYP1A2* gene mediated by Ah receptor–Arnt heterodimer☆,☆☆,★

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Abstract

We have identified an enhancer responsible for induction by 3-methylcholanthrene in the upstream region of the *CYP1A2* gene. The enhancer does not contain the invariant core sequence of XREs that are binding sites for the Ah receptor (AhR) and Arnt heterodimer. The enhancer did not show any inducible expression in Hepa-1-derived cell lines, C4 and C12, deficient of Arnt and AhR, respectively. On the other hand, bacterially expressed AhR–Arnt heterodimer could not bind to the enhancer. Mutational analysis of the enhancer revealed that a repeated sequence separated by six nucleotides is important for expression. A factor binding specifically to the enhancer was found by using gel shift assays. Bacterially expressed AhR–Arnt heterodimer interacted with the factor. A dominant negative mutant of the AhR to XRE activated the enhancer. Collectively, these results demonstrate that a novel induction mechanism is present in which the AhR–Arnt heterodimer functions as a coactivator.
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Keywords: CYP1A2; Coactivator; Inducible expression; Xenobiotic response; Ah receptor; Arnt; Enhancer

The cytochrome P450 (CYP) monooxygenases are a group of proteins responsible for the oxidation of drugs, environmental pollutants, and endogenous compounds such as steroids and fatty acids [1]. CYP1A2 is an inducible drug-metabolizing P450 and biotransforms foreign chemicals, especially carcinogenic heterocyclic amines such as 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), which are formed in meat and fish during cooking [2]. CYP1A2 is expressed preferentially in the liver [3]. However, other extrahepatic tissues such as the lung [4], esophagus [5], and brain [6] have been reported to express CYP1A2. Two different groups of inducers are known to induce CYP1A2. One includes various man-made chemicals such as 3-methylcholanthrene (MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) which act as ligands for the Ah receptor (AhR), a ligand-activated transcription factor [7,8].

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☆☆ The nucleotide sequence data of the upstream region of the rat *CYP1A2* gene will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with Accession No. AB035382.

★ *Abbreviations:* Arnt, Ah receptor nuclear translocator; AhR, aryl hydrocarbon receptor; CAT, chloramphenicol acetyltransferase; MC, 3-methylcholanthrene; XRE, xenobiotic responsive element.

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These inducers also induce a number of other drug-metabolizing enzymes such as CYP1A1, glutathione *S*-transferase Ya subunit, and a form of UDP-glucuronosyltransferase [7,8]. The other is CYP1A2-specific inducers represented by isosafrole [9]. Reports have indicated that induction of CYP1A2 by MC was caused partly by an increased transcription rate and partly by increased stabilization of the mRNA [10,11]. Recent studies, however, on mice lacking the AhR gene clearly demonstrate that the AhR is indispensable for the inducible expression of CYP1A2 as well as CYP1A1, and accordingly enhanced transcription of the *CYP1A2* gene is predominantly important for the induction, although the possibility cannot be ruled out that the AhR is involved in stabilization of the *CYP1A2* mRNA [12,13].

The stimulated AhR with inducers forms a heterodimer with Ah receptor nuclear translocator (Arnt) in the nucleus after dissociation from Hsp90, and activates transcription of its target genes by binding xenobiotic responsive element (XRE) localized in their control region [7,8]. XRE contains an invariant CACGC core sequence that is recognized by the AhR and Arnt heterodimer [14,15], and is known to be present in most, if not all, of MC-inducible genes hitherto examined ([7,8] and references therein).

In this paper, we describe a novel enhancer element in the upstream region of the rat *CYP1A2* gene which responds to stimulus by MC. The enhancer does not contain the XRE core sequence but contains a short repeated sequence conferring the inducibility to the gene. Furthermore, we present evidence that the factor which recognizes and binds the repeated sequence recruits the AhR–Arnt complex by interacting with it.

Materials and methods

Construction of plasmids. pIS-5600, containing a 6.7 kb genomic DNA fragment spanning from −5.6 kb upstream of the transcription-initiation site to the initiation codon (+1100) in the 2nd exon, was constructed from pMLCAT [16] and the *CYP1A2* gene [17]. Plasmids with various deletions were constructed by using Bal 31 exonuclease. These various deletion plasmids were designated as pIS-x (x indicates the length of the 5′ flanking sequence of the *CYP1A2* gene from the transcription-initiation site). pIS-2237x was constructed by converting the *Sph*I site (at the −2085 position) into the *Xho*I site with a synthetic linker. Various DNA fragments from the *CYP1A2* gene were inserted into the *Cla*I site of pMC53c which contains the *CYP1A1* promoter [18], and designated pMCD-x or pMCD-xr (x indicates the length of the 5′ upstream sequence of the *CYP1A2* gene from the transcription-initiation site, and r indicates reverse orientation). pGL3 promoter plasmid (Promega, Madison) was cleaved with *Bgl*II, and synthetic oligonucleotides for the two copies of sequences shown in Fig. 5 were inserted. For expression in cultured cells, human AhR and Arnt cDNAs were inserted in the *Xba*I site of pEFBOS vector [19]. To construct an expression plasmid for a dominant negative mutant of the AhR, AGA for Arg 39 in the basic sequence was changed to ATA for Ile by using synthetic oligonucleotides. For expression in yeast, human AhR and Arnt cDNAs were inserted in the *Eco*RI site (artificially generated using a synthetic linker) of pGAD424 (Clontech, Palo Alto) in which a sequence for activation domain of GAL4 and nuclear localization signal (*Xmn*I to *Bam*HI site) had been removed beforehand. A selection marker, LEU2, of Arnt expression plasmid was changed to TRP1. Reporter plasmids containing the *CYP1A2* enhancer or XRE or HRE [20] were constructed by inserting the corresponding synthetic oligonucleotides into the *Sma*I site of pZ7 [21] containing promoter of the yeast myo-inositol transporter 1 gene and the β-galactosidase gene. All constructions were confirmed by sequencing.

Cell culture, DNA transfection, and reporter assays. HepG2, Hepa-1, C4, and C12 cells were maintained as described [22,23]. The calcium phosphate precipitation method was employed for transfection as described [24]. Each of the test plasmids (5 μg) containing the CAT reporter gene was introduced into cultured cells with 1 μg of a plasmid consisting of the luciferase gene as an internal control for efficiency of transfection. The CAT assay was performed as described [25]. When the luciferase gene was used as the reporter, β-galactosidase-expression plasmid was used as an internal control for efficiency of transfection. All experiments were done at least 3 times.

Gel mobility shift analysis. Nuclear extracts were prepared from HepG2 cells grown in the presence or absence of 1 μM MC for 2 h by the method of Dignam et al. [26]. Gel mobility shift assays were performed as described [27].

Expression of AhR and Arnt in Escherichia coli and yeast. Chimeric proteins containing basic helix–loop–helix (bHLH) and PAS domains of the AhR or Arnt connected to the C-terminus of glutathione *S*-transferase, GST–AhR (amino acids 1–424) and GST–Arnt (amino acids 88–464), were coexpressed with thioredoxin in *E. coli*. After induction with 20 μM isopropyl-β-D(−)thiogalactopyranoside at 30 °C for 15 h, the chimeric proteins were purified with glutathione–Sepharose affinity column chromatography (greater than 80% purity), treated with thrombin to cut GST off, and used without further purification for gel shift assays. Yeast strain D452-1 (MATa leu2 ura3 his3 trp1) which was generated from D373-4B and D448-2 [28] was transformed with plasmids by the lithium acetate method [29]. Transformed yeast cells were treated with 3 μM β-naphthoflavon for 18 h for induction of β-galactosidase, and expressed β-galactosidase activity was measured as described [28].

Results

Upstream regions responsible for induction by MC

The CAT activity from a chimeric plasmid containing −5.6 kb upstream sequence of the *CYP1A2* gene and the CAT structural gene was induced 3.4-fold by the addition of MC as shown in Fig. 1A. This induction was not found when Hepa-1 and L929 cells were used as host cells (data not shown). These findings agree with the results of similar experiments using the human *CYP1A2* gene [30]. To identify *cis*-acting elements responsible for induction, we constructed a series of deletion plasmids as shown in Fig. 1A. A similar level of induction was observed until deletion from the 5′ end proceeded to −2237, and further deletion to −2063 largely reduced the induced CAT activity. This result shows an important sequence for the induction was localized between −2237 and −2063. We further constructed fine deletion plasmids to delineate the response element as shown in Fig. 1B. Induced CAT activity was gradually reduced with progressive deletion from −2205 to −2120.

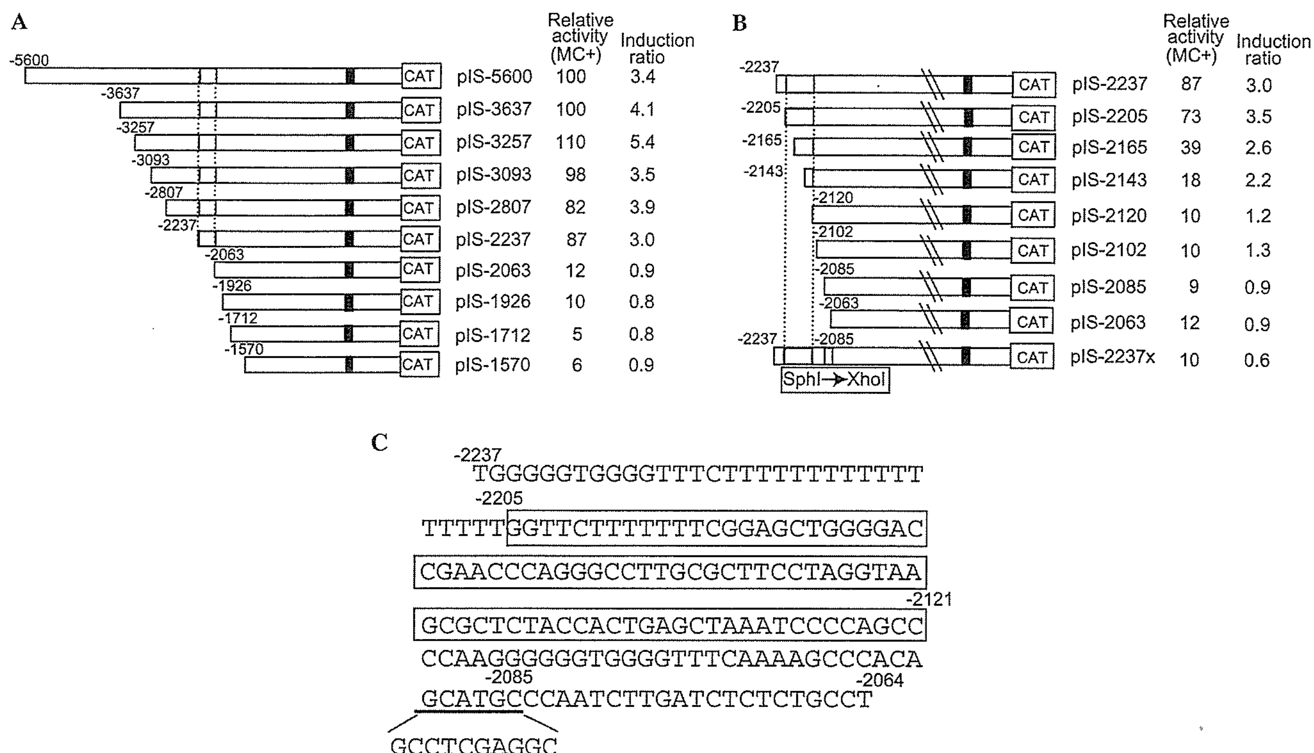

Fig. 1. Schematic representations of *CYP1A2*-CAT chimeric plasmids and their CAT activities in response to MC. (A) DNA fragments from the 5′-upstream region of the *CYP1A2* gene used to construct chimeric plasmids are represented by open bars. The first exon and regions responsible for inducible expression are denoted by filled and shaded boxes, respectively. HepG2 cells were transfected with each of the deletion plasmids (5 μg/plate) and an internal control plasmid (1 μg/plate) with the luciferase gene. Relative CAT activities are presented as percentages of the activity obtained with pIS-5600 in the presence of 1 μM MC. The extent of induction by MC is also shown as the ratio of the induced to the uninduced activity. Values represent means of at least 3 independent determinations. Nucleotide positions of the 5′-flanking sequence are numbered in negative from the start site. (B) Structure of chimeric plasmids with fine deletions and their CAT activities in response to 3-MC. Structure of each deletion plasmid and CAT activity are shown in the same way as in (A). (C) Nucleotide sequence of the regulatory region of transcription in the *CYP1A2* gene. The upstream regulatory region (−2205/−2120) is enclosed by boxes. The *Sph*I site in the downstream region is underlined. Inserted sequence by an *Xho*I linker is shown under the *Sph*I site.

The induction ratio decreased in accordance with the reduction of the induced CAT activity. Insertion of an *Xho*I linker into the *Sph*I site at −2085 completely abolished induced CAT activity, although the construct (pIS-2237x) contained the important region between −2205 and −2120. These results indicate that there are at least two regions responsible for the induction, −2205 to −2120, and a sequence around the *Sph*I site at −2085. The sequence from −2237 to −2064 is shown in Fig. 1C and contains no XRE core sequences (CACGC).

We investigated the transcriptional activity of the sequence (−2237 to −1603) on a heterologous promoter of the *CYP1A1* gene [18] as shown in Fig. 2A. The sequence inserted in the sense orientation showed very weak activity, and this activity was abolished when the sequence from −2237 to −2144 was deleted. On the other hand, a conspicuous induced activity was observed in the reverse orientation when the sequence from −2237 to −2144 was deleted (Fig. 2A). The reason for the orientation-dependent inhibition by the sequence from −2237 to −2144 is not known, and is necessary to be elucidated. Further deletions to −2085 abolished inducible expression completely. To delineate the 3′ end of the element indispensable for inducible expression, we constructed a series of deletion mutants as shown in Fig. 2B. Even deletions to −2036 (pDR-2036) still possessed inducible CAT activity. We synthesized two oligonucleotides which cover −2097 to −2073 and −2097 to −2079, and separately inserted them into the upstream region of the *CYP1A1* promoter as shown in Fig. 1C. The fragment from −2097 to −2073 gave rise to CAT activity in an MC-dependent and orientation-independent manner, indicating that this 25 bp element is an enhancer. Deletion from −2073 to −2078 completely abolished the activity (B sequence in Fig. 2C).

Involvement of the AhR and Arnt in the inducible expression of the CYP1A2 enhancer

Interestingly, the constructions as shown in Fig. 2C showed inducible expression in Hepa-1 cells as well as HepG2 (Fig. 3). Two Hepa-1-derived cell lines, C4 and C12 cells that are deficient in Arnt and AhR activity [22,23], respectively, were used to examine the

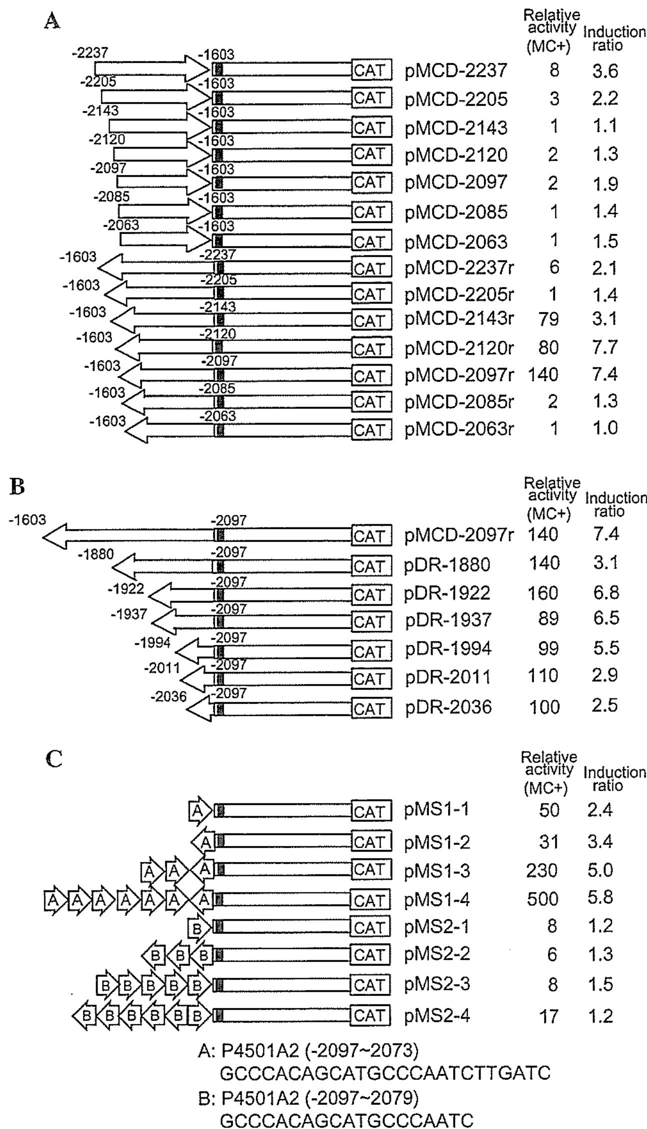

Fig. 2. Schematic representation of *CYP1A2* enhancer-CAT chimeric plasmids and their CAT activities in response to MC. (A,B) DNA fragments containing the *CYP1A2* enhancer were connected in sense or antisense orientation to pMC53c, which contains the *CYP1A1* promoter (up to −53 from the transcription-initiation site, see [18]) connected to the CAT structural gene. CAT activity was measured as described in Materials and methods, and normalized by the activity of pMC53c in the presence of MC. Bars show the *CYP1A1* sequence. Closed and open boxes show 1st exon and 1st intron, respectively. Arrows indicate length and orientation of DNA fragments of the *CYP1A2* gene. Nucleotide positions of the 5′-flanking sequence are numbered in negative from the start site. (C) Synthetic oligonucleotides of the *CYP1A2* enhancer were tandemly inserted in the upstream region of the promoter for pMC53c as in A and B. Right arrows represent the natural orientation relative to the transcription; left arrows the inverse orientation. CAT activity is shown in the same way as in A and B. Sequences of the inserted oligonucleotides are shown below.

involvement of the two transcription factors in the activity of the *CYP1A2* enhancer. As shown in Fig. 3, inducibility of CAT activity found in the parental cell line was completely abolished in C4 and C12 cells, although some constitutive expression was found particularly in C4 cells.

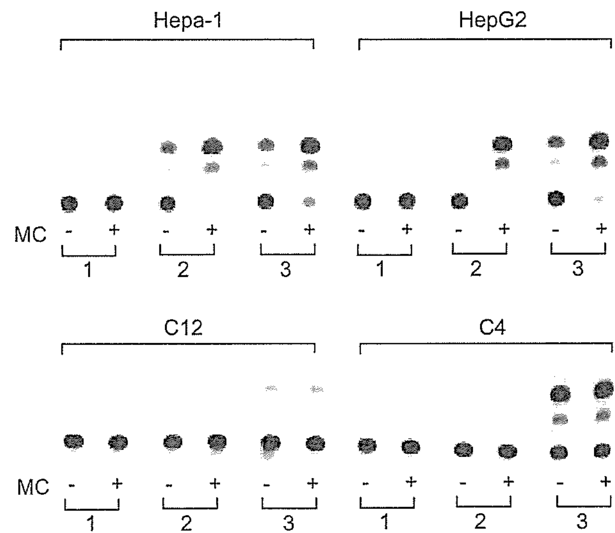

Fig. 3. Involvement of the AhR and Arnt in the inducible expression of the *CYP1A2* enhancer. Inducibility and enhancer activity of the *CYP1A2* enhancer and XRE were measured in mutant Hepa-1 cells, C4, and C12 cells. (1) pMC53c, (2) XRE4-pMC53, and (3) pMS1-4. Reaction products of the CAT activity were separated with thin-layer chromatography and a typical autoradiographic result is shown. Cells transfected with the test plasmids were grown in the presence (+) or absence (−) of 1 μM MC.

This result shows that both the AhR and Arnt are necessary for inducible expression of the *CYP1A2* enhancer.

We used an expression system in yeast to investigate the function of AhR and Arnt in the enhancer activity. As shown in Fig. 4A, a reporter gene containing four tandemly repeated XREs in the promoter region was activated about 20-fold by the addition of β-naphthoflavon as reported previously [31]. However, when a reporter gene containing the *CYP1A2* enhancer in the promoter region was used, the resultant β-galactosidase activity was at a similar level to those of negative controls containing no or HRE sequences in the promoter region. Binding activity of the AhR–Arnt heterodimer to the *CYP1A2* enhancer was investigated using gel mobility shift assays. Nuclear extracts from HepG2 cells contained an XRE-binding activity derived from the heterodimer only when cells were treated with MC (Fig. 4B) as reported [32]. The band was not effectively diminished by a large excess of unlabeled oligonucleotides carrying the *CYP1A2* enhancer (Fig. 4, lanes 8 and 9). Finally, we tested the binding activity of the AhR–Arnt heterodimer to the *CYP1A2* enhancer using AhR and Arnt which were bacterially expressed and purified (Fig. 4C). Although the purified proteins were mostly present in aggregate forms (data not shown), the preparation still possessed a strong binding activity to XRE (Fig. 4C, lane 5). On the other hand, no signal was detected when the oligonucleotides for the *CYP1A2* enhancer were used as the binding probe (Fig. 4C, lane 1).

Taken together, these results indicate that the AhR–Arnt heterodimer plays an essential role in the inducible

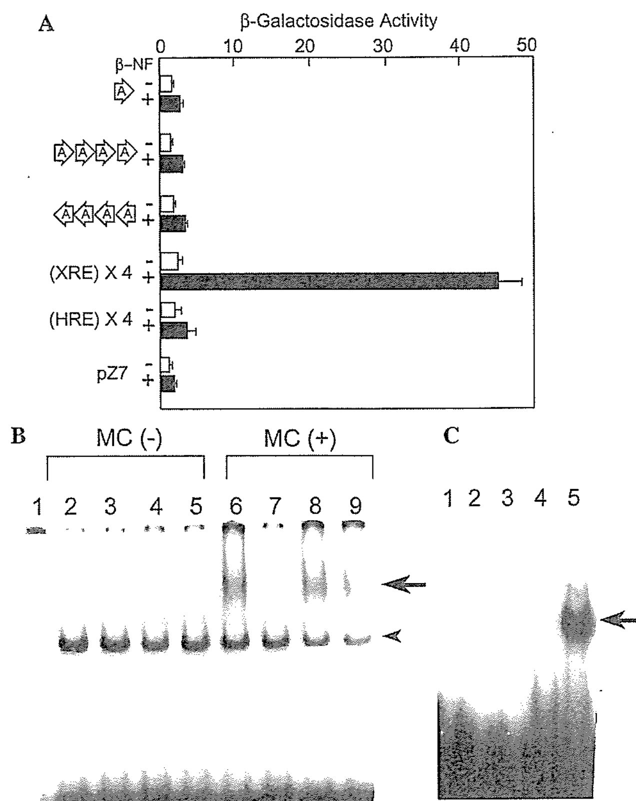

Fig. 4. Lack of binding activity of the AhR–Arnt heterodimer to the *CYP1A2* enhancer. (A) Expression of plasmids containing the *CYP1A2* enhancer in the yeast which expresses the AhR and Arnt. Arrows show number and orientation of the *CYP1A2* enhancer; the sequence is shown in Fig. 2C. Yeast cells were treated with 3 μM β-naphthoflavon at 30 °C for 18 h. β-Galactosidase activity is represented as described [28]. (B) Competition gel mobility shift assay of the *CYP1A2* enhancer with XRE using nuclear extracts of HepG2 cells. Labeled XRE sequence (20,000 cpm) was used as a probe. Lane 1, no protein; lanes 2–9, 10 μg of the nuclear extracts on protein base; lanes 3 and 7, 300-fold unlabeled XRE; lanes 4 and 8, 300-fold unlabeled *CYP1A2* enhancer (−2205 to −2073); lanes 5 and 9, 300-fold unlabeled *CYP1A2* enhancer (−2097 to −2073); lanes 2–5, nuclear extracts of untreated cells; lanes 6–9, nuclear extracts of MC-treated cells. An arrow and arrowhead show positions of specific and nonspecific bands, respectively. (C) Lack of binding activity of the *CYP1A2* enhancer to bacterially expressed AhR–Arnt heterodimer. Oligonucleotides for the *CYP1A2* enhancer (−2097 to −2073) and two oligonucleotides (−2097 to −2079 and −2087 to −2069) were used for labeled probes in lanes 1, 2, and 3, respectively. XRE was used for probe in lanes 4 and 5 with and without 100-fold excess unlabeled XRE, respectively. An arrow shows the position of the specific band. Incubation of the bacterially expressed AhR or Arnt separately with the XRE probe did not generate any shifted bands (data not shown).

expression of the *CYP1A2* enhancer, but that it does not directly bind to the enhancer sequence.

Mutational analysis of the CYP1A2 enhancer

We introduced point mutations systematically into the sequence of the *CYP1A2* enhancer in order to identify critical nucleotides for the inducible enhancer activity as shown in Fig. 5. The plasmids with mutations were transfected into HepG2 cells, and the expressed luciferase activity was measured. Consequently, an imperfect short repeat that mutated in the plasmids E, F, J, and K was found to be important for the inducible expression of the luciferase activity. Furthermore, the nucleotides changed in mutants D and H were found to be necessary for full activity of the enhancer because these mutants showed lower inducibility than the wild type. A notable enhancement in activity was observed in mutants H-I and I, suggesting the presence of an inhibitory sequence in the enhancer. Additional mutations in the repeated sequence were introduced into mutant G, used as the parental plasmid, which shows higher inducibility than the wild type (Figs. 5A and B). Only a mutation that gave rise to a perfect repeat showed comparable activity with mutant G while the other mutations in the repeated sequence abolished the expression. Six nucleotides of interspace between the units of the repeat were found to be essential for the inducible expression, since the expression was completely abolished by one base pair-deletion or addition (mutants N5 and N7 in Fig. 5B) in the repeated sequence. We searched a database for DNA sequences that contained the repeated sequence separated by 6 base pairs. Interestingly, a DNA sequence which satisfies the requirement was found in the upstream region of the rat *CYP1A1* gene (−259 from the transcription-initiation site) [33]. The element of the *CYP1A1* gene was found to exhibit an enhancer activity in response to MC as shown in Fig. 5A, although its activity was lower (approximately 60%) than that of the *CYP1A2* enhancer.

Binding factor to the CYP1A2 enhancer

Since the AhR–Arnt heterodimer did not directly bind to the *CYP1A2* enhancer, we looked for a factor that directly bound to the enhancer in nuclear extracts of HepG2 cells. A specific band was found (Fig. 6A, lane 2), and the intensity and position of the band was not changed by the treatment of cells with MC (data not shown). The band disappeared with a 50-fold excess of unlabeled oligonucleotides of the enhancer (Fig. 6A, lane 3). Similar competition experiments were performed using oligonucleotides, which had the same sequences as those used for construction of mutant plasmids shown in Fig. 5A as competitors. Mutated oligonucleotides with sequences D, E, F, H, J, and K could not compete efficiently with the probe (Fig. 6 upper panel). This result correlated well with the result of the enhancer activity of plasmids with mutated sequences (Fig. 5A). As shown in the lower panel of Fig. 6, the mutated sequences of J and K could not compete with the enhancer even in 400-fold excess. This result suggests that the 3′ unit of the repeat largely contributed to the binding affinity toward the factor. However, oligonucleotides used as binding probes lacking either the

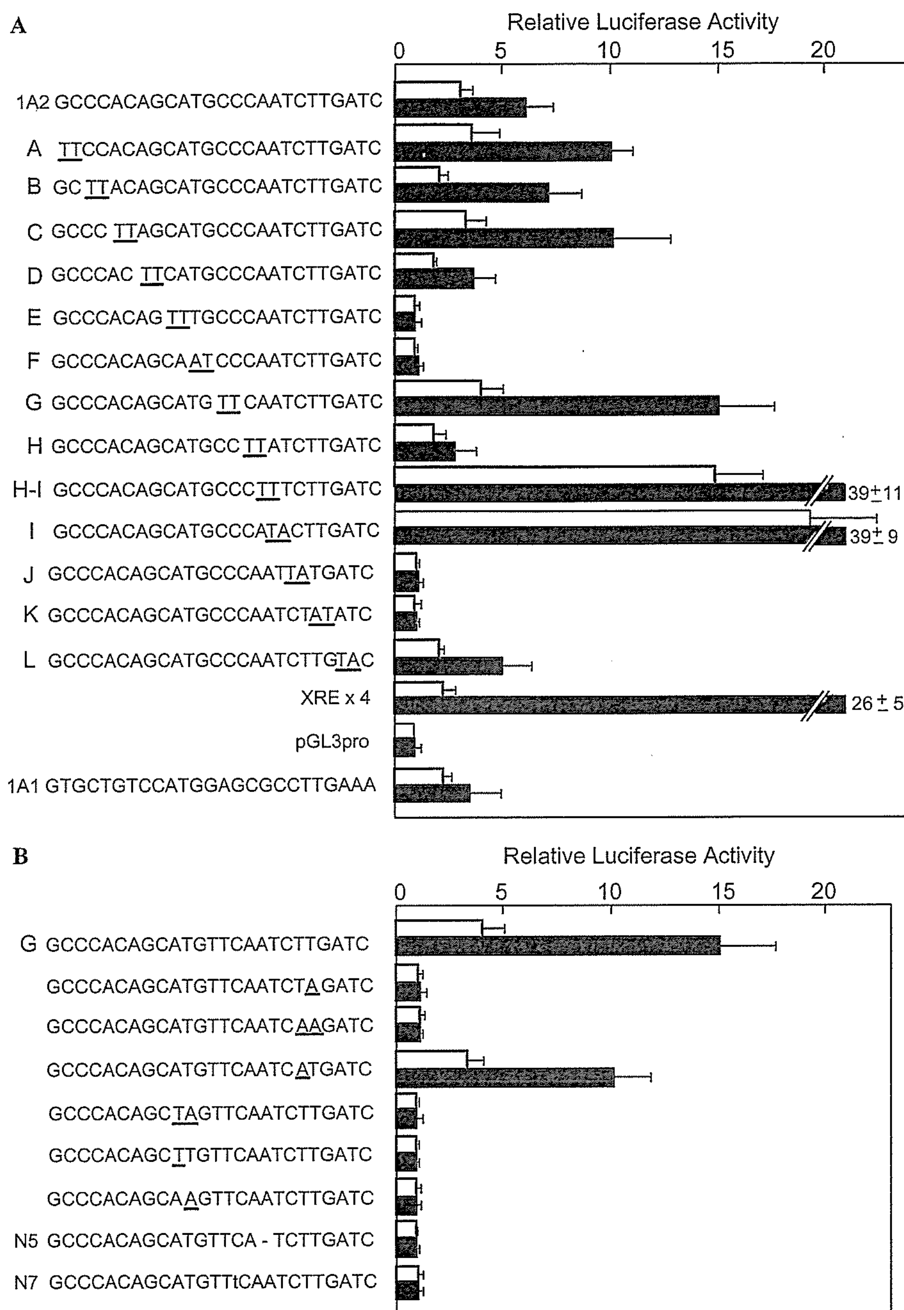

Fig. 5. Mutational analysis of the *CYP1A2* enhancer. (A) Effect of mutations in the *CYP1A2* enhancer on the transcriptional activity. Reporter plasmids (2 μg) containing two copies of the mutated sequences inserted upstream of the promoter region of pGL3pro were cotransfected with the internal control plasmid (3 μg) of lacZ, and the expressed luciferase activity was measured. Cells transfected with the test plasmids were grown in the presence (filled bars) or absence (open bars) of 1 μM MC for 40 h. Nucleotides different from the wild type *CYP1A2* enhancer are underlined. The uninduced luciferase activity of pGL3pro is used as a unit. (B) Mutational analysis of direct repeat in the *CYP1A2* enhancer. Mutated nucleotides are underlined. A deletion in N5 is denoted by a dash. An insertion of T in N7 is indicated with a small letter.

5′ or 3′ repeated sequence could not bind to the factor (data not shown).

Association of the binding factor with the AhR–Arnt heterodimer

To examine the interaction between the AhR–Arnt heterodimer and the factor binding to the *CYP1A2* enhancer, bacterially expressed AhR–Arnt was mixed with nuclear extracts of HepG2 cells, and the mixture was then used for gel mobility shift assays. As shown in Fig. 7A, addition of the AhR–Arnt resulted in decreased intensity of the band specifically bound to the enhancer. On the other hand, the intensity of bands of the GC box sequence bound by Sp1 was not affected by the AhR–Arnt complex, as shown in Fig. 7C, although Sp1 is

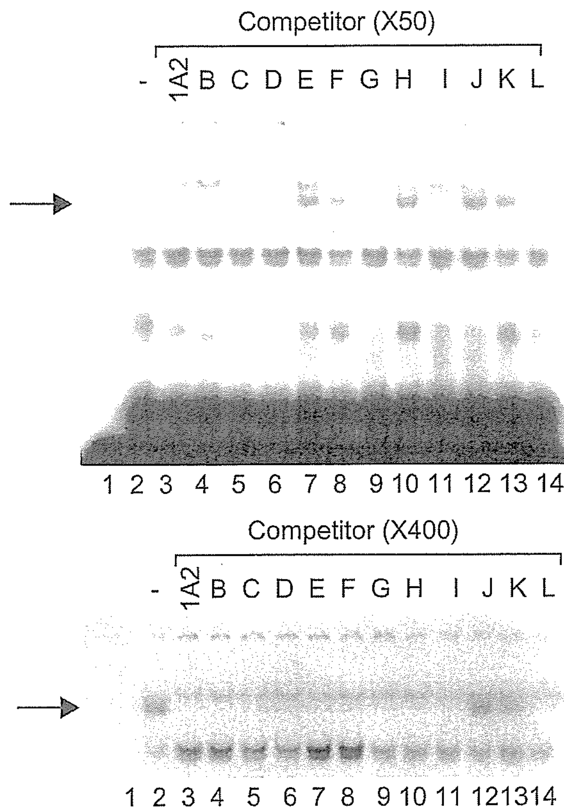

Fig. 6. Analysis of binding factors to the *CYP1A2* enhancer. Nuclear extracts of HepG2 cells treated with MC for 2 h were analyzed using oligonucleotides for the *CYP1A2* enhancer as a probe in the gel shift assay. An arrow shows the position of the specific band. Lane 1, no protein. lanes 2–14, 5 μg of the nuclear extracts on protein base; lanes 3–14, wild type competitor and mutated competitors B–L (they have the same sequences as those (mut B–L) shown in Fig. 5A), respectively. Fifty and 400-fold excess of competitors was used in the gel mobility shift assay on upper and lower panels, respectively.

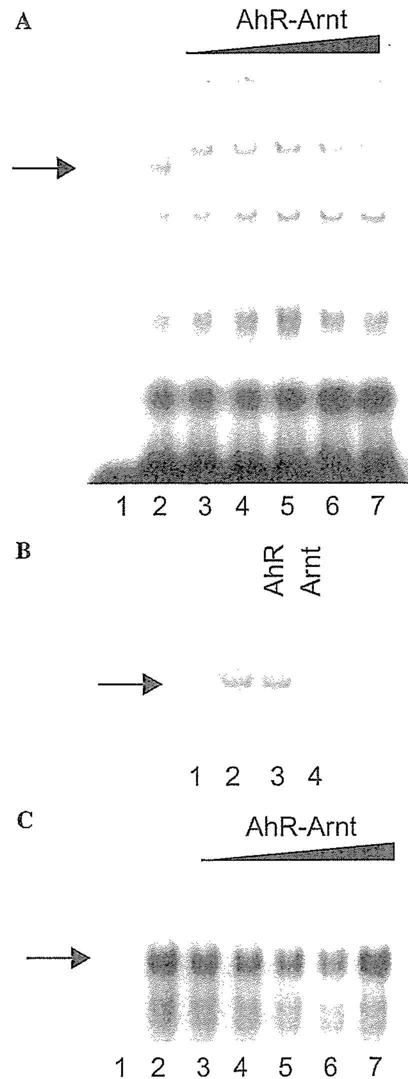

Fig. 7. Interaction of the binding factor to the *CYP1A2* enhancer with the AhR–Arnt complex. (A) Interaction of the enhancer-binding factor with AhR–Arnt. Nuclear extracts from HepG2 cells were mixed with increasing amounts of bacterially expressed AhR and Arnt, and analyzed by gel shift assays using the oligonucleotides of the *CYP1A2* enhancer as a labeled probe. Lane 1, no protein; lanes 2–7, 5 μg of the nuclear extracts on protein base; lanes 3–7, 0.15, 0.3, 0.45, 0.6, and 0.75 μg each of AhR and Arnt, respectively. An arrow shows the position of the specific band. (B) Association of the binding factor with Arnt or AhR. Bacterially expressed AhR or Arnt was separately incubated with HepG2 nuclear extracts and analyzed by gel mobility shift assays. Lane 1, no protein; lanes 2–4, 5 μg of protein; lanes 3 and 4, AhR and Arnt, respectively. (C) Interaction of Sp1 with AhR and Arnt. The oligonucleotide for BTE (a GC box, see [39]) was used as a probe. Experiments were done in a similar way to those in (A). The arrow shows the position of the specific band of Sp1 and BTE.

reported to possess some affinity to the AhR and Arnt [34]. This result may be explained by assuming that a fairly strong interaction occurs between the AhR–Arnt complex and the enhancer-binding factor, and that this interaction prevents the factor from migrating into acrylamide gels because almost all of the AhR–Arnt complex was present as aggregates which could not migrate into gels. We separately incubated the AhR or Arnt with the nuclear extracts and used them for gel shift assays. As shown in Fig. 7B, Arnt trapped the enhancer-binding factor, indicating that the AhR–Arnt heterodimer interacts with the factor through the Arnt protein.

Activation of the CYP1A2 enhancer with coexpression of the Arnt, AhR, and AhR mutant

We examined transactivation activity of the AhR and Arnt for the *CYP1A2* enhancer and XRE in HepG2 cells. The luciferase activity of the reporter plasmid containing the *CYP1A2* enhancer or XRE was enhanced by the coexpression of the AhR or Arnt, although synergistic activation by simultaneous expression of the two factors was not clearly observed (Fig. 8). To examine whether or not the DNA-binding capacity of the factors is necessary for the transactivation activity, we constructed a plasmid with a mutation, AGA for Arg39 to ATA for Ile in the basic sequence of the AhR, and cotransfected it with a reporter plasmid containing XRE

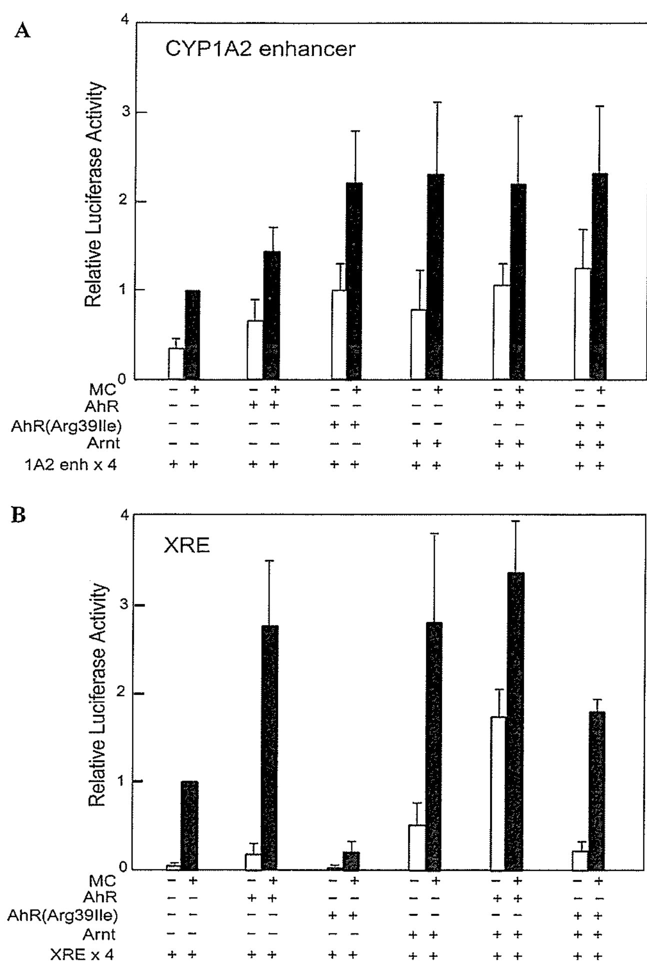

Fig. 8. Activation of the *CYP1A2* enhancer activity by transfection with AhR and Arnt expression plasmids. Reporter plasmids (1 μg) containing four copies of *CYP1A2* enhancer (A) and XRE (B) in the promoter region were transfected into HepG2 cells with various combinations of expression plasmids (1 μg each) for Arnt, AhR, and a dominant negative mutant (AhR Arg39Ile) of the AhR with a missense mutation in the basic sequence. The internal control plasmid (2 μg) of lacZ for efficiency of transfection was used, and total DNA was adjusted to 5 μg by adding the pEFBOS vector plasmid. The luciferase activity induced by the addition of MC from cells transfected with the only reporter plasmid is used as a unit. Cells transfected with plasmids were grown in the presence (+) or absence (–) of 1 μM MC for 40 h.

or *CYP1A2* enhancer sequences. As expected, the mutated AhR repressed 5.0-fold the luciferase activity of the reporter plasmid containing XRE sequences as shown in Fig. 8B. In contrast, the dominant negative mutant of the AhR activated the luciferase activity Fig. 8A more strongly than the wild type AhR, when the reporter plasmid containing *CYP1A2* enhancer sequences was used. This result clearly demonstrates that the DNA-binding activity of the AhR is not necessary for the transcriptional activation of the *CYP1A2* enhancer. In the yeast expression system used in Fig. 4, the mutant of the AhR induced no β-galactosidase activity of the reporter plasmid containing XRE sequences when it was coexpressed with Arnt (data not shown).

Discussion

We have identified two regions responsible for inducible expression of the rat *CYP1A2* gene by MC. The upstream one localized between −2205 and −2120 may function as a region that enhances the activity of the downstream enhancer. Tandem repeats of the sequence on a heterologous promoter did not exhibit any transcriptional activity (data not shown). The downstream one which consists of 25 base pairs showed an enhancer activity dependent on inducers such as MC, although the activity was not detected in the context of the *CYP1A2* promoter without the upstream region. A mutational analysis disclosed that two short repeated sequences in the enhancer sequence are important for enhancer activity. This feature of the *CYP1A2* enhancer is not shared with the classical XRE found in various MC-inducible genes; it contains an invariant core sequence, CACGC, which is recognized and bound by the AhR–Arnt heterodimer [14,15]. It is clear that the AhR–Arnt heterodimer could not directly bind to the *CYP1A2* enhancer, therefore suggesting the presence of a mediator between the DNA sequence and the AhR–Arnt heterodimer (Figs. 4 and 7). Finding that a dominant negative mutant of the AhR with a mutation in the basic region activates the *CYP1A2* enhancer but not XRE supports the indirect interaction between the AhR and the *CYP1A2* enhancer. We would like to propose that this novel type of XRE be called XRE II to distinguish it from the classical XRE. Database search for the enhancer sequence revealed that several genes contain this type of XRE (data not shown). It is interesting to note that the rat *CYP1A1* gene also contains XRE II. Functional analysis of the sequence revealed that it possesses an enhancer activity which responds to MC (Fig. 5A). Cooperation of both types of XRE is possibly involved in the inducible expression of the gene. This finding strongly suggests that XRE II is widely distributed in various promoters of MC-inducible genes.

The indirect binding of the AhR and Arnt heterodimer to the *CYP1A2* enhancer is reminiscent of coactivators such as CBP and p300 in the transcriptional mechanism [35]. Several PAS domain-containing proteins such as SRC1 and TIF2 are known to function as coactivators that act as bridges between enhancer-binding factors and general transcription factors in transcription activation [36,37]. Our results demonstrate that the AhR–Arnt complex does not only work as a sequence-specific transcription factor which directly binds to the classical XRE but could also act as a coactivator in the transcription via XRE II. These different transcription mechanisms mediated by the AhR–Arnt heterodimer are schematically depicted in Fig. 9. For detailed analysis of this transcription mechanism through the *CYP1A2* enhancer, isolation and characterization of the sequence-specific transcription factor

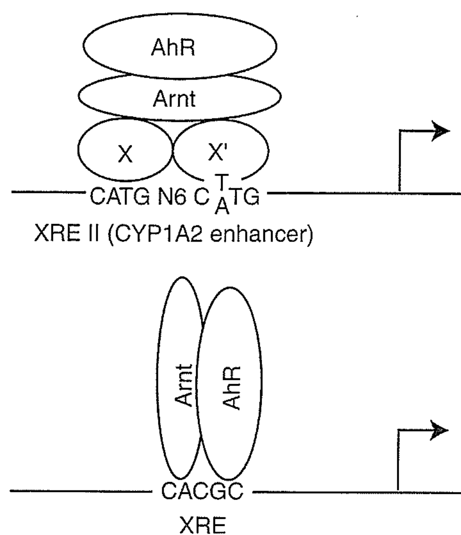

Fig. 9. Schematic representation of two types of transcription mechanisms mediated by the AhR–Arnt heterodimer. Upper, the AhR–Arnt heterodimer functions as a coactivator in the rat *CYP1A2* gene. X and X′ denote subunits of the binding factor to XRE II or *CYP1A2* enhancer. The dimeric structure of the binding factor is hypothetical. Lower, the classical model of induction of genes mediated by AhR–Arnt heterodimer. The AhR–Arnt heterodimer functions as the transcription factor that directly binds to XRE.

which directly bind to the *CYP1A2* enhancer are necessary, and studies along this line are now in progress.

It is reported that two regions (−2532 to −2423 and −2195 to −1987) in the human *CYP1A2* gene are involved in the inducible expression by MC. The upstream region contained an XRE sequence (termed X1) and no XREs were present in the downstream region although an analogous sequence (termed X2) to XRE is present [38]. Furthermore, several *cis*-acting elements such as AP-1-binding site, CAT box, HNF-1-binding site, and TATA box are reported to be present [38]. In the regions, no typical XRE II sequences were found, suggesting that different induction mechanism is present between the rat and human genes. However, it is also possible in the human gene that XRE II with a sequence deviated from that of typical XRE II may be present and activate the transcription by the aid of the reported DNA elements nearby localized.

Isosafrole did not induce the expression of reporter genes driven by the *CYP1A2* enhancer (data not shown), suggesting that the *cis*-acting element responsible for the induction by isosafrole and related CYP1A2-specific inducers is carried by other regions of the *CYP1A2* gene.

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Review Article

Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitously distributed environmental chemicals. PAHs acquire carcinogenicity only after they have been activated by xenobiotic-metabolizing enzymes to highly reactive metabolites capable of attacking cellular DNA. Cytochrome P450 (CYP) enzymes are central to the metabolic activation of these PAHs to epoxide intermediates, which are converted with the aid of epoxide hydrolase to the ultimate carcinogens, diol-epoxides. Historically, CYP1A1 was believed to be the only enzyme that catalyzes activation of these procarcinogenic PAHs. However, recent studies have established that CYP1B1, a newly identified member of the CYP1 family, plays a very important role in the metabolic activation of PAHs. In *CYP1B1* gene-knockout mice treated with 7,12-dimethylbenz[*a*]anthracene and dibenzo[*a,l*]pyrene, decreased rates of tumor formation were observed, when compared to wild-type mice. Significantly, gene expression of CYP1A1 and 1B1 is induced by PAHs and polyhalogenated hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin through the arylhydrocarbon receptor. Differences in the susceptibility of individuals to the adverse action of PAHs may, in part, be due to differences in the levels of expression of CYP1A1 and 1B1 and to genetic variations in the *CYP1A1* and *1B1* genes. (Cancer Sci 2004; 95: 1–6)

Most of the chemical carcinogens in the environment are chemically inert in themselves and require metabolic activation by cytochrome P450 (CYP) enzymes to more reactive metabolites in order to exhibit carcinogenicity in experimental animals and humans.[1,2] Of the 17 families of human CYPs identified to date, the CYP1, 2, and 3 family members play major roles in the metabolic activation of a variety of environmental carcinogens. It has been suggested that CYP1A1 and CYP1B1 are responsible for the activation of most carcinogenic polycyclic aromatic hydrocarbons (PAHs) to epoxide intermediates, which are further converted to more reactive diol-epoxides with the aid of epoxide hydrolase.[1,3] CYP1A2 converts aryl- and heterocyclic amines to *N*-hydroxylated metabolites that are subsequently modified by phase II enzymes, such as acetyltransferase and sulfotransferase, to ultimate carcinogens.[4] CYP2A6 is a key contributor to the activation of *N*-diethylnitrosamine and other tobacco-related nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*-nitrosonornicotine.[5] CYP2E1 activates *N*-dimethylnitrosamine and other low-molecular-weight procarcinogens, such as ethyl carbamate and vinyl carbamate.[6] CYP3A4, 5, and 7 activate aflatoxin B_1, aflatoxin G_1, and sterigmatocystin by forming the respective highly reactive epoxides.[4,7] Other human P450 members including CYP2B6, 2C8, 2C9, 2C18, 2C19, and 2D6 are known to play relatively minor roles in the metabolic activation of procarcinogens and promutagens.[2] Of the CYP4 family enzymes identified so far, CYP4B1 catalyzes activation of urinary bladder carcinogens, such as benzidine and 2-naphthylamine.[8]

PAHs are ubiquitously distributed carcinogens in the environment and their carcinogenic potentials have been extensively studied in experimental animal models.[1] Historically, CYP1A1 had been thought to be the sole enzyme responsible for the metabolic activation of most of the carcinogenic PAHs to reactive electrophiles in mice, rats, and rabbits.[1] However, recent studies have established that CYP1B1 also activates PAHs to reactive metabolites at rates similar to or even higher than CYP1A1 in experimental animals and humans. As a further cause for concern, human CYP1B1 has also been shown to metabolize 17β-estradiol to a 4-hydroxylated product, a chemical considered to cause breast cancer in women.[9] Both CYP1A1 and 1B1 are expressed mainly in extrahepatic organs and thus make a major contribution to the incidence of cancers in these organs, when PAHs and other carcinogens are ingested into an animal's body.[3] PAHs and polyhalogenated hydrocarbons such as TCDD induce several xenobiotic-metabolizing enzymes, including CYP1A1 and 1B1, through the arylhydrocarbon receptor (AhR) and the increased synthesis of these enzymes, as well as genetic variations in *CYP1A1 and 1B1* genes, may determine the different susceptibilities of individuals to carcinogenesis caused by PAHs. In this review, we summarize recent progress in the investigation of the roles of CYP1A1 and 1B1 in the metabolic activation of PAHs and their relevance to the occurrence of cancers in humans.

Metabolic activation of PAHs by P450 and epoxide hydrolase

Many studies have demonstrated that most carcinogenic PAHs are activated by the combined actions of CYPs and epoxide hydrolase to highly reactive diol-epoxides that initiate

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Abbreviations: P450, general term for cytochrome P450; CYP, individual forms of P450; AhR, arylhydrocarbon receptor; PAH, polycyclic aromatic hydrocarbon; B[*a*]P, benzo[*a*]pyrene; 7,12-DMBA, 7,12-dimethylbenz[*a*]anthracene; DB[*a,l*]P, dibenzo[*a,l*]pyrene; MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. The suffix "dihydrodiol" or "diol" is used for the prefix "dihydroxydihydro" for individual polycyclic hydrocarbons.

cell transformation.[1,10] Metabolic activation of the prototype carcinogen B[*a*]P for example, was extensively studied by the research groups of Jerina and Conney in the middle 1970's.[1] Historically speaking, the so-called K-region epoxide B[*a*]P-4,5-epoxide generated metabolically from B[*a*]P by CYPs was thought to be the ultimate carcinogenic metabolite.[10] However, the B[*a*]P-4,5-epoxide was found to be readily hydrolyzed by epoxide hydrolase to an inactive B[*a*]P-4,5-diol metabolite and was finally concluded not to play a major role in carcinogenesis by B[*a*]P.[1] Subsequent examination of several B[*a*]P metabolites revealed that (+)- and (−)-B[*a*]P-7,8-diol-9,10-epoxides, commonly referred to as bay region epoxides, were highly reactive towards DNA and, thus, they were concluded to be the ultimate carcinogenic metabolites of B[*a*]P.[1,10] The pathways leading to the formation of B[*a*]P bay region epoxides were extensively investigated in rat liver microsomes or reconstituted systems containing purified rat CYP and epoxide hydrolase (Fig. 1).[1,10] B[*a*]P is first oxidized by liver microsomes of MC-treated rats to (+)- and (−)-B[*a*]P-7,8 oxides, with the conversion rate of the (+) enantiomer being much higher than that of the (−) form. Subsequently, microsomal epoxide hydrolase rapidly hydrolyzes these oxides to produce (−)- and (+)-B[*a*]P-7,8-diol.[1,10,11] The resultant (−)- and (+)-B[*a*]P-7,8-diols are finally activated by CYPs to highly reactive bay region epoxides, namely (−)-B[*a*]P-7,8-diol-9,10-epoxide-1, (+)-B[*a*]P-7,8-diol-9,10-epoxide-2, (+)-B[*a*]P-7,8-diol-9,10-epoxide-1, and (−)-B[*a*]P-7,8-diol-9,10-epoxide-2.[1,12] Although these four diol-epoxides were all highly mutagenic when assessed in Ames *Salmonella* tester strains and Chinese hamster V-79 cells, the (+)-B[*a*]P-7,8-diol-9,10-epoxide-2 was identified as the most reactive in producing tumors in newborn mice.[1,11] Since (+)-B[*a*]P-7,8-diol-9,10-epoxide-2 had almost the same level of carcinogenicity as B[*a*]P itself or (−)-B[*a*]P-7,8-diol, this diol epoxide is considered to be the ultimate carcinogenic metabolite of B[*a*]P.[1,13]

The bay region theory has also been applied to other PAHs to explain their carcinogenic activation by CYPs and epoxide hydrolase.[1] For example, DB[*a,l*]P and 7,12-DMBA are converted to their proximate carcinogenic metabolites, namely DB[*a,l*]P-11,12-epoxide and 7,12-DMBA-3,4-oxide, respectively, by CYPs (Fig. 2). These epoxides are hydrolyzed by epoxide hydrolase to form DB[*a,l*]P-11,12-diol and 7,12-DMBA-3,4-diol, which are finally oxidized again by CYPs to the ultimate carcinogenic metabolites, DB[*a,l*]P-11,12-diol-13,14-epoxide and 7,12-DMBA-3,4-diol-1,2-epoxide, respectively (Fig. 2).[1,14]

Other typical PAH compounds considered to be activated in accordance with the bay region theory include benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*c*]phenanthrene, benzo[*g*]chrysene, chrysene, 5,6-dimethylchrysene, and 5-me-

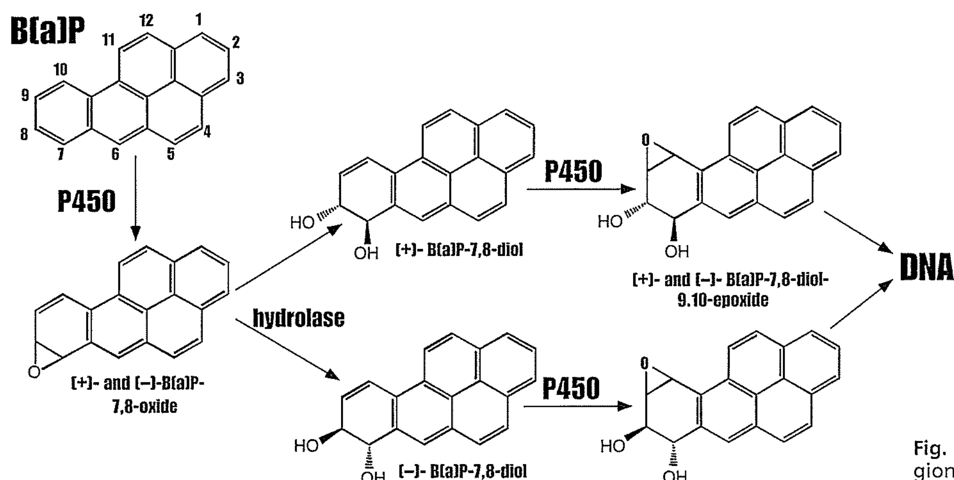

Fig. 1. Metabolic activation of B[*a*]P to bay region epoxides by P450 and epoxide hydrolase.

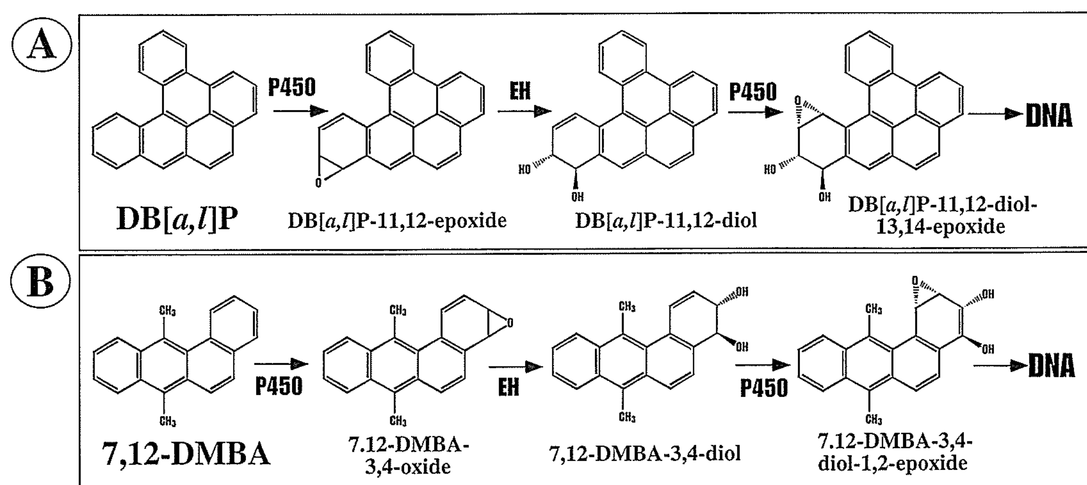

Fig. 2. Metabolic activation of DB[*a,l*]P (A) and 7, 12-DMBA (B) to bay region epoxides by P450 and epoxide hydrolase.