Experimental Cell Research 253, 365–371 (1999)
Article ID excr.1999.4605, available online at http://www.idealibrary.com on IDEAL®

MINIREVIEW

Nuclear Factor Y (NF-Y) and Cellular Senescence

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NF-Y, also termed CBF, is a major CCAAT-binding transcription factor that specifically recognizes the consensus sequence 5′-CTGATTGGYYRR-3 or 5′-YYRRCCAATCAG-3′ (Y = pyrimidines and R = purines) present in the promoter region of many constitutive, inducible, and cell-cycle-dependent eukaryotic genes. The functional NF-Y is a heterotrimeric protein, consisting of three different subunits, A, B, and C. Each of the three subunits contains two or three distinct protein-interacting domains for trimer formation and for interacting with other nuclear proteins. Only the trimeric NF-Y, and not the individual subunit, possess DNA-binding activity. The transcriptional activity of NF-Y can be regulated by differential expression, alternative splicing, protein–protein interactions, and cellular redox potential. The regulation of thymidine kinase (TK) and dihydrofolate reductase (DHFR) genes in human diploid fibroblasts serves as an example of how NF-Y may have a role in replicative senescence by regulating age-dependent G1/S genes. © 1999 Academic Press

Key Words: **NF-Y; G1/S; cell senescence.**

INTRODUCTION

The CCAAT box is one of the most common *cis* elements present in eukaryotic promoters. Analysis of 502 unrelated gene promoters indicates that the CCAAT box is present in about 30% of them [1]. Several CCAAT-binding proteins have been described, including c/EBP, CTF/NF-1, Y-box factors, HSP-CBF, and NF-Y. Among them, NF-Y (nuclear factor for Y box) is the most ubiquitous and specific acting as a key proximal promoter factor in the transcriptional regulation of a whole array of different eukaryotic genes. Unlike other CCAAT-binding proteins, NF-Y has an absolute requirement of CCAAT pentanucleotide as well as a strong preference for specific flanking sequences. NF-Y, originally identified as the protein recognizing the MHC class II conserved Y box in Eα promoters [2], has been shown to be identical to CP1 and CBF that binds to the CCAAT box in the adenovirus major late promoter and α2(1) collagen promoter, respectively. NF-Y is also homologous to yeast CCAAT-binding protein HAP2/3/5 [reviewed in Refs. 3, 4]. The criteria commonly used to determine whether NF-Y is involved in the regulation of a particular gene include gel mobility shift assay and competition analysis, gel mobility supershift assay using anti-NF-Y antibodies, *in vivo* footprinting analysis, and transient transfection reporter gene assay using a dominant negative NF-YA mutant. A comparison of 178 promoter sequences that contain at least one bona fide NF-Y-binding site reveals that (i) the consensus NF-Y-binding sequence is 5′-CTGATTGGYYRR-3′ or 5′-YYRRCCAATCAG-3′; (ii) the NF-Y site has a mean position at −89 in TATA-containing promoters, and at −66 in TATA-less promoters as measured from the transcription initiation site; and (iii) more than 60% of CCAAT boxes in eukaryotic promoters are in antisense orientation [4]. Since genes that harbor NF-Y sites include constitutive, inducible, and cell-cycle-dependent genes, the mechanism of regulating the expression of these vastly different genes cannot simply be due to NF-Y DNA binding. It is likely that additional factors will be involved in the action of NF-Y. Indeed, recent studies from several laboratories have suggested that NF-Y interacts, either functionally or physically, with other transcription factors or nuclear proteins both *in vitro* and *in vivo* [5–9].

The hallmark of cellular aging is the failure of senescent cells to initiate DNA synthesis during the progression of the cell cycle [10]. Alterations of gene regulation at the late G1/S boundary in the cell cycle could be a major contributing factor to the loss of dividing potential during cell senescence. Indeed, most, if not all, G1/S boundary genes including thymidine kinase (TK), dihydrofolate reductase (DHFR), and E2F1 are down-regulated during cell senescence [11–15]. Since NF-Y has been shown to be involved in regulating some

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A NF-YA

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YA_HS    MEQYTANSNSSTEQIVVQAGQIQQQQGGVTAVQLQTEAQVASASGQQVQTLQVVQGQPL
YA_MM    MEQYTTNSNSSTEQIVVQAGQIQQQQ-GGVTAVQLQTEAQVASASGQQVQTLQVVQGQPL
HAP2_SC  ----------------MSADETDAKFHPLETDLQSDTAAATSTAAASRSPSLQEK---PI

YA_HS    MVQVSGGQLITSTGQPIMVQAVPGGQGQTIMQVPVSGTQGLQQIQLVPPGQIQIQGGQAV
YA_MM    MVQVSGGQLITSTGQPIMVQAVPGGQGQTIMQVPVSGTQGLQQIQLVPPGQIQIQGGQAV
HAP2_SC  EMPLDMGKAPS-----------PRGEDQ----R-VTNEEDLFLFNRLRASQNRVMDSLEP

YA_HS    QVQGQQGQTQQIIIQQPQTAVTAGQTQTQQQIAVQGQQVAQTAEGQTIVYQPVNADGTIL
YA_MM    QVQGQQGQTQQIIIQQPQTAVTAGQTQTQQQIAVQGQQVAQTAEGQTIVYQPVNADGTIL
HAP2_SC  QQQ-SQYTSSSVSTMEPSADFTSFSAVTTLPPPPHQQQQQQQ------QQ--Q-----

YA_HS    QQVTVPVSGMITIPAASLAGAQIVQTGANTNTTSSGQGTVTVTLPVAGNVVNSGGMVMMV
YA_MM    QQVTVPVSGMITIPAASLAGAQIVQTGANTNTTSSGQGTVTVTLPVAGNVVNSGGMVMMV
HAP2_SC  QQLVV-----------Q---AQYTQNQPNLQSDVLG------------------------

YA_HS    PGAGSVPAIQRIPLPGAEMLEEEPLYVNAKQYHRILKRRQARAKLEAEGKIPKERRKYLH
YA_MM    PGAGSVPAIQRIPLPGAEMLEEEPLYVNAKQYHRILKRRQARAKLEAEGKIPKERRKYLH
HAP2_SC  ------------------TAIAEQPFYVNAKQYYRILKRRYARAKLEEKLRISRERKPYLH

YA_HS    ESRHRHAMARKRGEGGRFFSP------KEKDSPHMQDPN--QADEEAMTQIIRVS-----
YA_MM    ESRHRHAMARKRGEGGRFFSP------KEKDSPHMQDPN--QADEEAMTQIIRVS-----
HAP2_SC  ESRHKHAMRRPRGEGGRFLTAAEIKAMKSKKSGASDDPDDSHEDKKITTKIIQEQPHATS

YA_HS    ---------
YA_MM    ---------
HAP2_SC  TAAAADKKT
```

NF-YB

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YB_HS    MTMDGDSSTTDASQLGISADYIGGSHYVIQPHDDTEDSMNDHEDTNGSKES-FREQDIYL
YB_MM    MTMDGDSSTTDASQLGISADYIGGSHYVIQPHDDTEDSMNDHEDTNGSKES-FREQDIYL
HAP3_SC  ------------------MNTNESEHVSTSPEDTQENGGNASSSGSLQQISTLREQDRWL

YB_HS    PIANVARIMKNAIPQTGKIAKDAKECVQECVSEFISFITSEASERCHQEKRKTINGEDIL
YB_MM    PIANVARIMKNAIPQTGKIAKDAKECVQECVSEFISFITSEASERCHQEKRKTINGEDIL
HAP3_SC  PINNVARLMKNTLPPSAKVSKDAKECMQECVSELISFVTSEASDRCAADKRKTINGEDIL

YB_HS    FAMSTLGFDSYVEPLKLYLQKFREAMKGEKGIGGAVTATDGLSEELTEEAFTNQLPAGLI
YB_MM    FAMSTLGFDSYVEPLKLYLQKFREAMKGEKGIGGAVSATDGLSEELTEEAFTNQLPAGLI
HAP3_SC  ISLHALGFENYAEVLKIYLAKYRQQQ--------------ALKNQLMYEQDDEEVP----

YB_HS    TTDGQQQNVMVYTTSYQQISGVQQIQFS
YB_MM    TADGQQQNVMVYTTSYQQISGVQQIQFS
HAP3_SC  ----------------------------
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NF-YC

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YC_HS    MSTEGGFGGTSSSDAQQSLQS---------------------------------------
YC_MM    MSTEGGFGGTSSSDAQQSLQS---------------------------------------
HAP5_SC  -MTDRNFSPQQGQGPQESLPEPEGPQPSTMIQREEMNMPRQYSEQQQLQENEGEGENTRL

YC_HS    ------------------------------------------------------------
YC_MM    ------------------------------------------------------------
HAP5_SC  PVSEEEFRMVQELQAIQAGHDQANLPPSGRGSLEGEDNGNSDGADGEMDEDDEEYDVFRN

YC_HS    ---------------FWPRVMEEIRNLT------VKDFRVQELPLARIKKIMKLDEDVKM
YC_MM    ---------------FWPRVMEEIRNLT------VKDFRVQELPLARIKKIMKLDEDVKM
HAP5_SC  VGQGLVGHYKEIMIRYMQELINEIESTNEPGSEHQDDFKSHSLPFARIRKVMKTDEDVKM

YC_HS    ISAEAPVLFAKAAQIFITELTLRAWIHTEDNKRRTLQRNDIAMAITKFDQFDFLIDIVPR
YC_MM    ISAEAPVLFAKGAQIFITELTLRAWIRTEDNKRRPLQRNDIAMAITKFDQFDFLIDIVPR
HAP5_SC  ISAEAPIIFAKACEIFITELTMRAWCVAERNKRRTLQKADIAEALQKSDMFDFLIDVVPR

YC_HS    DELKPPKRQEEVRQSVTPAEPVQYYFTLAQQPTAVQVQGQQQGQQTTSSTTTIQPGQIII
YC_MM    DELKPPKRQEEVRQSVTPAEPVQYYFTLAQQPTAVQVQGQQQPQQTTSSTTTIQPGQIII
HAP5_SC  RPLPQ-------------------------------------------------------

YC_HS    AQPQQGQTTPVTMQVGEGQQVQIVQAQPQGQAQQAQSGTGQTMQVMQQIITNTGEIQQIP
YC_MM    AQPQQGQTTPVTMQVGEGQQVQIVQAQPQGQAQQTQSGTGQTMQVMQQIITNTGETQQIP

YC_HS    VQLNAGQLQYIRLAQPVSGTQVVQGQIQTLATNAQQITQTEVQQGQQQFSQFTDGQQLYQ
YC_MM    VQLNAAQLQYIRLAQPVSGTQVVQGQIQTLATNAQQITQTEVQQGQEQFSQFTDGQQLYQ

YC_HS    IQQVTMPAGQDLAQPMFIQSANQPSDGQAPQVTGD
YC_MM    IQQVTMPAGQDLAQPMFIQSANQPSDGQTPQVTGD
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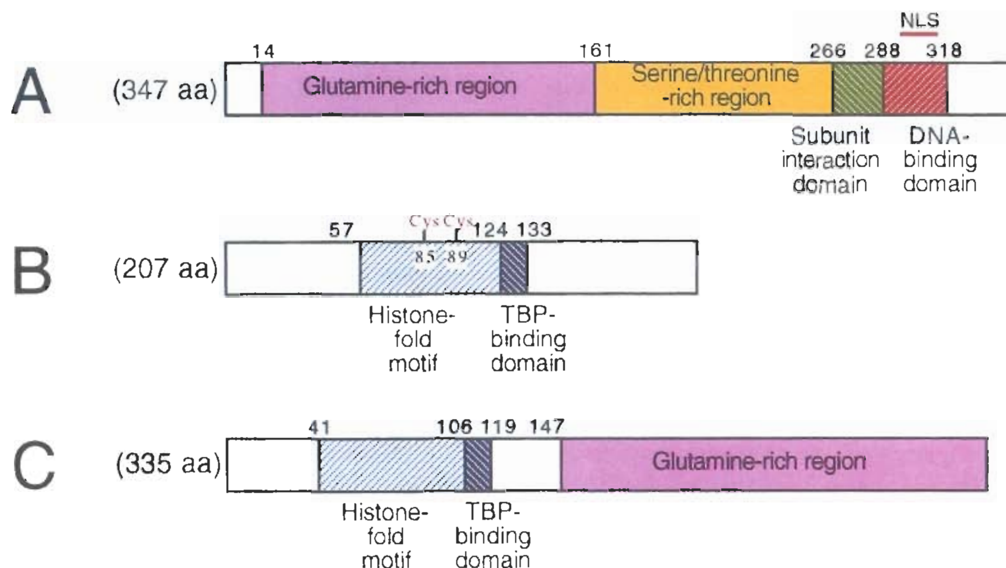

FIG. 1. (A) Sequence alignment and motif structure of NF-Y. Amino acid sequence alignment of three different NF-Y subunits from yeast (SC), mouse (MM), and human (HS) using the Clustal W program. A putative nuclear localization signal in NF-YA is boxed. The two conserved cysteine residues (aa 85 and 89 in human NF-YB) are also boxed. (B) Domain structure of human NF-Y subunits, A, B, and C. The long form NF-YA is shown in both (A) and (B).

of these G1/S genes, it is possible that NF-Y may have a physiological role in replicative senescence.

SEQUENCE COMPARISON AND MOTIF STRUCTURE OF NF-Y SUBUNITS

Alignment of amino acid sequences of three NF-Y subunits from yeast, mouse, and human shows that several regions in each of these polypeptides are highly conserved (Fig. 1A). This, together with mutational analysis, has led to the definition of domain structure in these subunits (Fig. 1B). NF-YA has at least two subdomains for protein binding: a Q-rich domain and a subunit interaction subdomain. NF-YB also has two subdomains for protein binding: histone-fold motif and TATA-binding protein (TBP)-binding subdomain. NF-YC has three subdomains for protein binding: histone-fold motif, TBP-binding domain, and Q-rich domain. The Q-rich domain in NF-YA and in NF-YC resembles that of Sp1 [16] and is involved in the transcriptional activation [17]. The Q-rich domain in NF-YA is also the site for alternative splicing that generates NF-YA isoforms [18]. Both NF-YB and NF-YC contain a histone-fold "handshake" motif, originally identified in histones 2A and 2B. This motif is characterized by three alpha helices connected with two loops and is responsible for H2A/H2B dimer formation [19]. A TBP-binding subdomain has been identified at the C-terminus end of the histone-fold motif in both NF-YB and NF-YC [20]. A bipartite nuclear localization signal (NLS) containing clustered basic amino acid residues is present in NF-YA at positions 292 to 312. This NLS is located within the NF-YA signature DNA-binding domain (aa 288–318). As shown in Fig. 1, histone-fold motif in NF-YB and NF-YC and subunit interaction domain and DNA-binding domain in NF-YA are conserved from yeast to human. The two subunits, NF-YB and NF-YC, first form a dimer mediated through their histone-fold motif. The dimer provides a suitable groove for NF-YA to dock using its subunit interaction domain that leads to the creation of a functional NF-Y heterotrimeric protein. The sequence specificity of NF-Y, however, appears to depend not only on NF-YA alone but also on the histone-fold alpha1 helix of NF-YB [21].

FACTORS THAT REGULATE NF-Y TRANSCRIPTIONAL ACTIVITY

The presence of Q-rich domains and histone-fold motifs gives NF-Y the potential to interact not only with other transcription factors but also with nuclear proteins such as TBP and TBP-associated factors in the transcriptional machinery. Such interactions could have added additional layers of fine tuning the transcriptional activity of NF-Y in regulating various genes. For example, NF-Y has been shown to stabilize E2F binding on the E2F1 promoter and synergize the action of E2F via *trans*-activation domains [9]. The cell cycle regulation of the *cdc25C* gene has been shown to be mediated by the

periodic repression of the glutamine-rich activators NF-Y and Sp1 [7]. The synergistic interaction between sterol regulatory element-binding protein and NF-Y at the farnesyl diphosphate synthase promoter has been demonstrated [8] and the cooperative interactions of Sp1 and NF-Y have been shown to be responsible for the upregulation of the expression of the MHC class II-associated invariant chain gene [6].

In addition to transcription factors such as Sp1, several nuclear proteins have recently been identified to interact with NF-Y [20, 22–25]. The presence of histone acetyltransferases hGCN5 and PCAF (p300/CBP-associated factor) has been demonstrated in the crude NF-Y-binding complex isolated from HeLa cells [22]. This finding suggests, for the first time, that NF-Y, by recruiting the histone acetyltransferases or deacetylases, may be involved in chromatin remodeling and that histone acetylation or deacetylation may modulate the NF-Y transcriptional activity. The regulation of the *MDR1* gene by histone acetylation/deacetylation using specific deacetylase inhibitors has been shown to be mediated by NF-Y [23]. High mobility group proteins (HMG) are nonchromosomal proteins containing a 9-amino-acid repeat motif (the A-T hook) that binds to the minor groove of many AT-rich sequences. HMG proteins can stabilize transcription factor–DNA binding through such binding. *In vitro* gel mobility shift assay suggests that HMG1 proteins can modulate NF-Y-binding activity, and that the effect is dictated by the sequence flanking the CCAAT box rather than direct NF-Y-HMG1 interaction [24, 25]. Many cell-cycle-dependent genes, including TK, cdc2, cyclin A, cyclin B1, and RNR R2, do not contain TATA box in their promoters [4]. In these cases, it is possible that NF-Y can serve a structural role by recruiting TBP and/or TAFIIs to connect upstream activators with the general polymerase II transcription machinery. A direct interaction of NF-YB and NF-YC with TBP has recently been demonstrated *in vitro* [20].

Earlier studies have shown that NF-Y-binding activity is sensitive to sulfhydryl reagents such as diamide and *N*-ethylmaleimide [12]. An oxidation of either one of the two conserved cysteine residues in NF-YB (aa 85 and 89 in Fig. 1A) would render NF-YB incapable of forming a dimer with the NF-YC subunit [26]. The physiological implication of this finding is that, in addition to interacting proteins, the cellular redox state such as glutathione content or thioredoxin level can modulate the NF-Y-binding activity *in vivo*. The physiological effects of NF-Y *in vivo* can also be influenced by differential expression of NF-YA and alternative splicing of NF-YA pre-mRNA. Thus, human NF-YA can exist either as the long form (347 aa) or the short form (318 aa) due to alternative splicing [18]. Lymphoid and epithelial cells express these two forms differently, which may explain why the promoter activity of the Type 2 CD10/NEP gene behaves differently in these two cell types [27]. In addition, the protein level of NF-YA, but not NF-YB and NF-YC, appears to vary in certain cell types under different physiological states [28–30].

NF-Y AND THE REGULATION OF G1/S GENES

Many G1/S genes contain multiple CCAAT boxes or E2F sites in their promoter region (Table 1). For example, the human TK gene promoter contains two inverted CCAAT boxes with flanking regions matched closely to consensus NF-Y-binding sequences. This feature is shared by other G1/S genes, including the R2 subunit of ribonucleotide reductase (RNR R2), histone H2A, H2B, and H3, PCNA, E2F1, and cdc2. With the exception of cdc2 and E2F1, which also contain E2F sites, the multiple CCAAT boxes arranged in tandem represent the only prominent motif structure in all these genes [31]. In some cases, the CCAAT box and its flanking regions form long repeats, similar to those in the TK promoter. The most striking example is the three almost identical 20-bp repeats in the RNR R2 subunit gene. The involvement of NF-Y in regulating TK and RNR R2 has been confirmed by various criteria [32–34]. A possible involvement of NF-Y in regulating cdc2, cyclin A, H2B, H3, E2F1 genes has also been suggested [4, 9, 35]. Table 1 also lists seven G1/S genes that contain one or more E2F sites (consensus sequence 5′-TTTCGCGC-3′) in their promoters. The E2F site is the key *cis* element that controls the expression of DHFR, TS, DNA polymerase α, E2F1, and possibly cyclin A and cdc2 [31]. The DHFR and TS promoters contain only E2F-binding sites whereas the promoters of DNA polymerase α, histone H1, cyclin A, cdc2, and E2F1 contain both an E2F site and at least one CCAAT box. The CCAAT pentanucleotide in DNA polymerase α, H1, cyclin A, and E2F1 also appears to be a part of a NF-Y consensus sequence. E2F1 is the key transcription factor that recognizes the E2F *cis* element. The promoter region of E2F1 that contains the CCAAT box has a configuration that closely resembles that in the TK promoter [31]. Indeed, NF-Y has been shown to cooperate with E2F factors in mediating the promoter activity of E2F1 genes [9]. Taken together, NF-Y is likely to be involved in the regulation of E2F1 gene expression. If this is the case, NF-Y may have a pivotal role in regulating G1/S genes either directly via NF-Y-binding sequence or indirectly via E2F-binding site.

NF-Y IN CELLULAR SENESCENCE

Normal diploid fibroblasts have a limited doubling potential in tissue culture [36]. Thus, human fibroblasts such as the IMR-90 strain will replicate for about 50–60 times and cease to divide, albeit still viable and metabolically active. The remarkable consistency of the lifespan of these cells in culture, which

appears to be inversely related to the age of the donor, and the species specificity of the lifespan have made them a useful model for studying the biochemistry of cellular aging [reviewed in Ref. 14]. Many of the early and mid-G1 genes are fully inducible by serum in senescent cells, indicating that senescent cells still retain the ability to receive growth stimulatory signals [11–13]. However, a global attenuation of gene expression appears to occur at the late G1/S boundary in senescent cells [11–15]. Thus almost all the genes listed in Table 1 have been shown to be suppressed in senescent cells following serum stimulation. The proteins encoded by these genes are either enzymes such as TK, DHFR, RNR, TS, and PCNA that are necessary for DNA biosynthesis or histones that are necessary for maintaining chromosomal integrity during division. It is therefore conceivable that a suppression of any of these genes could lead to a defect in DNA synthetic machinery and thus renders cells incapable of entering the S phase of the cell cycle.

The age-dependent attenuation of TK and DHFR gene expression has been shown to be controlled at the transcriptional level [12, 37]. Using a TK promoter fragment containing a CCAAT box as a probe, a sequence-specific DNA-binding complex, termed CBP/tk (CCAAT binding protein for tk gene), has been shown to be prominently induced in young cells, but not in senescent or progeria cells [12, 38]. Competition analysis, gel mobility supershift assay, and DNA affinity chromatography suggest that either CBP/tk contains NF-Y as the major component [30, 38] or CBP/tk may be identical to NF-Y [33]. Among three NF-Y subunits, the protein level of NF-YA, but not its mRNA, is strikingly reduced in senescent cells [30]. This decrease in NF-YA levels could explain why senescent cells have little or no CBP/tk-binding activity for TK gene expression (Fig. 2). Differential expression of NF-YA subunits at the protein level, but not at the mRNA level, has also been reported during cell cycling [28] and during monocyte to macrophage differentiation [29]. The E2F-binding activity measured with DHFR promoter fragments correlates with DHFR gene expression in IMR-90 cells during senescence [37]. Within the E2F family, transcription factor E2F1 shows a clear age-dependent expression at both the mRNA and the protein level [37, 39] and appears to be the key transcription factor that is responsible for the down-regulation of DHFR genes in senescent cells (Fig. 2). In view of the likelihood that E2F1 gene expression is controlled by both NF-Y and E2F [9], the decrease in NF-YA in senescent cells could be responsible for the attenuation of E2F1 gene expression.

Although the age-dependent decrease in NF-YA could account for the decrease in the CBP/tk-binding activity in senescent cells, the biochemical behavior of NF-YA, however, does not completely correlate with that of CBP/tk-binding activity in human cells. Thus, serum induces a more than 10-fold increase in CBP/tk-binding activity, but only a two- to threefold increase in NF-YA protein levels (Fig. 2). The half-life of NF-YA appears to be at least five times longer than that of CBP/tk-binding activity in young IMR-90 cells [30]. These discrepancies suggest that the CBP/tk complex may contain factors other than NF-Y. This notion is consistent with recent findings that the NF-Y-binding complex isolated from HeLa cells contains other nuclear proteins [22].

TABLE 1

G1/S Genes That Contain the CCAAT Box or the E2F Site as the Prominent *cis* Elements in Their Promoter Regions

G1/S gene[a]	CCAAT box[b]	E2F site[c]
TK	++	
RNR R2	+++	
PCNA	++	
H2A	+++	
H2B	++++	
H3	++	
cdc2	+++	++
E2F1	+++	++++
DNA pol α	+	++
H1	+	+
cyclin A	+	+
TS		+
DHFR		++

[a] All the human G1/S genes listed here have been shown to be down-regulated in human cells during senescence [31].

[b] Each + symbol indicates one CCAAT box, either sense or antisense orientation, located within 400 bp upstream from ATG.

[c] Each + sign indicates an E2F site located within 400 bp upstream from ATG. The E2F sites in E2F1 and in DHFR promoters are arranged in imperfect palindromes.

CONCLUDING REMARKS

Biochemical and molecular biological studies have shown that suppression of CBP/tk- and E2F-binding activities is responsible, respectively, for the suppression of TK and DHFR gene expression in senescent cells [12, 37]. Since NF-Y either is the major component of CBP/tk or is identical to CBP/tk, the striking attenuation of the DNA-binding component of the NF-Y protein, NF-YA, may explain, at least in part, why the expression of the TK gene is suppressed in senescent cells. It will be of interest to investigate whether the age-dependent expression of other CCAAT box-containing genes listed in Table 1 is also due to the decrease in NF-YA. The expression of the E2F1 gene, the key transcription factor in the E2F family, may also be controlled by NF-Y [9]. It is therefore possible that the attenuation of the NF-YA subunit may account for the age-dependent suppression of G1/S genes containing an E2F site. The cause for the differential expression of NF-YA in human cells during senescence

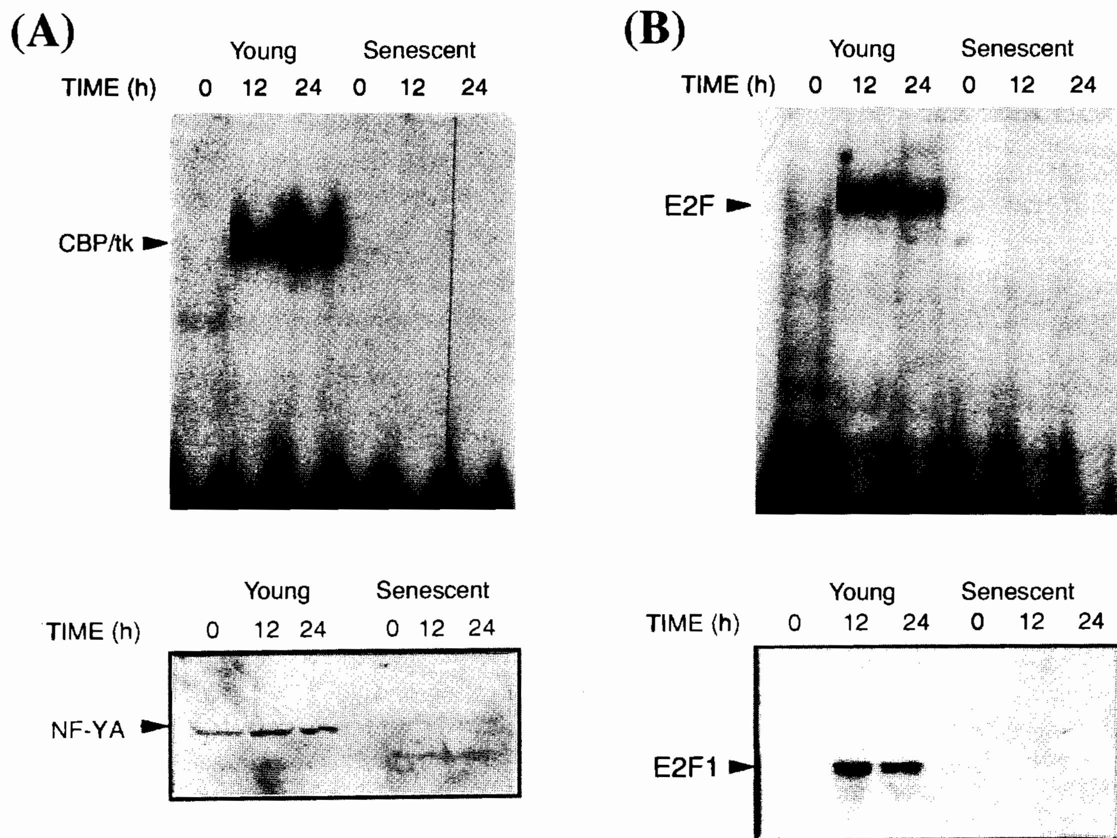

FIG. 2. (A) Comparison of the CBP/tk-binding activity and NF-YA protein level in young and senescent IMR-90 human cells at 0, 12, and 24 h after serum stimulation (adapted from Ref. [12]). (B) Comparison of the E2F-binding activity and E2F1 protein level in young and senescent IMR-90 human cells at 0, 12, and 24 h after serum stimulation (adapted from Ref. [37]).

is unclear. In view of the possible role of NF-Y in regulating many of these age-dependent genes, it is clearly important to understand how each of the NF-Y subunits, particularly the A subunit, is regulated in human diploid cells during senescence.

The work from the authors' laboratory has been supported by United States Public Health Service Grants from the National Institute on Aging, NIH (RO1 AG03578), and the National Cancer Institute, NIH (RO1 CA49695). We apologize to authors whose papers have not been cited due to space limitations.

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Received April 9, 1999
Revised version received July 1, 1999