Introduction

Japan has been experiencing the fastest growth of its older population in the world, with the proportion of people aged over 65 years old reaching 20% in 2005. In parallel with the increasing number of older persons, the number of older people affected by various forms of dementia is also rising.

Although there are several cholinesterase inhibitors available worldwide for treating patients with Alzheimer's disease (AD) (Blennow *et al.*, 2006), only donepezil has been licensed so far in Japan. In terms of maximizing the effect of donepezil for retarding the disease progression, adherence of the patients to the pharmacotherapy is crucial. Other than the cessation of the medication due to adverse drug reactions such as gastrointestinal irritations (Birks, 2006), the continuation of donepezil can be hampered by patients' lack of competence to take the drug regularly owing to their memory problems or inadequate support from their caregivers. With the aim of examining what factors can lead to the discontinuation of donepezil, we surveyed the background of patients when they started the medication and examined its association with the discontinuation of the drug during the follow-up period.

Methods

The study was conducted in patients in the outpatient clinic in the Department of Geriatrics, Nagoya University Hospital. AD was diagnosed according to the DSM-IV criteria (American Psychiatric Association, 1994).

Patients started donepezil at 3mg daily. If tolerated for one or two weeks, the doses were increased to 5mg daily. The severity of dementia in patients at the point of discontinuation of treatment was evaluated by their attending doctors with the clinical dementia rating (CDR) (Hughes *et al.*, 1982; Morris, 1993).

Using a retrospective chart review from 1 July 2003 to 30 June 2005, the prescription of donepezil and the reasons for discontinuing the prescription were determined. The lack of efficacy was determined by the attending doctors mainly based on caregivers' information.

The Pearson χ^2 test for categorical data and the independent t-test for continuous data were used to compare the characteristics of the patients. A Kaplan-Meier analysis was conducted to investigate the continuation of the prescription of denepezil. Logistic regression analysis was performed to investigate the factors associated with the discontinuation of donepezil.

A p value < 0.05 was considered significant. The analysis for this study was generated using SAS statistical software, version 9.1 (SAS Institute Inc., Cary, North Carolina).

Results

Out of 264 patients, 140 (53.1%) discontinued taking donepezil during the observation period. The mean age of the continued group and the discontinued

Table 1. Patients' backgrounds

	ALL	CONTINUED	DISCONTINUED	P VALUES
Age (years old)	79.6 ± 6.5	79.5 ± 6.7	79.8 ± 6.4	0.775
Male/female	87/177	41/84	46/93	0.171
Duration of prescription (days)	231.4 ± 145.6	359.4 ± 98.8	105.1 ± 43.7	<0.001
Number of patients of CDR 0.5	9	3 (33.3%)	6 (66.7%)	
Number of patients of CDR 1	165	91 (55.2%)	74 (44.8%)	
Number of patients of CDR 2	58	21 (36.2%)	37 (63.8%)	
Number of patients of CDR 3	32	10 (31.3%)	22 (68.8%)	

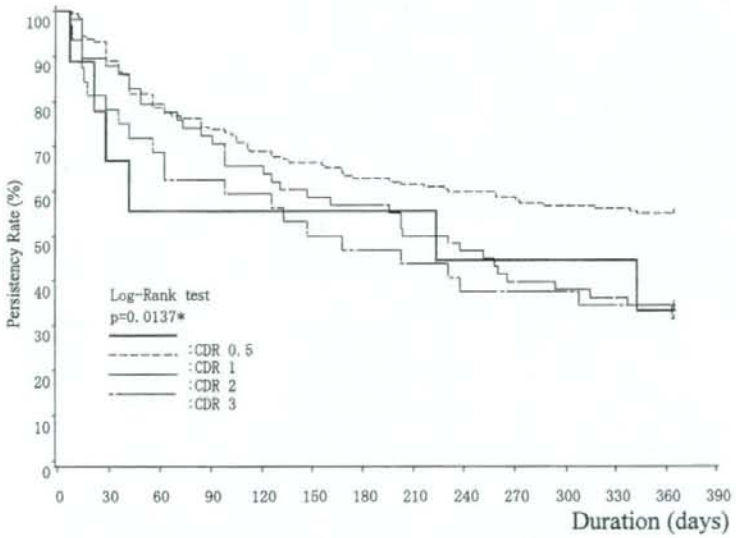

Figure 1 Kaplan-Meier analysis showing that the patients with more severe cognitive impairment represented by the CDR score (= 3) discontinued taking donepezil earlier and more frequently.

group did not differ significantly (79.5 ± 6.7, 79.8 ± 6.4, respectively) (Table 1). The percentages of the patients having concomitant treatment with antipsychotics, benzodiazepines or antidepressants were 25.6% (32/125) and 23.7% (33/139) for continued and discontinued patients, respectively. Kaplan-Meier analysis showed that the patients with more severe cognitive impairment

Table 2. Logistic regression analysis adjusted for age

	ODDS RATIO	95% CI	P-VALUE
CDR	1.713	1.170–2.508	0.006
Living alone	1.073	0.492–2.338	0.860

Note: CI = confidence interval.

as represented by their CDR score (CDR = 3) discontinued taking donepezil earlier and more frequently (Figure 1). The durations of the treatment with donepezil were 236.6 ± 139.5 days, 252.3 ± 141.1 days, 205.2 ± 147.9 days, and 187.3 ± 148.8 days for CDR 0.5, 1, 2, 3, respectively.

The reasons for discontinuation were changes in the doctors treating the patients (n = 71), ineffectiveness (n = 16), gastrointestinal side effects (n = 11) and others (n = 41).

To determine the variables significantly associated with discontinuation, logistic regression analysis was performed. The variables selected for this analysis were patients' age, gender, CDR, and living alone. The analysis showed that CDR was the only significant variant affecting discontinuation (Table 2).

The reasons for discontinuation at each CDR severity level are shown in Table 3. In patients with CDR = 1 or 2, whose dementia was relatively less severe, the changes of doctors were the most frequent reason for discontinuation. However, in the patients with CDR = 3, ineffectiveness of the medication was the major reason for discontinuation.

Discussion

In the current study, the prescriptions of donepezil given to AD patients were frequently discontinued. The discontinuation was observed earlier and more frequently in patients with severe AD (CDR = 3). The main reason for the cessation was the perceived ineffectiveness of the medication. However, in less severe AD patients (CDR = 1, 2), the prescription of donepezil from the specialist was stopped mostly because of referral to the local primary care physicians. In the current study the continuation of the prescription by the local primary care physicians was not traced. Our previous study showed that the attitudes of the majority of general practitioners in Japan were reluctant to follow patients with dementia (Umegaki *et al.*, 2007). The continuation of the prescription after the hand-over should be investigated in future studies. Previous surveys showed that the rate of discontinuation of the cholinesterase inhibitor prescription to patients with senile dementia of Alzheimer's type (SDAT) was 30–50%, which is similar to the present results (Evans *et al.*, 2000; Frankfort *et al.*, 2005; Mauskopf *et al.*, 2005; Suh *et al.*, 2005).

Under the current National Health Insurance-based medical practice in Japan, donepezil (5 mg) is licensed only for patients with mild to moderate AD. This means that patients with an advanced stage of AD (CDR = 3) are not

Table 3. Reasons for the discontinuation of donepezil

	SIDE EFFECTS	DETERIORATION OF BPSD	INEFFECTIVENESS	DIFFICULTY OF COMPLIANCE	CHANGES OF DOCTORS
CDR 0.5	2 (33.3%)	0	0	0	4 (66.7%)
CDR 1	5 (10.4%)	3 (6.3%)	1 (2.1%)	1 (2.1%)	38 (79.2%)
CDR 2	3 (11.5%)	0	1 (3.8%)	0	22 (46.2%)
CDR 3	1 (5.6%)	3 (1.7%)	6 (33.3%)	1 (5.6%)	7 (38.9%)

P value by χ^2 analysis = 0.002.
BPSD = behavioral and psychiatric symptoms of dementia.

indicated for treatment by donepezil. However, due to the lack of options for alternative pharmacotherapy, donepezil tends to be used irrespective of dementia severity. The application of a higher dosage (10 mg) of donepezil to severe AD patients has recently been licensed, and is expected to be beneficial for AD patients in the advanced stages. In the current survey, age of the patients, gender and living alone were not significantly associated with discontinuation. In addition, very few patients discontinued because of poor drug adherence. This may be due to the fact that donepezil was not prescribed from the outset to those patients who were considered incapable of taking the drug regularly.

The current study has several limitations. First, because the survey was implemented in the outpatient clinic of a university hospital, the results may not be generalizable to other clinical settings. In university hospitals there are many referral cases, and hence the patients are in a more advanced stage of dementia relative to other clinical settings. This may have contributed to the higher rate of discontinuation than we had expected. Also, because of higher expectations for drug efficacy in patients and their caregivers, a poor response to denepezil treatment may have led to the discontinuation of pharmacotherapy. Therefore, the results need further substantiation by extending the survey to other clinical settings. Another limitation of this study is that we could not trace the patients who were transferred to other settings. Those transferred cases that were counted as discontinued in this survey do not reflect the number of patients who stopped donepezil treatment.

In summary, in the memory clinic of a university hospital, donepezil was more frequently discontinued than we had expected, and the rate of discontinuation was higher in patients with advanced dementia. In terms of seamless pharmacological treatment, drugs that can be beneficial for patients in the advanced stages of dementia are greatly needed.

Conflict of interest

None.

Description of authors' roles

H. Umegaki designed the study, performed the statistical analysis and wrote the paper. A. Itoh contributed to the study design and collected data. Y. Suzuki and T. Nabeshima supervised the study design and data collection.

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生活（読書，趣味，嗜好）

鈴木　裕介*
すずき　ゆうすけ

- 認知症も他の生活習慣病と同様に個々の生活様式が発症要因に深く関わっている可能性が示唆されるようになってきた．
- 生活習慣による認知症予防の共通の機序として考えられるのは，①認知機能における蓄え（cognitive reserve），②血管因子仮説，③ストレス原因仮説などである．
- 適量の飲酒習慣は認知症発症に予防的に働くかもしれない．
- 喫煙は認知症発症のリスクを高めるという認識が一般的である．
- 報告により内容，頻度は異なるが，さまざまな余暇活動は年齢，性別，教育歴に関係なく認知症の発症に予防的に働く可能性がある．

Key Words　生活習慣，認知機能の蓄え，血管因子，ストレス，飲酒，喫煙，余暇，知的活動，社会とのネットワーク

はじめに

加齢が認知症の危険因子であるという認識は今も昔も変わるところではない．認知症研究の進歩により病理的側面での解明はかなり進んできたといえる．臨床の場面においてもプライマリーケアにおける病的なもの忘れに関する認識やスクリーニング法はかなり浸透したため，もの忘れを主訴に来院する患者さんやご家族に接するとき，「歳をとればだれでももの忘れはしやすくなりますよ，たぶん年のせいでしょ」では済まされない時代になった．近年，疫学研究の成果により，徐々に認知症の危険因子における外的要因が明らかになってきた．その結果によると認知症も他の生活習慣病と同様に個々の生活様式が発症要因に深く関わっている可能性が示唆されるようになってきた．認知症の発症要因は一部の遺伝性の疾患を除けば，身体・精神的な内因と環境（外的）要因の双方が関わっているためその一方の要因のみに介入するだけで発症を予防できるものではないが，生活習慣というのは長年にわたり蓄積することによりボディーブローのように徐々に脳に（正あるいは負の）影響を与え，結果として発症あるいはその予防に寄与することが推察される．本稿では今までの研究成果から類推される認知症の予防的側面における生活習慣について概観してみる．

□ 生活習慣としての認知症予防の機序

生活習慣を主に分類すると社会的，精神的，身体的な要素に分けられる．これらの要素を認知機能の維持，あるいは認知症の発症予防という視点から考察した場合，それぞれが異なる機序を介して作用するというよりはむしろ，認知症発症に予防的に働くであろうと考えられる共通の機序への作用が考えられる．これら共通の要因として考えられるのは，①認知機能における蓄え（cognitive reserve），②血管因子仮説，③ストレス原因仮説などである．血管性の因子に関しては血管性認知症のみに特異的な要因と考えがちであるが，アルツハイマー型などの神経変性による認知症における血管障害の高率な合併やアルツハイマー型認知症自体の発症機序に血管因子が直接関わっている可能性も近年指摘されており[1]，認知症全般にわたりその関与が示唆されてよいものである．それぞれの仮説について概説する．

□ 認知機能蓄え仮説（cognitive-reserve hypothesis）

動物（ラット）による実験において環境が認知

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機能に与える影響が証明されている．ラットを複数でおもちゃや遊具（トンネルとか梯子など）のある環境で飼育した場合，単独で何もない環境で飼育されたラットと比較して海馬にNGF（神経成長因子）含量が有意に多く，トータルの運動量に差はないにもかかわらず作業空間記憶を評価する行動実験において成績が良かった[2]．実験の検討項目（分子，細胞，形態）によらず，脳機能の可塑性を規定するのは対象動物の活動性であるという報告[3]もこの仮説を支持するものである．近年，血管，神経新生という観点から成人の脳における可塑性が報告されるようになった．Chruchill らによると，成人脳において精神活動における刺激はシナプス新生に，身体運動はシナプス以外の血管などの新生に寄与する可能性が示唆されている[4]．神経新生は齧歯類のみでなく哺乳類や人間の成人脳でも確認されている．実際，教育歴とか職業から判断する限り認知機能の蓄えが多いことは認知症の症状の臨床的な発現には抑制的に働いていることが示唆されている[5]．

□ 血管因子仮説（vascular hypothesis）

前述したように血管障害の認知症における関与は何も血管性認知症に限ったものではない．疫学的に中高年期の高血圧がその後の認知症の発症リスクとなることを示唆するデータは多い[6,7]．血圧のみでなく動脈硬化がアルツハイマー型を含む認知症との関連性は神経病理学的知見から疫学研究の結果に至るまではばひろく支持されている．

□ ストレス原因仮説

社会的に活発で他者との接触も多い生活を維持している場合，自己評価や社会的能力などにおいて良好な心理環境につながりストレスのレベルも低いというのが一般的な考え方である．ストレスとアルツハイマー型認知症発症リスクとの関連が示唆されている[8]．アルツハイマー病理に深く関わっている海馬はストレス反応の経路であるHPA Axis（hypothalamic-pituitary-adrenocortical axis）の一部でもある．glucocorticoid cascade 説に従えば，ストレスにより誘発された corticosterone の過剰分泌は海馬の corticostroid 受容体に down regulation をかけ受容体数が減少した結果，海馬から副腎皮質への抑制がかからずさらに corticosterone の過剰分泌を助長する結果，海馬ニューロンが失われるということになる．実際に，認知症やうつ病患者において cortisol の高値と認知機能障害，海馬萎縮との関連が示唆されている[9]．

□ 社会性，余暇活動と認知症リスクの関連

表1において社会活動や余暇と認知症発症に関する縦断研究の結果をまとめてみた．これらはほとんどがエントリー時に認知症を認めなかった高齢者の縦断的な追跡研究の結果である．これらの研究の結果は社会性や余暇活動の認知症予防の意義を示唆するのに十分な論拠ではあるが，同時に観察縦断研究が包含する本質的な限界を認めないわけにはいかない．ほとんどの研究がエントリーから追跡まで3年以上の間隔があり，エントリー時には認知症は認めないこと（認知症の有無の評価についても厳格な基準が適用されているわけではない）になってはいるが，社会とのつながりや余暇活動の乏しさは認知症の発症前リスクなのか，認知症の早期の症状によるものかの区別に困難である．しかしながらすべての調査においてベースラインでの認知機能は調整したうえでの解析である．適切な交絡因子をコントロールすることも重要な課題である．健康に関する因子はおそらくは他の測定に含まれていない要因も含めて相対的に健康に関するリスクを構成しているので，ライフスタイルに大きく影響する性別や年齢，教育歴のみならず余暇活動への参加の動機や意欲に大きく影響する身体障害，慢性疾患，抑うつなどもコントロールすることが望まれる．

□ 生活習慣と認知症発症リスク

生活習慣といった場合には生活におけるあらゆる習慣をあらわすため，食事や運動なども含まれることになるが，本稿においてはそれ以外の個人の指向を反映する習慣と認知症発症リスクあるいは予防の可能性について知見を紹介する．

1．嗜　好

食事の一部とも考えられるがアルコール摂取と

表1　生活習慣（食事，運動を除く）と認知症発症リスクに関する報告（縦断観察研究のみ）

国名	年齢	人数	観察期間	調査項目	調整因子	結　果
ドイツ	>65	422	5～8年	人間関係，婚姻状態		単身あるいは寡婦は認知症のリスク高い
フランス	>65	2040	3年	社会参加，文化活動	飲酒，認知機能，身体機能，社会階層	旅行，日曜大工，編み物，庭いじりがリスク軽減
フランス	>65	3675	5年	婚姻状態，社会との絆満足度，参加活動	飲酒，うつ，ネットワークレジャー	未婚が認知症，アルツハイマー型認知症のリスク高 社会とのネットワーク，レジャーは関連なし
スウェーデン	>75	1203	3年	婚姻状態，生活状況 社会との絆　満足度	認知機能，うつ，ADL，血管疾患	独身，独居，低い満足度，社会とのつながりに乏しいことが認知症のリスク高める
スウェーデン	>75	732	6年	精神，社会，余暇活動 活動の参加頻度	認知機能，身体機能，合併症，うつ	精神，社会，生産活動への参加頻度高いほど認知症の発症率が低い
アメリカ	>65	1172	1～7年	13の活動，レジャー活動 知的，社会的活動スコア	身体機能，職業，うつ，血管疾患，高血圧，糖尿病	活動スコア（レジャー，知的　社会的）が高いと認知症のリスクが低い
アメリカ	>65	801	4～5年	知的活動への参加，頻度	健康状態（自己申告） うつ，認知機能，身体機能	活動参加スコアが高いほどアルツハイマー発症頻度が低い
アメリカ	>75	469	5.1年	知的活動，参加時間	うつ，慢性疾患，身体精神機能	読書，ボードゲーム，楽器演奏が認知症，アルツハイマー型認知症ともに発症リスク軽減
アメリカ	>65	801	4.5年	聖職者の知的活動スコア	教育歴，ベースライン認知機能	知的活動スコアが高いとアルツハイマー発症低い

認知症発症との関連は以前より報告されている．適量のワインあるいはアルコール摂取の認知症リスク軽減については以前より報告されてきた．たとえばボルドー（フランス）での調査によれば1日に3～4杯（250～500 m*l*）のグラスワインを飲用する群は非飲用群と比較した場合，年齢，性別，教育歴，職業，ベースラインでの認知機能で調整した後でも認知症/アルツハイマー型認知症の発症に関するオッズ比が0.19/0.28であった[10]．カナダの高齢者6434人を5年間追跡した調査によると年齢，性別，教育歴で調整した場合，週1回以上のワインの摂取（オッズ比：0.49），毎日のコーヒー摂取（オッズ比：0.69）がアルツハイマー発症の予防因子である可能性が示唆された[11]．これが純粋にワイン自体の効果によるものか，ワインの産地においてなお適量の飲用にコントロールできる人はおしなべて食事や他の生活習慣においても中庸を維持できることによるのかは定かではない．アルコールの依存性や過量による有害作用を考えれば，非飲用者に予防に効果があるかもしれないからといってむやみに勧めるべきではない．かつてはタバコの常用については脳内のニコチン受容体への刺激を介して神経保護的に働くため，認知症の予防効果があるのではという報告もあったが[12]，高血圧や動脈硬化などの血管因子への明らかに有害な作用，あるいは高齢期の喫煙者において考えられる選択効果（喫煙者は認知症の好発年齢に達する前に他の病因である程度淘汰されている可能性）から判断して到底予防のための嗜好として推奨されるものではない．近年は喫煙が認知症発症のリスクという考えが定着しており，MRIを用いた脳内灰白質密度の比較では，喫煙者は非喫煙者と比較してアルツハイマー型認知症の初期病変部位である後部帯状回，楔前部の密度が有意に低下しているという報告がある[13]．

2．余暇活動

認知機能を刺激するような余暇活動が認知症の発症予防に効果的であるとの指摘は以前よりされ

てきた．2004年のレビューによるとこれらの活動に従事することが認知症の発症リスクを低くするとの縦断研究の報告は7件ある[14]．余暇活動の内容は報告により異なるが，旅行，日曜大工，編み物，庭いじり，ボードゲーム，楽器演奏，読書，ダンスなどは年齢，性別，教育歴に関係なく認知症の発症に予防的に働く可能性がある[15,16]．スウェーデンにおける双子の追跡調査によると余暇活動の多寡がそれより20年以上経た後の認知症の発症リスクを規定することが示唆されている[17]．しかしながらその後さらに対象群を増やして認知症の正確なスクリーニングによる比較をしたところ，余暇の調査後30年以上を経て認知症を発症した群は知的刺激となる余暇の数が有意に少ないことが示されたが，この結果は教育歴で調整すると統計的に有意なものではないことも報告された[18]．長期的な読書習慣の認知症予防効果については統一した見解が得られてはいないが，短期的（6ヵ月）な音読と計算課題を課した高齢者群では前頭葉機能の改善効果がみられたとする報告がある[19]．

3．生活環境

心理的，社会的ネットワークの形成が認知症の予防に効果があることは経験的に示唆されてきた．地域の開業医に通院する認知症患者195名と非認知症患者229名の後ろ向き調査によると，年齢，性別，居住地域，教育歴，両親の認知症の有無，喫煙習慣で調整した後ロジスティック回帰分析を行ったところ，相談者の数，スポーツ活動への参加，文化的活動への参加（30歳，50歳，調査の10年前の3時点）はそれぞれ独立して認知症発症に予防的に働いていることが示唆された[20]．残念ながら生活習慣の認知症予防効果についてのRCT（無作為割り付けによる介入研究）の成果は現時点ではみあたらない．実際に認知症発症における1次予防効果を生活習慣の視点から追跡するには非常に長い追跡期間を要し，生活習慣も個人の生活状況（個人，地域，社会）により刻々と変遷する可能性もあり，仮に認知症の発症の有無を確認できたとしても，生活要因に関する結果の解釈の妥当性にはおのずから限界が生じるものである．**図1**に過去の縦断観察研究における認知機能低下，認知症の発症リスクと生活習慣との関連の結果を示した．この結果によると認知機能低下，認知症，アルツハイマー型認知症のすべてにおいておおむね社会，身体，精神活動が発症に対して予防的に作用している傾向が伺われる．しかしながらそれぞれの報告において活動の強度による定義が一貫しているわけではなく，いわゆるpublication bias（ある報告が発表された場合，それに追随する結果の報告が出やすい傾向）の影響を排除し得ないのも事実である．

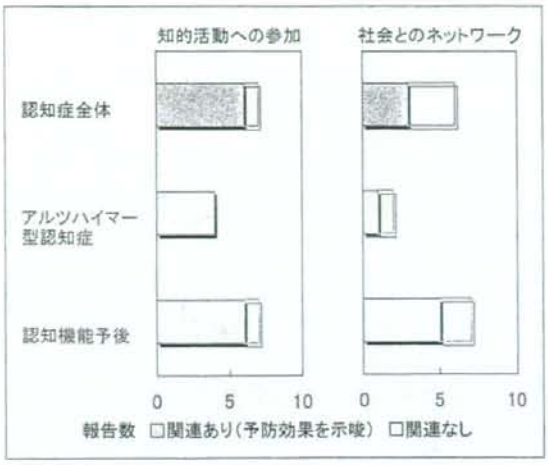

図1　知的活動，社会とのネットワークと認知症予防効果

□ 生活習慣の改善は本当に認知症予防に有効か？

これまでの研究成果にみられる調査時の生活習慣と追跡期間における認知機能との関連はどのように説明できるのか．まず認知症発症前の個人の認知能力が社会参加や活動性に影響を与える可能性がある．この能力に関しては一部の調査ではベースラインでの教育歴や精神活動能力で調整してあるが厳密にもともとの認知能力の結果への影響は否定できない．開始時における生活習慣の変化（社会活動，つながりの欠如）などが認知症の臨床的な発症前の症状の結果として起こっている可能性も指摘できる．これらについては開始時の認知機能を対象の一部で厳密に評価しまったく認知機能に問題のないことを確認したり，追跡期間の早期で認知症を発症した群を除いて解析したり

することによって関連の妥当性を確認した報告もある．一卵性双生児の調査のように厳密に生物学的背景を調整したうえで生活習慣と認知症の発症について縦断的に検討することには多くの現実的な困難が存在する．そもそも生活習慣は個人の人生のステージによって変化するのが常であり，どの時期のどのくらいの期間の生活習慣が後の認知症発症に影響するかを規定することは不可能に近い．とはいえ生活習慣における一定の指向性は認知症発症リスクを規定する因子であることは，過去の報告をレビューする限り間違いはなさそうである．

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Molecular characterization of senescence marker protein-30 gene promoter: Identification of repressor elements and functional nuclear factor binding sites

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Abstract

Background: Senescence marker protein-30 (SMP30), whose expression declines during aging in rat liver, has been proposed as an important aging marker. Besides apoptosis, SMP30 also protects cells against various other injuries by enhancement of membrane calcium-pump activity. The mechanism of this differential gene expression mechanism is not known. DNA-protein interactions, mutation analysis and luciferase reporter assay studies have been performed to elucidate the mechanism of transcriptional regulation of SMP30 gene.

Results: We have characterized up to -2750 bp of the promoter by DNA-protein interactions studies. Twenty eight transcription factor binding sites have been identified by DNase I footprinting and electrophoretic mobility shift assay (EMSA). Transient transfection of 5' and 3' -deleted promoter-reporter constructs and luciferase assay illustrated the region between -128/+157 bp is sufficient to drive promoter activity. We have mapped an essential regulatory region between -513 to -352 bp which causes a drastic decline of reporter activity. This region contains CdxA, GATA2 and SRY transcription factor binding sites. Individual mutation of these three sites showed increase in reporter activity. Mutation in SRY site (-403/-368) showed maximum increase in reporter activity among these three sites. Therefore, we suggest that SRY like protein may be acting as a strong repressor of SMP30 gene along with CdxA and GATA-2. We also report that mutation of both Sp1 (172/-148 bp) and a C/EBPβ (-190/-177 bp) transcription binding site located adjacent to each other on SMP30 gene promoter, causes a significant enhancement in reporter activity than individual mutation, thus may be causing the repression of SMP30 promoter activity.

Conclusion: These studies provide novel insights into the mechanism that regulate SMP30 gene expression.

Background

Senescence marker protein-30 (SMP30), a 34 kDa protein, is preferentially expressed in hepatocytes and renal tubular epithelia. SMP30 is unique in that, its expression is

maintained at a high level throughout the tissue maturation process, then decreases in an androgen-independent manner during senescent stages in both sexes [1,2]. Analysis of murine genomic clone revealed that SMP30 is organized into seven exons and six introns spanning approximately 17.5 kb. The full length cDNA fragment (1.6 kb) contains an open reading frame of 897 bp encoding 299 amino acids. Cloned SMP30 promoter is approximately 3 kb in length and up to -1.5 kb of upstream promoter region has been sequenced [2]. We have further sequenced upstream region -3001 to -1502 bp, and the sequence is available from the NCBI database under the accession number EU251064. SMP30 knockout mice though are viable and fertile have reduced body weight and life span. SMP30 deficiency in mice causes an accumulation of neural lipids and phospholipids in liver and shortens the life span [3]. SMP30 plays an important role in maintaining calcium homeostasis as it blunts down cell death caused by intracellular accumulation of calcium by enhancing plasma membranes calcium pumping activity [1]. It also plays a profound role in rescuing cells from cellular injuries such as apoptosis and hypoxia [4]. Besides, SMP30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy [5]. Recently, we have also reported the alteration of SMP30 expression in hyperthyroidism [6]. Considering the immense importance of SMP30 in aging and in general physiology of an organism, it is highly essential to understand the mechanism of SMP30 gene expression. Regulation of gene expression at transcriptional level is mediated by the interaction of trans-acting factors with *cis*-acting DNA sequences on the promoter region of the genes. Thus, the cross-talk between trans-acting regulatory factors and *cis*-acting regulatory elements may be important for regulation of SMP30 gene expression. The identification of *cis*-regulatory elements are therefore central and detailed analyses of *cis*-regulatory mechanisms controlling critical transcription factor will be required in order to understand the transcriptional regulation of SMP30 gene. Previously, we reported DNase I footprinting on SMP30 promoter up to -800 bp upstream of the transcription start site and identified eight nuclear factor DNA binding sites in this region excluding -513 to -352 bp [7]. The aim of the present study is to characterize and decipher the mechanism of SMP30 gene expression and regulation. In elucidating the mechanism that endow potent and regulated expression of SMP30, detailed characterization of the promoter is highly desirable. To characterize SMP30 promoter we carried out DNA-protein interaction study by DNase I footprinting studies from -800 bp to -2750 bp and electrophoretic mobility shift assay. In this region about twenty eight putative transcription factor binding sites have been identified of which ten transcription factor binding sites were confirmed by competitive EMSA. Further, to access the transcriptional mechanism of SMP30 gene expression and regulation, we have carried out for the first time 5' and 3' serial deletion of SMP30 promoter and subsequently cloned into luciferase reporter vector. Transient transfection and luciferase assay illustrated the region of SMP30 promoter between -128/+157 bp (Luc-6), having significant promoter activity. Progressive deletion study confirmed the presence of a repressor element between -513 bp to -352 bp. DNase I footprinting assay was carried out to chalk out the repressor elements, which revealed the presence of three DNase I protected sites. Analysis of these sequences with TFSEARCH showed the binding of CdxA, GATA2 and SRY transcription factors. Transient transfection of individual site-directed mutated constructs into RAG cells and luciferase assay showed an increase in reporter activity for all the three mutated constructs.

Since mutation of SRY region (-403/-368) showed maximum reporter activity, we suggest SRY along with CdxA and GATA-2 may be acting as a major negative regulator of this gene. Binding of SRY, GATA-2 and CdxA to their respective sites were confirmed by competitive EMSA. Another interesting feature of SMP30 gene promoter is location of Sp1 and C/EBPβ transcription factor binding sites adjacent to each other. Here, we also report that though the presence of these two transcription factor binding sites to minimal promoter region (Luc-5) did not show any significant change in reporter activity as compared to Luc-6, but mutation of both the transcription factor binding site enhanced the reporter activity significantly by 23%. This suggests either direct or indirect interaction between Sp1 and C/EBPβ occurs at transcriptional level in presence of other regulatory factor in SMP30 promoter which causes repression in SMP30 gene promoter activity.

Results

Identification of DNase I protected sites on SMP30 promoter

We have previously identified eight transcription factor binding sites within 0.8 kb mouse SMP30 promoter fragment by DNase I footprinting and EMSA [7]. In this study we further investigated the transcription factor binding sites by DNase I footprinting assay on SMP30 promoter region between -2750/-777 bp using rat liver nuclear extract. The Primers used for footprinting study of the above mentioned regions are shown in table 1. Within this region, twenty eight DNase I protected sites were identified (Table 2 and 3) and a representative of three DNase I footprinting sites of the regions -1208/-777 bp, -1491/-1205 bp and -2028/-1626 bp are shown (Figure 1, 2 and 3).

Table 1: Primers used for footprinting from -777 to -2750 kb.

Serial No	Region Amplified	Primers: Sense (SS) and antisense (AS)
1	-2008 to -777	SS: CAGCATTCCTGGTAGAAACAGGTCC
2	-2008 to -777	AS: GTCCTACACATGGGTGGGCAAATG
3	-1276 to -1039	SS: GCTTCCCAGAGTTCGGCCATTGTTG
4	-1276 to -1039	AS: GTCTTGCAAGCGATGTGTGTGG
5	-1491 to -1205	SS: CCCTTCCCAAGGTTCTCTGC
6	-1491 to -1205	AS:GGTTTTCCCATTGTGACGACGTCGG
7	-1695 to -1391	SS: CACTTGCTTTAACTCCTGCAGC
8	-1695 to -1391	AS: GCTTCTTCATCTTACCCACC
9	-2028 to -1626	SS: GACACACCAGGTGAGCACTGTAC
10	-2028 to -1626	AS: GGTAACTGGAAGTACCCAGC
11	-2190 to -1865	SS: CAAGGCCAGCATGGACTGC
12	-2190 to -1865	AS: GAAGACCTTGGTGGCAGCAG
13	-2448 to -2112	SS: GGTATGCATGCATGCAGTGC
14	-2448 to -2112	AS: GAGCCAATCACCTCCAGGTG
15	-2750 to -2283	SS: GAACGGCAAAGTTAGTATGAGGCC
16	-2750 to -2283	AS: GAGACAGTCCTCAAGTAGCCTGC

Confirmation of identified transcription factor binding site through electrophoretic mobility shift assay (EMSA)

All the DNase I protected sequences were analyzed in transcription factor data base (TFSEARCH, Japan) which revealed binding sites sequence homology to multiple transcription factors. To demonstrate the specificity of transcription factors binding sites to the DNase I protected regions, we carried out EMSA and/or supershift assay. We synthesized oligonucleotides (both strands) corresponding to the protected sites (Table 2) and prepared radiolabeled duplexes for EMSA studies. Five DNase I protected sites were identified in the region -1208/-777 bp of which FP 4 and FP 5 have been confirmed by EMSA (Figure 4 and 5). To FP 4 site binding of C/EBPβ was confirmed by competition with cold C/EBP oligonucleotide and also by C/EBPβ antibody shift experiments. Binding of GATA-1 to FP 5 is confirmed by competition experiments. Four DNase I protected sites were detected in the region -1491/-1205 bp. Though TFSEARCH revealed the binding GATA-3, GATA-1, GATA-2 and AML-1a in order of decreasing binding affinity, only AML-1a competed with FP 6 site (Figure 6). Similarly TFSEARCH revealed the binding of Lyf-1 and GATA-1 to FP8, only cold GATA-1 oligonucleotide competed with FP 8 but not Lyf-1 thus confirming the binding of GATA-1 to FP8 (Figure 7). Three DNase I protected sites were detected in -2028/-1626 bp region. Binding of no transcription factor up to 80% was observed in TFSEARCH to FP 10, thus it may be a novel transcription factor binding site (Figure 8). Binding of SRY to FP 11 is confirmed by competition studies (Figure 9). Table 3 shows all the DNase I protected sites confirmed by EMSA (data not shown).

Elucidation of mechanism of SMP30 gene expression and regulation

To elucidate the mechanism of SMP30 gene expression, both 5' and 3' deletion constructs were sub-cloned into pGL3 luciferase plasmid and transiently transfected into RAG cells. The 5' -serially deleted constructs are -920/+157(Luc-1), -710/+157(Luc-2), -513/+157(Luc-3), -352/+157(Luc-4), -240/+157(Luc-5) and -128/+157(Luc-6). The 3' -serially deleted constructs are +157/-128 and +104/-128. The expression pattern of 5' -serially deleted constructs is shown in figure 10. The 5' -deleted -128/+157 bp construct (Luc-6) showed highest reporter activity among others. In order to delineate the basal promoter activity further, we carried out transfection of 3' -deletion construct into RAG cells. The construct -128/+104 bp showed ~ 28% reduction in reporter activity (Figure 11). Thus, the region between -128/+157 bp is essential in

Table 2: DNase I protected sites.

Footprint	DNase I protected region	Transcription factor
FP 4	TGTGGGTTAAGC*TATTGCAAAACTCCAACATCTGATCTTGGGGCTT*	C/EBP β
FP 5	ACCCCTCCAC*ACACATCGCTT*GCAAGACAAACTGTGGGTT	GATA1
FP 6	*CACCCCAATCCGGCTGAGACTGCTCTGTGAGTAGC*	AML-1A
FP 8	CTTGGTGGGTAAGATGAAGAAG*CTAGATTTGGGCGAAGGC*	GATA1
FP 10	TTTGCAAGCGTTGGCCTGCTGCCACCAAGGTCTTCCC	Novel
FP 11	TAAACCAAAT*CAAATAAAGGCATTTTTCTTCCCCTTCC*	SRY

Table 3: DNase I protected sites between -777 to -2750 (data not shown)

Footprint	DNase I protected region	Transcription factor
-953 to -913	GGGCCAATTTTTAACAGCCAATGAAAATGGCAAATGCTACACA	CdxA
-995 to -954	GGGGCTTATCTACGATTGATAGCATGAAGC	GATA-X
-1123 to -1088	CCACCAGTTTGCAGCCAGAATTCCTGGTAGAAACAG	CdxA
-1374 to -1351	CCCCTGGGAAGCTAGATTTGTTCA	SRY
-1440 to -1419	CCCTGTGAATAACCGGGACAGG	HSF2
-1546 to -1520	CCCCGGGCCGGCGGCTACCTATCTGCC	GATA2
-1584 to -1550	AGAAGGGTGAGCCCTCAGGATCGCTAGTCTCTGCC	GATA2
-1621 to -1587	GCTGAAAAATGAAAGGACAGCGTGGGCACCCGTAG	Cdxa
-1983 to -1956	GGCTATGTCATTTAGAATCGTTTATTCC	Oct-1
-1994 to -1981	TAGCTATGTTTGGC	SRY
-2076 to -2051	CCTATAAAATAAAGGGAAAAGAACACC	TATA
-2111 to -2082	ACCTGGAGGTGATTGGCTCTGAGTTTCACC	GATA1
-2134 to -2109	GGGGAGTGAGTCAGTGGTAGTGCACC	Ap1
-2167 to -2141	TGAAACTGCCAAGAAATAATGTCTTAG	CdxA
-2384 to -2347	TAAATAGGTTTTTAAAAAGAAAAAGAAAAAATGGGGCA	SRY
-2414 to -2381	AAGCAAAGCCCTCACACACATTAAAATGAGTAAA	CdxA
-2491 to -2449	GAGATGACAATTACATCAAATAATAAAAGTTATATTTACATCA	CdxA
-2604 to -2562	TGGTGAGCTTATTTAATCTCAGAGACTGAAAACATTCTAGGCC	HNF-3b
-2675 to -2646	GCTTGGCGAGGAGTTTTAACCCAGAACAGC	SRY
-2691 to -2654	AGAAGCCAGATTGAATCAGTTGCTTGGCGAGGAGTTTTAACCC	AP1
-2704 to -2691	CCAAGAGAAGAAGCC	Novel
-2721 to -2703	GAGCAATTCAGGAGAGGCC	Nkx-2.5

determining the promoter activity of SMP30 gene and sequence between +104 bp to +157 bp plays a detrimental role during transcription.

Identification of SRY, GATA-2 and CdxA like transcription factor as a repressor element present between -513 and -352 bp

The reporter assay of 5' -serially deleted constructs showed a drastic decline (~41%) of activity of Luc-4 (-352/+157 bp) as compared to Luc-3 (-513/+157 bp). DNase I footprinting study of the region between -513 bp and -352 bp revealed three distinct DNase I protected sites such as Luc 3–1, Luc 3–2 and Luc 3–3 (Figure 12). TFSEARCH revealed the binding of CdxA to Luc 3–1, GATA-2 to Luc 3–2 and SRY to Luc 3–3. To pin point the transcription factor which act as a repressor we prepared site directed mutated constructs for all the three sites. Transient transfection of these mutated constructs was carried out along with wild type Luc 3 (-513/+157) (Figure 13). Mutation of Luc3-1 leads to increase in reporter activity by 29%, Luc3-2 by 27% and Luc 3–3 site leads to maximum increase in reporter activity about 59%. This result revealed SRY, CdxA and GATA-2 as the major repressor elements. To further establish this fact, we did EMSA study using both wild type and mutated oligonucleotide of Luc3-3 site. The wild type Luc3-3 oligonucleotide competed with SRY specific cold oligonucleotide consensus, but mutated 3–3 site did not show any binding, thus establishing the binding of SRY to this site (Figure 14). The competitive EMSA using radiolabeled Luc3-2 oligonucleotide showed 100% competition with GATA-2 (Figure 15), while EMSA using radiolabeled Luc 3–1 which has 85% homology revealed that although there is competition by unlabeled Luc 3–1 oligonucleotide, no significant competition is observed using consensus CdxA site. Considering the high homology to Cdx A consensus binding site, Luc 3–1 site may be interacting with CdxA like transcription factor with slight deviation in binding sequence (data not shown).

Table 4: Primers used for 5' and 3' deletion.

Luc-1 (SS)	ACAGGTACCCAAATGCTACAGCGCTGG
Luc-2 (SS)	ACAGGTACCAATGTCTACTGGGGTAG
Luc-3 (SS)	ACAGGTACCCATGCAAGGAAGCAAG
Luc-4 (SS)	ACAGGTACCCCTCATACCTGCCATTATC
Luc-5 (SS)	ACAGGTACCTACCAAGCCTCTGGCTG
Luc-6 (SS)	ACAGGTACCGAATGAGGGAGAGGTG
Luc-SMP-XhoI (AS)	ACACTCGAGGCAAGACAGGAGGTGATTG
Luc-Exon-SMP-XhoI	ACACTCGAGCGTCTTCAGTCAACTTACC

Sp1 and C/EBPβ causes repression of SMP30 promoter activity

SMP30 gene promoter has a Sp1 and a C/EBPβ transcription factor binding site adjacent to each other. Sp1 site spans between -172 to -148 bp and C/EBPβ spans between -190 to -177 bp. Here we report that, presence of these two sites in the minimal promoter region did not cause any significant change in reporter activity (that is, there is no significant change in Luc-5 as compared to Luc-6). But site-directed mutation of both the transcription

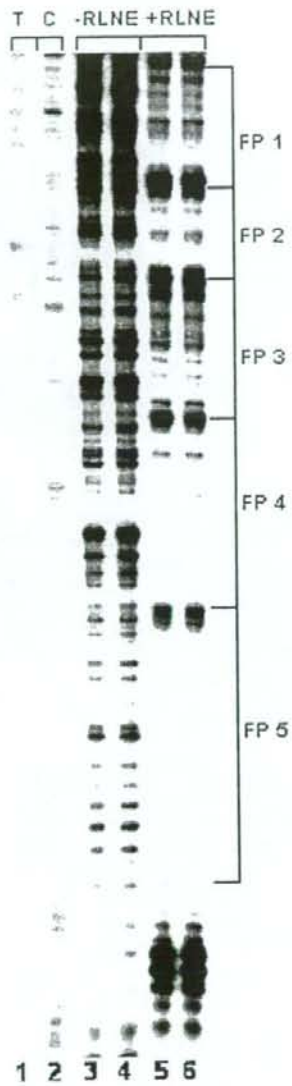

Figure 1
DNase I footprinting analysis of SMP30 promoter between -1208 bp to -777 bp region: *Lane 1–2*, represent sequencing ladder T and C; *Lane 3–4*, represent DNA treated with DNase I in absence of RLNE; *Lane 5–6*, represent DNA treated with DNase I in presence of 50 μg RLNE. The DNase I protected sites are marked on right site.

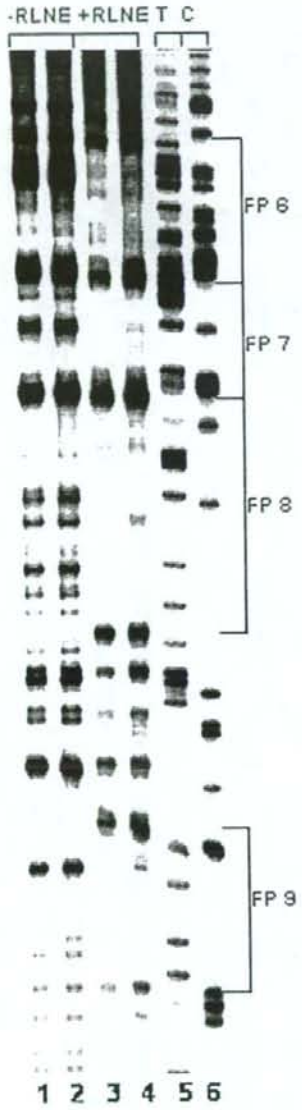

Figure 2
DNase I footprinting analysis of SMP30 promoter between -1491 bp to -1205 bp respectively: *Lane 1–2* represent DNA treated with DNase I in absence of RLNE. *Lane 3–4*, represent DNA treated with DNase I in presence of 50 μg RLNE and *Lane 5–6*, represent sequencing ladder T and C.

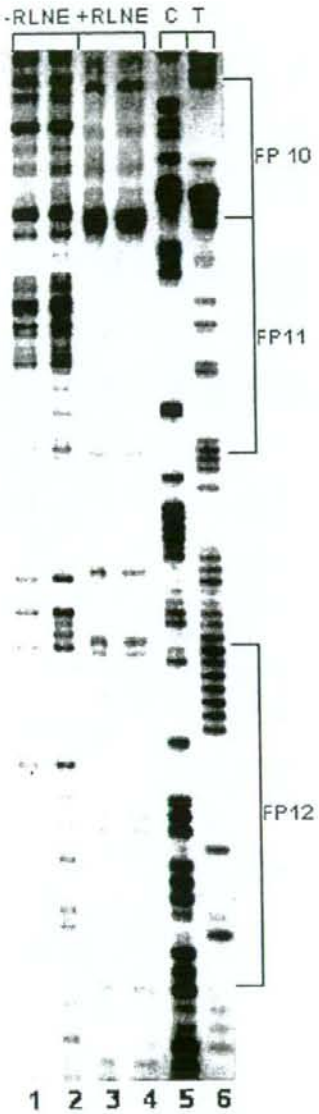

Figure 3
DNase I footprinting analysis of SMP30 promoter between -2028 to -1626 bp respectively: *Lane 1–2* represent DNA treated with DNase I in absence of RLNE. *Lane 3–4*, represent DNA treated with DNase I in presence of 50 μg RLNE *Lane 5–6*, represent sequencing ladder C and T.

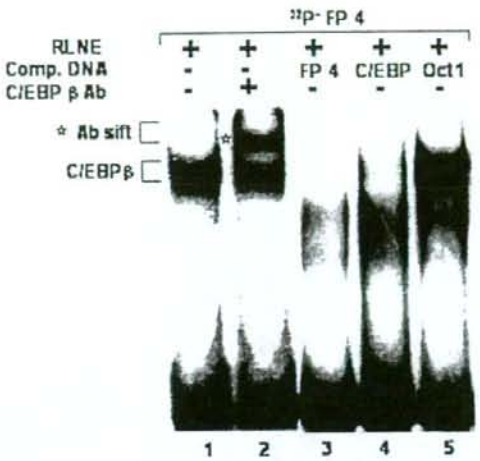

Figure 4
Electrophoretic mobility shift assay to confirm the binding of C/EBP transcription factor to DNase I protected site FP 4: *Lane 1*, labeled FP 4 oligonucleotide duplex with 6 μg RLNE; *Lane 2* C/EBPβ antibody; *Lane 3–5*, describe the competition with 100 fold molar excess of unlabeled homologous self, C/EBP consensus, and nonspecific Oct 1 oligonucleotide duplex respectively. Antibody shift is seen with C/EBPβ antibody.

factor binding site caused a significant increase in reporter activity (~23%) (Figure 5A). Individual mutation of only Sp1 and C/EBPβ did not contribute to any significant change in reporter activity. Mutation of Sp1 site reduced the reporter activity by only 16% and mutation of C/EBPβ lead to enhancement of reporter activity by only 14% (Figure 16). Binding of Sp1 to the region between -172 to -148 bp is confirmed by competitive EMSA done in presence of 100 fold molar excess of cold Sp1 consensus (Figure 17). EMSA was also carried out using labeled mutated Sp1 oligonucleotide, which showed no DNA-protein interaction, thus confirming the inability of Sp1 to bind to the mutated site. Binding of C/EBPβ to the region -190/-177 bp is confirmed by EMSA and antibody shift experiments using C/EBPβ antibody (Figure 5C). EMSA study carried out with labeled mutated C/EBP oligonucleotide also yielded a DNA-protein complex. But this complex is not due to binding of C/EBPβ as confirmed by competition with C/EBPβ consensus and antibody shift experiments (Figure 18).

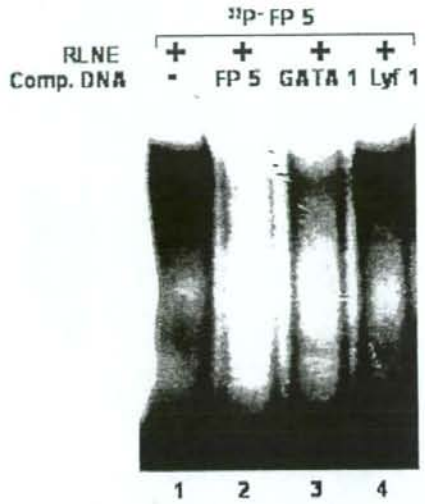

Figure 5
Electrophoretic mobility shift assay to confirm the binding of GATA1 transcription factor to DNase I protected site FP 5: *Lane 1*, labeled FP 5 oligonucleotide duplex with 6 μg RLNE; *Lane 2–4*, describe the competition with 100 fold molar excess of unlabeled homologous self, GATA1 consensus and Lyf-1 consensus.

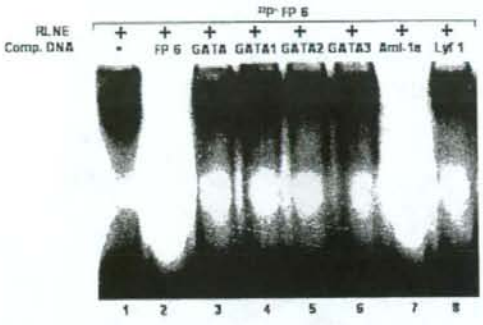

Figure 6
Electrophoretic mobility shift assay to confirm the binding of Aml-1a transcription factor to DNase I protected site FP 6: *Lane 1*, labeled FP 6 oligonucleotide duplex with 6 μg RLNE; *Lane 2–8*, describe the competition with 100 fold molar excess of unlabeled homologous self, GATA consensus, GATA1 consensus, GATA2 consensus, GATA3 consensus, Aml-1a and nonspecific Lyf-1 oligonucleotide duplex respectively.

Discussion

The multiple biological functions of SMP30 in diverse target cells require its expression to be regulated precisely. It is suggested that the transcriptional regulation of a particular gene is a complex process which usually involves interaction between multiple *cis*-acting regulatory elements and their cognate protein factors [8,9]. A growing list of transcription factors has been shown to function as either transcriptional activator or repressor in different gene promoter. In this study we analyzed the transcriptional regulation of SMP30 gene by DNase I footprinting, EMSA and functional characterization by transient transfection, reporter assay of 5' and 3' -serially deleted promoter reporter constructs and site-directed mutagenesis. We have earlier reported eight nuclear factor binding sites on SMP30 gene promoter [7]. In this report twenty eight new DNase I footprinting sites were identified using rat liver nuclear extract. We also demonstrate that the 5' -flanking regions of SMP30 gene possess a functional promoter when transfected into RAG cells. The results of 5' and 3' -deletion analysis illustrated the region -128/+157 bp possesses significant reporter activity. The presence of a TATA sequence (-29 ATAAAA -25) and a CAAT box (-72 CCAAT -68) were previously reported respective to the transcription start site [2]. Our results suggest that the TATA and CAAT box located between -128 bp and +157 bp plays an important role in determining the promoter activity and sufficient to drive SMP30 gene expression. 3' – deletion from +157 bp to +104 bp resulted in ~28% decrease in basal promoter activity, thus indicating that this region is essential for SMP30 gene expression. An interesting feature of SMP30 promoter is the presence of C/EBPβ binding site adjacent to Sp1 binding site. Sp1 is a ubiquitous DNA-binding protein with three zinc finger at its C-terminal that activates the transcription of many cellular and viral genes [10]. SMP30 promoter possess a Sp1 binding site between -172 bp to -148 bp. C/EBPβ belongs to CCAAT-enhancer-binding protein family of transcription factors, involved in different cellular response like in control of cellular proliferation, growth and differentiation, metabolism, immune response and many others. C/EBPβ binding site spans between -190 bp to -177 bp on SMP30 promoter. This spatial arrangement of C/EBPβ and Sp1 is critical as Sp1 is known to recruit C/EBPβ to cryptic C/EBP site [11]. Presence of these two sites in the minimal promoter region represented as Luc-5 did not show any significant change in luciferase activity as compared to the Luc-6, but mutation of both Sp1 and C/EBPβ significantly enhanced the reporter gene activity to about 23%. Thus, it is reasonable to believe that direct or indirect interaction between Sp1 and C/EBPβ in presence of some other regulatory factor occurs at transcriptional level

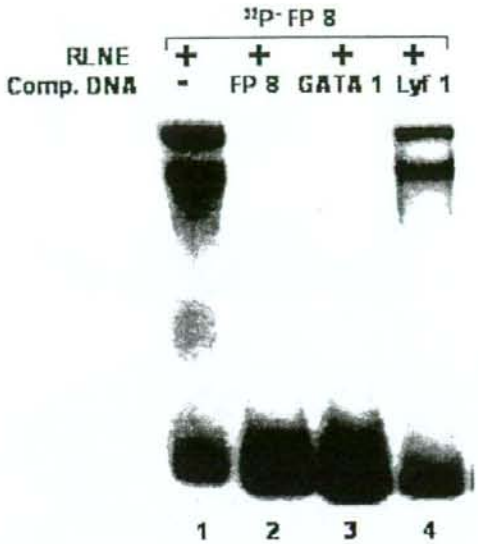

Figure 7
Electrophoretic mobility shift assay to confirm the binding of GATA1 transcription factor to DNase I protected site FP 8: *Lane 1*, labeled FP 8 oligonucleotide duplex with 6 μg RLNE; *Lane 2–4*, describe the competition with 100 fold molar excess of unlabeled homologous self, GATA1 consensus, and Lyf-1 consensus.

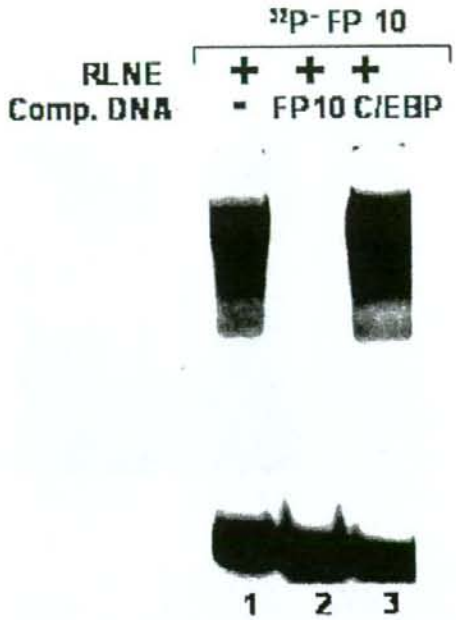

Figure 8
Electrophoretic mobility shift assay of DNase I protected site FP 10: *Lane 1*, labeled FP 10 oligonucleotide duplex with 6 μg RLNE; *Lane 2–3*, describe the competition with 100 fold molar excess of unlabeled homologous self, and non specific C/EBP consensus. Binding of no transcription factor was observed in TFSEARCH data base to this site.

in SMP30 promoter which causes a repression in SMP30 promoter activity. Transient transfection of 5' -deletion fragments revealed the presence of a repressor element between -513 to -352 bp, as deletion of this region caused 41% decrease in reporter activity. Our DNase I footprinting study showed three putative transcription factor binding sites within this region (Figure 12). In order to confirm the potential repressor among these DNase I protected sites, we carried out site directed mutagenesis studies of these three sites and subsequent transfection along with wild type (Luc-3). This result suggested a significant enhancement of reporter activity of Luc 3–3 mutated fragment by ~59%, Luc3-2 by 27% and Luc 3–1 by 29%. (Figure 13). TFSEARCH indicated the binding of SRY to wild type site Luc 3–3, GATA-2 to Luc 3–2 and CdxA to Luc 3–1 sequences, which is confirmed by competitive EMSA. Earlier reports depicted the tissue specific expression of SRY in testes [12] where it involve in testes determination and differentiation in mammals. Though expression of SRY in substantia nigra of adult male rodents in tyrosin hydroxylase expressing neurons has also been reported but its expression in liver and kidney is still obscure [13]. So the identified transcription factors might be SRY like proteins which bind to a similar binding site as SRY. The affinity of SRY for double-stranded DNA varies with DNA sequence and shares a conserved DNA binding domain (HMG-box) NACAAT [14]. SRY is reported to bind and negatively regulates the androgen receptor gene promoter [15]. We also suggest that GATA-2 and CdxA might be interacting directly or indirectly with SRY to bring about repression of SMP30 gene.

Conclusion

Transcription factors Sp1, C/EBPβ, SRY, GATA-2 and CdxA, binding within -513 of SMP30 promoter, have significant role in regulation of SMP30 gene expression.

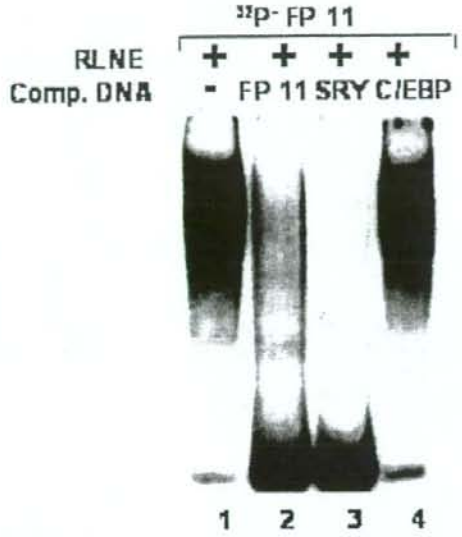

Figure 9
Electrophoretic mobility shift assay to confirm the binding of SRY to DNase I protected site FP 11: *Lane 1*, labeled FP 11 oligonucleotide duplex with 6 μg RLNE; *Lane 2–4*, describe the competition with 100 fold molar excess of unlabeled homologous self, SRY and non specific C/EBP consensus.

Methods

Preparation of nuclear extract

Nuclear extract from liver of adult (5 months) male rats (Fisher 344) were prepared as described previously [16]. Briefly, liver slices were homogenized in 4 volumes (w/v) of ice-cold buffer containing 0.25 M sucrose, 15 mM Tris-HCl (pH 7.9), 16 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.15 mM spermine, and 0.15 mM spermidine; supplemented with the following protease inhibitors: 0.1 mM PMSF, 2 μg/ml leupeptin, 5 μg/ml aprotonin. After centrifugation for 10 minutes at 2000 × *g*, the pellets were resuspended in 4 volumes of ice-cold buffer (10 mM HEPES; pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and protease inhibitors). The nuclei were pelleted down by centrifugation for 10 minutes at 4000 × *g*, and resuspended in ice cold buffer of 10 mM HEPES (pH 7.9), 0.75 mM $MgCl_2$, 0.5 M KCl, 0.5 mM EDTA, 12.5% glycerol and protease inhibitors. After incubation on ice for 30 minutes with continuous agitation, the supernatants containing the nuclear extracts were collected by centrifugation for 30 minutes at 14,000 × *g*, frozen in liquid nitrogen and stored in -70°C until used. All manipulations were carried out at 4°C. Protein concentrations were determined by the Bradford protein assay reagent (Sigma, USA).

DNase I footprinting

DNase I footprinting was carried out as described before [7]. Briefly, end-labeled DNA fragments (50 fmoles) were incubated with 50 μg of rat liver nuclear extract and 2 μg of poly (dI-dC) in binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5%

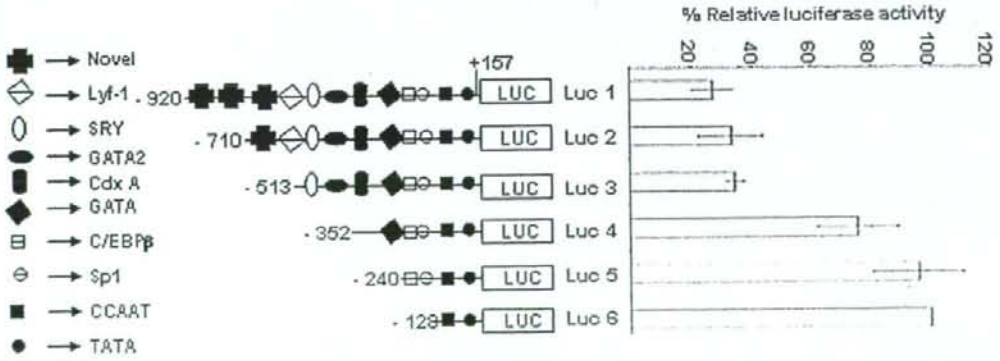

Figure 10
Relative luciferase activity of different 5' -serially deleted SMP30 promoter-reporter constructs were transfected in RAG cells. The results were obtained after normalization with β-galactosidase activity. All transfections were repeated in duplicates and the results are expressed as the mean of five different experiments ± S.D. On left, a schematic representation of all the 5' -deleted luciferase constructs used for transfection is depicted. Approximate locations of transcription factor binding sites are shown.