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Purification and Characterization of OTF-l, A Transcription Factor Regulating Cell Cycle Expression of a Human Histone H2b Gene: Demonstration of its Functional Identity with NF-III, a Factor Required for Efficient Initiation of Adenovirus DNA Replication

Colin F. Fletcher

Purification and Characterization of OTF-1, A Transcription Factor Regulating Cell Cycle Expression of a Human Histone H2b Gene: Demonstration of Its Functional Identity with NF-III, a Factor Required for Efficient Initiation of Adenovirus DNA Replication.

Colin F. Fletcher

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Dum puer alveolo furatur melia cupido furanti digitum cuspidae fixit apis. Sic etiam nobis brevis et pertura voluptus quam petimus tristi mixta dolores nobis.

From L. Cranach the Elder,

Cupid and the Bees

After Theocritus, Idyll 19

ACKNOWLEDGEMENTS

I am grateful to my advisors, Robert G. Roeder and Nathaniel Heintz, for the opportunity to pursue the research described herein, and for their generous support. I am also grateful to all the people, who are too numerous to mention, who have made my stay here both pleasant and productive.

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ABSTRACT

An octamer-binding transcription factor, OTF-1, which stimulates transcription of a human histone H2b gene, has been purified from HeLa nuclear extracts. This purification was achieved through the use of DNA affinity chromatography, and the factor was unambiguously identified by renaturation of activity following SDS-polyacrylamide gel electrophoresis. The purified factor retained the ability to efficiently stimulate H2b transcription in a reconstituted *in vitro* system. This effect was dependent on an intact octamer element and was observed in the absence of other H2b promoter elements (except the TATA motif).

This activity is absent from nuclear extracts prepared from cells synchronized in G2. However, the apparent mass and binding activity of the factor are unchanged in S and G2. Because this factor can stimulate transcription in a G2 extract, we suggest that the modulation of activity is due to either *in vivo* constraints on binding or covalent (or other) modification(s).

We have demonstrated that this factor is identical, by a number of criteria, to NF-III. Therefore, OTF-1 is able to stimulate the initiation of replication of adenovirus DNA. This effect was shown to be dependent on the presence of an intact NF-III binding site in the adenovirus origin of replication.

Finally, preliminary data suggesting that we have isolated a cDNA clone for this factor are presented and discussed.

INTRODUCTION

Perspectives and Rationale

The ability to grow and reproduce is a fundamental property of living organisms. The elucidation of the mechanism(s) which regulates proliferation is one of the most compelling areas of current biological research. Cytological, biochemical, and genetic descriptions of the growth and reproduction of somatic cells have shown that there are three important aspects of cell proliferation which, in fact, constitute two separate cycles (reviewed in Prescott, 1976, and Pardee et al., 1978). Thus, the essential requirements for cell reproduction include 1) growth, i.e. a doubling of the structural and functional components of the cell, 2) replication of DNA and 3) cell division, including the distribution of the replicated chromosomes into the two daughter cells. These events can be grouped into two cycles - a growth cycle and a chromosome cycle - which may be subject to different regulatory control, and are certainly comprised of distinct biochemical events. A number of empirical observations and experimental studies have demonstrated that these two cycles are clearly distinct, yet causally interdependent.

It was essentially the chromosome cycle which was described by Howard and Pelc when they originally suggested that proliferation could be divided into four stages (Howard and Pelc, 1953). These were named G1, or Gap 1, which is the period following division but before the DNA is replicated, S, during which DNA is synthesized, G2, the period following DNA replication during which the cell prepares for mitosis, and D or M, the phase during which the cell physically divides. One of the first suggestions of how this process is regulated was provided by Hertwig (Hertwig, 1908). While his proposal, that attainment of a particular growth state triggers division, has not been supported (Mazia, 1961, Mitchison, 1971), it has been

demonstrated that there is a coupling of overall growth and initiation of DNA replication (Killander and Zetterberg, 1965, 1965a). For instance, it has been demonstrated that variation in length of G1 between two daughter cells can be correlated to uneven partitioning of total mass during M such that the smaller sibling takes longer to enter S phase (Killander and Zetterberg, 1965a, Pringle and Hartwell, 1981). Lack of growth due to either nutritional deficit or drug block will prevent progression through the cell cycle (reviewed in Prescott, 1976). It should be noted that while growth may be required (to some degree) for division, it is also true that the growth cycle can continue in the absence of cytokinesis. This is exemplified by multinucleated cells, for instance the slime mold Physarum (Tyson and Sachsenmaier, 1984), and by experiments in which cells blocked in S phase continue to grow (Fournier and Pardee, 1975).

Interestingly, the period of greatest variation in the cycle occurs during G1; in fact there is very little variation in the length of S, G2 and M in a given cell type under most growth conditions (reviewed in Prescott, 1976, and Pardee et al., 1978). In addition, it is clear that non-proliferating cells are arrested in G1. [The designation G_0 describes quiescent or growth arrested cells (Lajtha, 1963)]. Studies with normal cells under a variety of growth conditions, with defined growth factors, and with temperature sensitive cell cycle mutants (Hartwell, 1971, Marcus et al., 1985) suggest that the cell must complete a linear sequence of biochemical events during G1 to become committed to the initiation of DNA synthesis. These observations have led to the conclusion that this phase is the focal point of regulatory control; as such the cell must pass through a series of "checkpoints" before the commitment to replication is made (initiation of the chromosome cycle). The final point before commitment has been termed the restriction point (Pardee, 1974). Once the cell becomes committed to the initiation of DNA synthesis, it has essentially made a decision to reproduce, as it is extremely

unusual for cells to become arrested in S, G2 or M. In sum, one can conclude that the regulation of cellular reproduction resides principally in control of the growth cycle.

Therefore, investigations of regulation of proliferation commonly focus on one of two transition points - that is, either the entry of quiescent cells into the cycle (G_0->G1) or the progression of continuously cycling cells through G1 (G1->S). It is not clear how the decision of quiescent cells to enter the cell cycle is related to the commitment to divide made by continuously cycling cells (Baserga, 1968). In fact, while cells in G_0 can be shown to be biochemically and kinetically distinct from cells in G1 (Baserga, 1976), there is some evidence that quiescent cells are progressing through the cell cycle, albeit at an extremely slow rate (Dell'Orco et al., 1975, Rubin and Steiner, 1975). It is a matter of debate at this point as to whether regulation of transit through G1 is mediated by reversible entry into G_0 (dependent on biochemical and environmental events) or if it is mediated by a stochastic event. The characterization of the regulator as being a probabilistic event follows from mathematical descriptions of the entry of sister cells into S phase and the deviation of cycling times of individual cells in relation to population behavior (reviewed in Smith and Martin, 1974, and Brooks, 1981, Brooks et al., 1980). The lack of physical evidence for such a mechanism makes such explanations unsatisfying. Moreover, alternate mathematical models which "suggest a biochemical basis for the G1->S transition" have been proposed (Murphy et al., 1984). On the other hand, the search for substances (such as cAMP) which are modulated in a physiologically relevant way, and their characterization as putative regulators, has not proceeded past simple correlation (for example, Abell and Monahan, 1973, Sheppard, 1973). Clearly, the complete biochemical description and dissection of a particular cell cycle regulated event is a prerequisite to the definition of the relevant regulatory circuits.

Therefore, it has been the interest of our laboratory to elucidate the cascade of biochemical events which culminates in the entry of a cell into S phase. Our strategy has been to identify and characterize an end substrate for this cascade, and with that molecule in hand, define the regulatory activities which impinge on it. Given that histone genes are coordinately expressed primarily upon entry into and during S phase (reviewed in Maxson et al., 1983), obvious final substrates are the factors regulating this induction of gene expression.

In 1884 Albrecht Kossel described a class of proteins which could be extracted with dilute acid from goose erythrocytes. He concluded that these were basic proteins which were able to bind to DNA. For reasons that remain obscure to this day, he designated them histones (Kossel, 1884). Subsequently it has been shown that histones are a major component of chromatin, in fact they form complexes with DNA in a mass ratio of 1:1 (reviewed in Elgin and Weintraub, 1975). Initially histones were fractionated into lysine and arginine rich subgroups (Stedman and Stedman, 1950); finer analysis revealed that histones can be separated into 5 subtypes, H1, H2a, H2b, H3 and H4 (reviewed in Isenberg, 1979). The description in the 1970s of the "beads on a string" chromatin structure and the characterization of the nucleosome revealed that DNA is packaged around a multi-subunit complex composed of two molecules each of H2a, H2b, H3 and H4 and one molecule of H1 (Olins and Olins, 1974, reviewed in Elgin and Weintraub, 1975, and McGhee and Felsenfeld, 1980). The ubiquitous presence of histones as fundamental structural components of eukaryotic chromatin is reflected in the extreme conservation of primary sequence of each type across species (reviewed in Isenberg, 1979). In contrast, the gene structure of the histone family has diverged extensively (Kedes, 1979, Hentschel and Birnsteil, 1981, Maxson, 1983). The organization varies from the highly reiterated tandem repeat present in sea urchin to the moderately reiterated repeats of *Drosophila* and *Xenopus* to the dispersed single copy gene pair

arrangement of yeast. The genes in higher eukaryotes are organized in clusters which are not tandemly arrayed. These differences have been attributed to different requirements for expression during development of these various organisms, but this is the subject of much controversy (discussed in Hentschel and Birnsteil, 1981). Other suggestions, such as the idea that coordinate regulation of the histone genes is dependent on the tandem repeat structure, or that conservation of gene sequence by recombination operates through the repeat, have not been borne out. While there are some examples of developmentally regulated expression of repeats (see below), the wide variation in structure and organization of this family makes analysis of any single motif difficult.

This gene family is subject to a number of different levels of regulation. Not only must the various subtypes be coordinately expressed, in the proper ratio to one another, but the proper histone:DNA ratio must be maintained as well (Han et al., 1987). In addition, there are many interesting observations of stage and tissue-specific expression of subtype variants (Isenberg, 1979, Zweidler, 1980, Maxson et al., 1983a, Graves et al., 1985). Examples include the variants (CS, alpha, and gamma) which are encoded in separate clusters in sea urchin and expressed at different times in development. There also exist several tissue-specific variants, notably the erythroid and spermatocyte specific H1's (Weintraub, 1978, Harborne and Allan, 1986, Shires et al., 1975, Lieber et al., 1986). These variants can be correlated with non-transcribed or extremely condensed chromatin (Pehrson and Cole, 1981, Roche et al., 1985). While it is thought to be due to differential transcription, the regulation of these subtype variants is not completely understood. An additional feature of histone gene expression is the fact that cell cycle regulation is acquired during the course of embryogenesis. (Again, there are examples of variants which are expressed continuously during the cell cycle [Wu and Bonner, 1981, Wu et al., 1982, Urban and Zwiedler, 1983, Zwiedler, 1984, Maxson et al.,

1983]). In contrast to our lack of understanding of these other regulatory mechanisms, a great deal has been learned about the cell cycle regulation of histone gene expression.

The original observation (in cultured cells) of histone protein synthesis being restricted to S phase (Robbins and Borun, 1967) was extended by the demonstration that this was due to transcriptional and post-transcriptional effects (Butler and Mueller, 1973, Borun et al., 1975, Plumb et al., 1983). A 3 to 5 fold S phase specific increase in mRNA half-life combined with a 3 to 5 fold induction of transcription results in a 20 to 25 fold increase in steady state mRNA levels (Heintz et al., 1983). Regulation of mRNA half-life appears to be dependent on the unique stem loop structure found at the 3' end of almost all histone genes (reviewed in Marzluff and Pandey, 1988, Krieg and Melton, 1984, Stauber et al., 1986, Levine et al., 1987, Capasso, et al., 1987, note also Wells and Kedes, 1985). It has been shown that this structure is formed most efficiently in S phase cells, and that it is a signal for regulation only while the mRNA is being translated on soluble polysomes (Graves et al., 1987, Lüscher and Schümperli, 1987). It has been previously established that the transcriptional regulation is dynamic (Heintz et al., 1983, Graves and Marzluff, 1984), that it results in a tight coupling of histone transcription and DNA synthesis (Borun et al., 1975, Pederson, 1976, Heintz et al., 1983, Sittman et al., 1983), and that it likely involves *trans*-acting factors which operate by binding to promoter proximal DNA sequences (Heintz and Roeder, 1984, Capasso and Heintz, 1985, Hanly et al., 1985, Artishevsky et al., 1985, Dailey et al., 1986, Sive et al., 1986, Sive and Roeder, 1986, Seiler-Tyuns and Peterson, 1987). Our strategy, then, has been to analyze the various histone promoters in order to identify a *cis*-acting transcriptional regulatory element, and so be able to identify and isolate the cognate *trans*-acting factor. The work I will describe below has focused on a human histone H2b promoter.

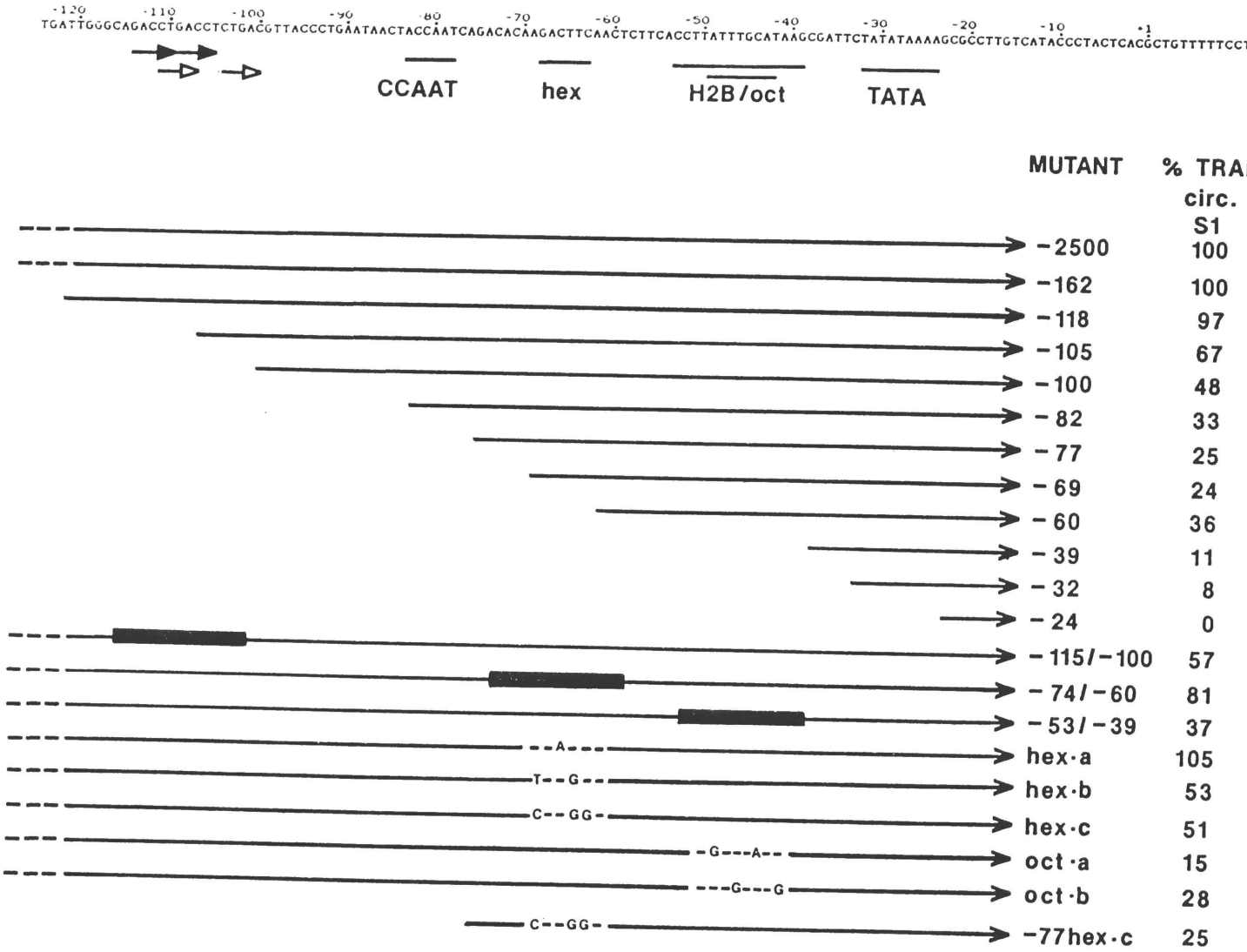
Background

The initial characterization of the human histone H2b gene promoter was carried out by Hazel Sive (Sive et al., 1986). A variety of mutated promoters were constructed, consisting of 5' and 3' deletions, linker insertions and point mutations. Analysis of these constructs by *in vitro* transcription assays allowed the identification of numerous discrete functional promoter elements. These include a series of unique direct repeats centered around -105, a CAAT box located near -80, a hexamer (GACTTC) found in many histone gene promoters located around -65, and a histone H2b specific consensus sequence located at -45, in addition to a TATA motif centered at -30. The sequence of the promoter and a summary of Hazel's results are presented in Figure 1. This analysis demonstrated that, for the core promoter elements, the TATA motif was essential for basal transcription of the promoter while the cap site was dispensable. The direct repeats and the CAAT box each stimulate transcription approximately two fold in the *in vitro* assay. Although the function of the octamer motif is not obvious in the deletion series, a double base substitution mutation in this sequence has a pronounced effect on transcription. In contrast, point mutations in the hexamer had little discernable effect. This analysis did not address the question of regulation of transcription during the cell cycle.

In vivo analysis of the contributions of the histone H2b promoter elements to transcription during the cell cycle was performed by Franca LaBella (LaBella et al., 1988). To assay the specific function of each element, fusion genes were constructed which consisted of a subset of the promoter mutations described above coupled to the bacterial chloramphenicol acetyl transferase gene. The particular constructs used included 5' deletions to -147 (wt), -118 (wt), -100 (DR-),

FIGURE 1. Summary of H2b Promoter Analysis.

Construction and analysis of these mutants was described in Sive et al., 1986. Each mutant was analysed in at least three different transcription assays. Signals were quantitated densitometrically, and the results are presented as % of wild type transcription. Endpoints of 5' deletions, substituted sequences and nucleotide alterations are indicated. Not presented are the results of deletions from the 3' end, which indicated that sequences downstream of -19 were dispensible for *in vitro* transcription.


-120 -110 -100 -90 -80 -70 -60 -50 -40 -30 -20 -10 +1
TGATTGGGCAGACCTGACCTCTGACGTTACCCTGAATAACTACCAATCAGACACAAGACTTCAACTCTTCACCTTATTTGCATAAGCGATTCTATATAAAAGCGCCTTGTCATACCCTACTCACGCTGTTTTTCCT
CCAAT
hex
H2B/oct
TATA
MUTANT
% TRANSCRIPTION
circ.
linear
S1
S1
runoff
-2500 100 100 100
-162 100 100 100
-118 97 nd 96
-105 67 nd 81
-100 48 79 73
-82 33 nd 56
-77 25 37 48
-69 24 nd 38
-60 36 50 69
-39 11 22 22
-32 8 nd 16
-24 0 0 0
-115/-100 57 59 64
-74/-60 81 112 102
-53/-39 37 38 34
hex·a 105 117 nd
hex·b 53 49 nd
hex·c 51 45 nd
oct·a 15 9 nd
oct·b 28 24 nd
-77hex·c 25 30 nd

-82 (DR-), and -77 (CAAT-), and point mutants in the hexamer (Hex-) and octamer (Oct-). Because an identical, unregulated mRNA was transcribed from every construct, it can be assumed that the effect of each promoter mutation on transcription efficiency is reflected in the accumulation of mRNA. These constructs were transfected into 293 or Hela cells, which were then synchronized at the G1/S boundary by a single block with aphidicolin. Total RNA was prepared from the transfected cells either during the block or three hours after release, and analysed by an S1 nuclease protection assay. Co-transfection of an unregulated fusion gene provided an internal control of transfection efficiency, and thus allowed comparison of the promoter mutations. A representative experiment is depicted in Figure 2. In this experiment, total RNA was hybridized to a labelled DNA fragment spanning the fusion gene from -147 to +290, and then digested with S1 nuclease. Hybridization of the probe to mRNA derived from the H2b fusion gene protects a DNA fragment of 290 nucleotides, while hybridization of the probe to mRNA from the internal control fusion gene protects a fragment of 250 nucleotides. Therefore, the upper band in the gel corresponds to RNA from the H2b fusion gene, and the lower band corresponds to RNA from the internal control gene. As is evident from this analysis, deletion of the upstream elements reduces transcription equally at the G1/S boundary and in S phase. However, while the level of transcription is decreased, there is still an induction of 4-5 fold upon entry into S phase. In contrast, a double point mutation in the octamer does not affect transcription at the G1/S boundary, but it does prevent the induction of transcription upon entry into S phase. The quantitation of several transfection experiments is presented in Table 1. These data demonstrate that the subtype-specific sequence is necessary and sufficient to mediate the induction of transcription of the H2b gene upon entry into S phase. These results were in agreement with my observation of a differential activity of the

FIGURE 2. Expression of H2b Promoter Mutants in Synchronized 293 Cells.

Cells were co-transfected with H2b/CAT and pSV2/CAT and synchronized as described in the text. Total RNA was extracted ~40 hours after transfection, and 5 ug were S1 mapped using the probe described in the text. Names of the transfected mutants are indicated over each pair of lanes. (0,3) are the hours after release into S phase when the RNA was extracted. The large and small arrows indicate the H2b/CAT and the pSV2/CAT protected bands, respectively.

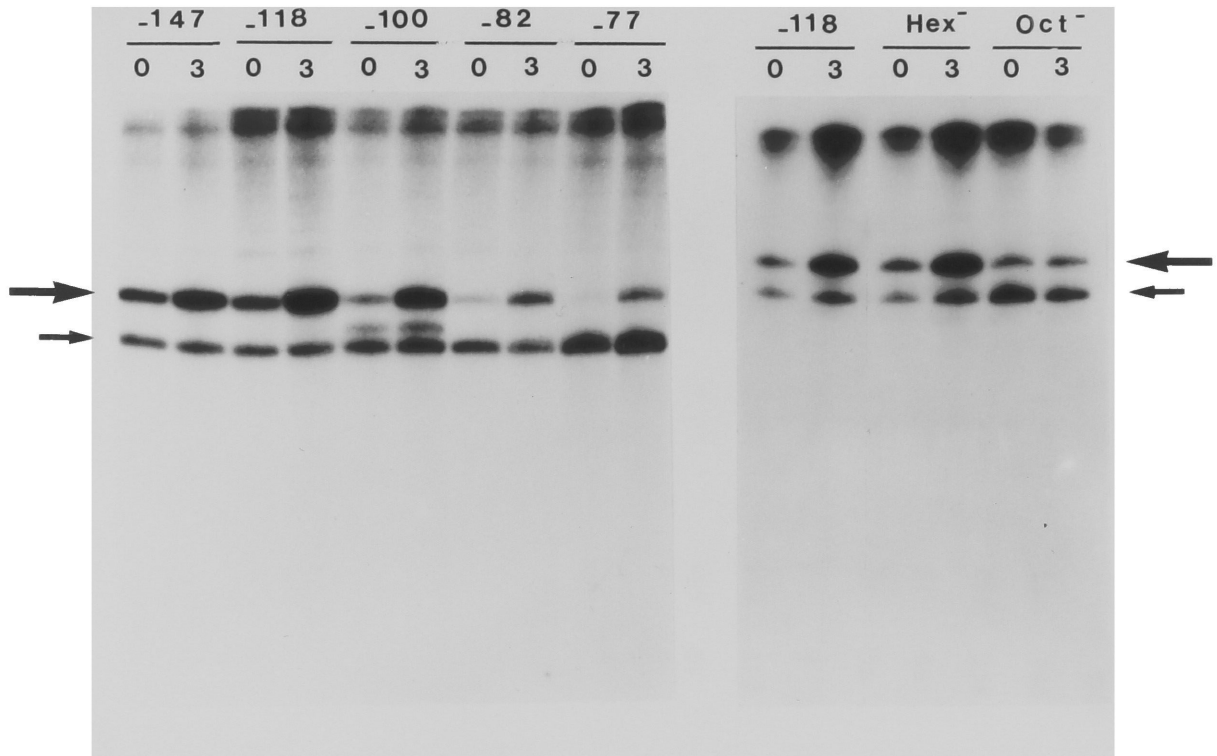

-147
-118
-100
-82
-77
0 3 0 3 0 3 0 3 0 3
-118
Hex⁻
Oct⁻
0 3 0 3 0 3

TABLE 1

IN VIVO TRANSCRIPTION OF H2B PROMOTER MUTANTS

MUTANT	T=0	T=3	T=3/T=0	%IN VIVO T=0	%IN VIVO T=3	%IN VITRO
-147 (4)	0.95	3.71	3.90	100	100	100
-118 (3)	1.20	5.13	4.28	126	138	97
-100 (3)	0.55	2.47	4.50	57	66	48
-82 (3)	0.18	0.99	5.50	19	26	33
-77 (3)	0.05	0.21	4.16	5	6	25
HEX- (2)	1.15	4.16	3.60	118	112	51
OCT- (5)	1.21	1.17	0.97	127	31	15

octamer element as tested by *in vitro* transcription in extracts prepared from cells synchronized either in S or G2 (described below), and support the idea that the cognate transcription factor would regulate the transcription of the H2b gene through this element.

RESULTS

CHAPTER 1

Introduction

Analysis of the DNA sequences controlling RNA polymerase II transcription in higher eukaryotes has revealed the complexity of enhancers and promoters (reviewed by Dynan and Tjian, 1985, Serfling et al., 1985). In many cases, these promoters and enhancers have been shown to be composed of several discrete, independently active DNA sequence elements. Although our knowledge of DNA sequences controlling transcription initiation is quite extensive, our understanding of the nature and mechanism of action of the factors which operate through these sequences is rather rudimentary. *In vitro* transcription studies have demonstrated that accurate initiation by RNA polymerase II requires a number of soluble factors that are likely to be common to most cell types and that are required by all class II genes analyzed (reviewed in Sawadago and Roeder, 1985, Reinberg et al., 1987). One of these, the TATA recognition factor TFIID, binds directly to a proximal promoter element (Parker and Topol, 1984, Sawadago and Roeder, 1985a, Nakajima, Horikoshi and Roeder, unpublished data), while the others function at later steps in initiation (Samuels et al., 1982, Hawley and Roeder 1986, Reinberg et al., 1987). In addition, there have been identified a variety of factors which bind to distal promoter or enhancer elements. Only in a few cases have the transcriptional activities of these factors been demonstrated directly in cell free systems (Parker and Topol, 1984a, Gidoni et al., 1985, Sawadago and Roeder, 1985a, Carthew et al., 1985, Moncollin et al., 1986, Jones et al., 1987, Bohman et al., 1987). Of the known transcription factors, it is clear that some are important for the expression of a large number of genes (Sp1, CTF), while others appear to operate on a rather limited set of specific, and often related, genes (HSTF, USF/MLTF). It is also evident that a

subset of these transcription factors mediate the induction or repression of specific genes in response to specific physiological cues. In no case do we understand the process which results in a change of activity of such a transcriptional regulatory protein. Similarly, we do not understand the mechanism(s) by which regulatory proteins alter the rate or extent of initiation by RNA polymerase II, although a cooperative interaction between USF and TFIID has been observed (Sawadago and Roeder, 1985a). The ability to reproduce transcriptional regulation *in vitro* offers the opportunity to examine both of these facets of regulated transcription.

Our interest has been to elucidate the mechanisms regulating histone gene transcription during the cell cycle. Our initial study in this area demonstrated that the *in vivo* transcriptional regulation of a human histone H4 gene could be reproduced *in vitro* using nuclear extracts from synchronized populations of HeLa cells (Heintz and Roeder, 1984). Subsequent studies of both the H4 and H2b genes demonstrated that the maximal level of transcription *in vitro* was dependent upon several promoter proximal DNA sequences, which function through distinct transcription factors (Hanly et al., 1985, Sive et al., 1986, Sive and Roeder, 1986, Dailey et al., 1986). Although the regulatory element for histone H4 has not been identified, we have demonstrated definitively that the H2b subtype-specific consensus sequence (Harvey et al., 1982, Perry et al., 1985) is necessary and sufficient for the induction of transcription during the transition from G1 to S phase (LaBella et al., 1988).

In this study, we describe the purification of a 90 kDa protein that binds specifically to the H2b consensus element. This purification was achieved through the use of DNA affinity chromatography and the factor was unambiguously identified by renaturation of activity following sodium dodecyl sulfate polyacryl-amide gel electrophoresis (SDS-PAGE). The purified factor retained the ability to efficiently stimulate H2b transcription in a reconstituted *in vitro* system. This effect

was dependent upon an intact octamer element and could be observed in the absence of the other H2b promoter elements (except the TATA motif). Furthermore this activity was not detected in nuclear extracts prepared from cells synchronized in G2. From these data we conclude that we have purified the *bona fide* H2b transcriptional regulatory factor.

Transcription of H2b Promoter Mutants in Crude Extracts From Cells Synchronized in S or G2.

My initial experiments, referred to above, were aimed at determining, by *in vitro* analysis, the activity of the various elements of the H2b promoter at different stages in the cell cycle. We reasoned that if the elevated S phase transcription resulted from the activation of a single transcription factor, the corresponding binding site would display a mutant phenotype only in extracts prepared from S phase cells. Furthermore, comparison of the relative levels of transcription from the various promoter mutants in each extract would not require matched extracts. Our strategy for preparing the extracts was to block cells in sequential S phases and then, following release from the second block, prepare extracts at a point in mid-S and at a point in G2. Transit of the cells through S was monitored by thymidine incorporation. Because the mutant phenotypes were more pronounced with supercoiled templates (Sive et al., 1986, Hanly et al., 1985), and in order to simplify template preparation, the selected promoter mutants were fused to a G-minus cassette (Sawadago and Roeder, 1985). Transcription through the G-minus cassette generates discrete transcripts from a circular template when used in conjunction with 3' O-methyl GTP and RNase T1. These constructs are detailed in Figure 3. The results of a representative *in vitro* transcription experiment are shown

FIGURE 3. Construction of Promoter Mutant/Cassette Fusions.

The parental wild-type promoter fragment was derived from the previously described 3' deletion M13 clone MP10-16.3 (Sive et al., 1986), which contained sequences from -178 to -19. Ligation of a Hind III linker to the 5' end of the G minus cassette (Sawadago and Roeder, 1985) allowed these two fragments to be cloned into pUC 12 as diagrammed. Other promoter fragments were derived from 5' deletions or point mutants of the H2b promoter described by Sive et al., 1986. These included a deletion of the promoter elements upstream of -59 and a double point mutant of the octamer sequence. A deletion of the entire region upstream of -36 was constructed using the Hinf I site centered at -35.

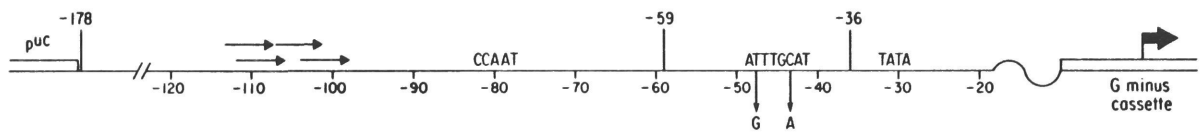
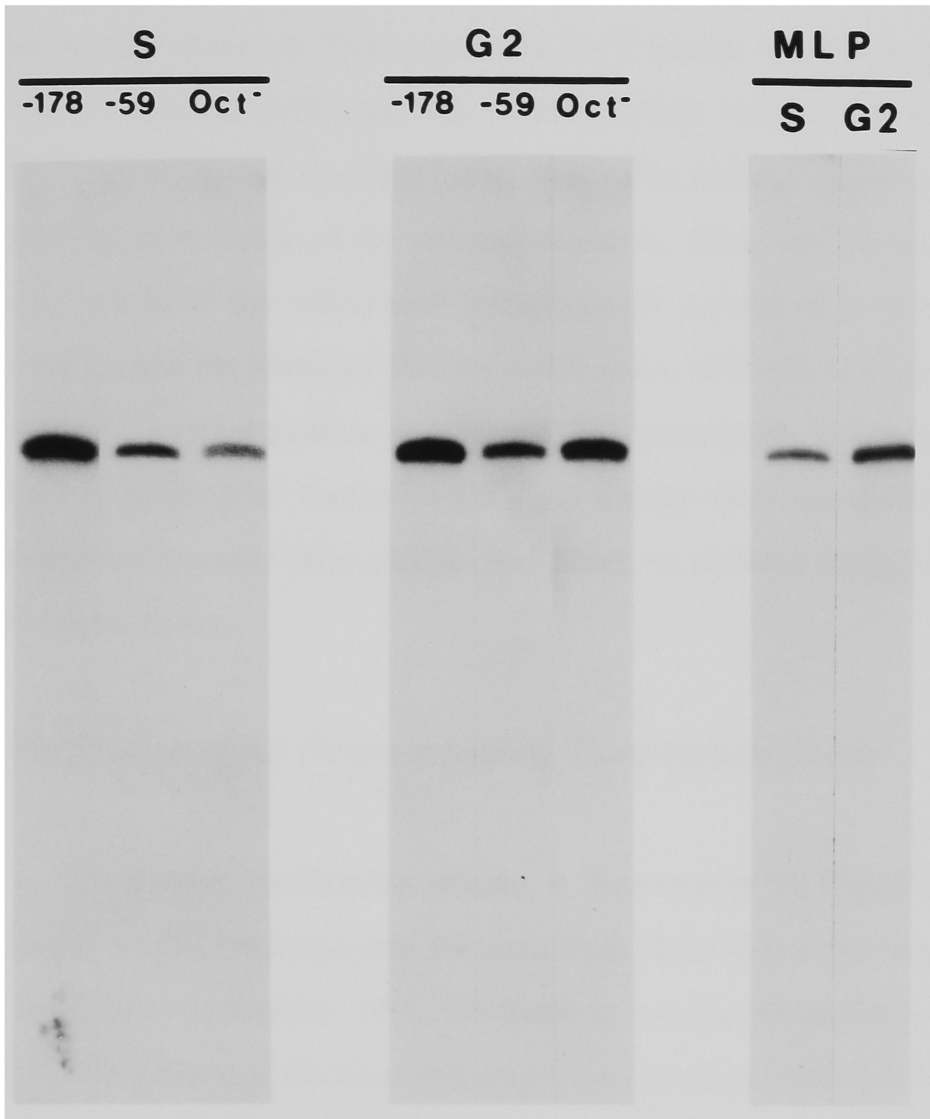

-178
pUC
-59
-36
CCAAT
ATTTGCAT
TATA
-120
-110
-100
-90
-80
-70
-60
-50
-40
-30
-20
G
A
G minus
cassette

FIGURE 4. In vitro Transcription With Extracts From Cells Synchronized in Either S-phase or G2.

Cell synchronization, extract preparation, and *in vitro* transcription was carried out as described in Experimental Procedures. In this case 120 ug (S) and 90 ug (G2) of protein were used, giving equivalent activity on the wild type template. The templates used are indicated over each lane. The constructs are detailed in the previous figure. As is apparent, there is a pronounced difference in the relative transcription of the Oct^- template in the two extracts when compared to the other two templates.

When normalized relative to the control adenovirus major late promoter lacking upstream regulatory elements (Sawadago and Roeder, 1985a)(shown in right-most lanes), the H2b gene was transcribed several fold more efficiently in the S extract than the G2 extract.


S
-178
-59
Oct-
G2
-178
-59
Oct-
MLP
S
G2

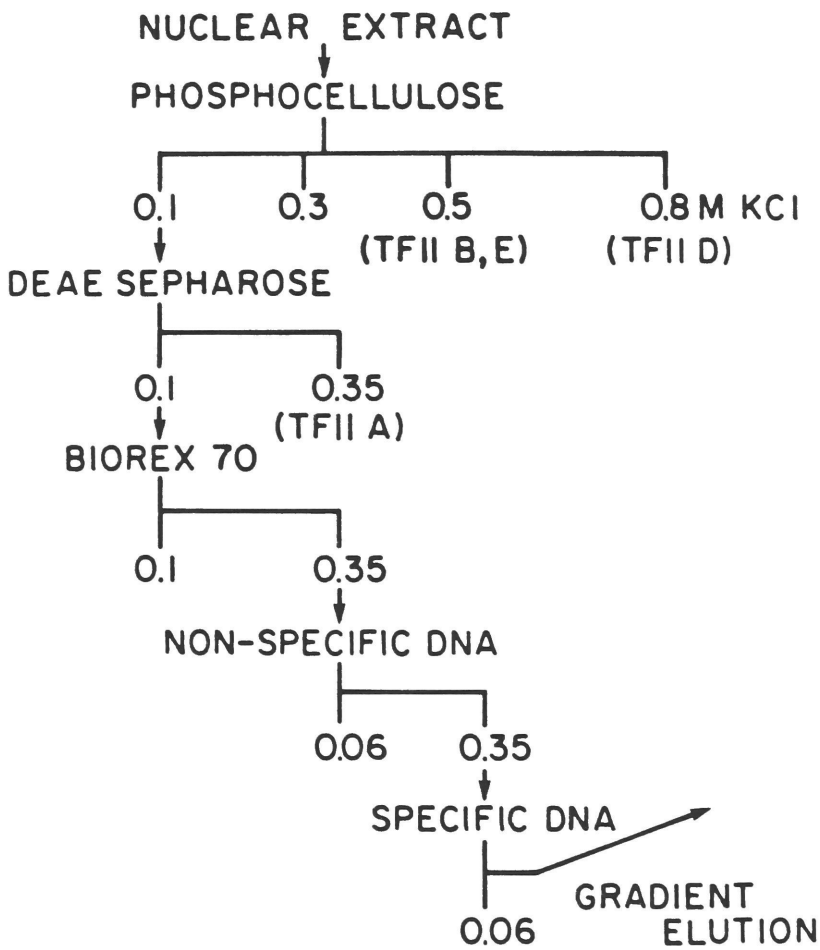
in Figure 4. The templates were the wild type (WT) which includes promoter sequences to -178, a 5' deletion mutant (-59) which removes all the promoter elements except the H2b subtype-specific sequence and the TATA box, and a double point mutant (Oct^-) which inactivates the core octamer contained within the H2b subtype specific sequence. Transcription in the S extract was clearly reduced for both the 5' deletion (-59) and the octamer point mutants. In contrast, transcription in the G2 extract was significantly reduced by deletion of the upstream elements, but not by mutation of the octamer sequence. These results refine our previous observation of the differential transcriptional activity of S and non-S extracts on the histone H4 promoter (Heintz and Roeder, 1984, Hanly et al., 1985) and demonstrate *in vitro* regulation of histone H2b transcription. Encouraged by these results, and by our prior demonstration of an activity which specifically binds to the H2b octamer sequence (Sive and Roeder, 1986), we initiated the purification of this transcription factor.

Purification of the Octamer-binding Transcription Factor

The current purification scheme is diagrammed in Figure 5. The starting material for this procedure was prepared from HeLa cells according to the procedure of Heintz and Roeder, 1984. We chose to use phosphocellulose (P-11) and DEAE CL-6B columns in the beginning stages for a variety of reasons. First, the higher salt steps (0.5 and 0.8 M KCl) of the P-11 column provide the general factors (TFII B, E and D)(Matsui et al., 1980, Samuels et al., 1982, Sawadago and Roeder, 1985) required for *in vitro* transcription reconstitution. Second, the DEAE column both removes contaminating nucleic acids and nucleotides and separates the octamer-binding transcription factor (OTF-1) from the general transcription factor TFIIA (ibid.). At this stage the binding activity needed only to be concentrated

FIGURE 5. Purification Scheme for the Octamer Binding Transcription Factor.

Extract preparation was performed essentially as described by Heintz and Roeder, 1984. The initial two columns were loaded in tandem and then separated and eluted as indicated. As noted, the higher salt steps of the phosphocellulose column contained the general transcription factors used in the reconstitution assay. The Biorex column was loaded at pH 7 and eluted as indicated. The DNA columns were loaded at 60 mM KCl, pH 7.9.


NUCLEAR EXTRACT
PHOSPHOCELLULOSE
0.1
0.3
0.5
(TFII B,E)
0.8 M KCl
(TFII D)
DEAE SEPHAROSE
0.1
0.35
(TFII A)
BIOREX 70
0.1
0.35
NON-SPECIFIC DNA
0.06
0.35
SPECIFIC DNA
GRADIENT
ELUTION
0.06

before the DNA affinity steps. Our initial attempts at purification were hampered by the rather variable binding of this factor to any type of ion exchange column. We found, however, that after dialysis against buffer at pH 7 the material bound well to a Biorex 70 column equilibrated at pH 7. This column was then step-eluted at 350 mM KCl and the resulting material dialyzed to 60 mM KCl and pH 7.9.

The strategy we have used in the DNA affinity chromatography exploits the differential chromatographic behavior of the binding factor on a non-specific DNA matrix versus on a DNA matrix containing the octamer sequence (Carl Wu, personal communication, Rosenfeld and Kelly, 1986). The octamer-binding factor was first bound to a non-specific DNA column (sonicated salmon sperm DNA) at low salt (60 mM KCl) and then step-eluted with 350 mM KCl. After dialysis to 60 mM KCl, the eluate was then applied to a specific oligonucleotide DNA affinity column. This matrix was prepared by coupling a 22 bp synthetic oligonucleotide (see Methods) to CnBr activated Sepharose CL-2B (Pharmacia). This column was then washed with 2 volumes of input buffer and eluted with a gradient of 70 to 700 mM KCl. The yield and fold purification of the binding material is summarized in Table 2.

Identification of the Octamer-binding Transcription Factor (OTF-1) as a 90 kDa Protein

By eluting the oligonucleotide affinity column as described, we were able to assay across the gradient and to identify candidate polypeptides as those which co-eluted with the functional activity. Figure 6 shows such an analysis. Panel A shows a DNase protection assay, which indicated the presence of the octamer-binding activity in fractions 44-50. Panel B shows a transcription reconstitution assay

TABLE 2

	Volume (mls)	Protein (mgs)	Activity	Specific Activity	Yield step	Yield total	Purification step	Purification total
Nuclear Extract	100	1,000	100 U*	0.100				
Phosphocellulose/ DEAE Flowthrough	120	360	30	0.083	.30	.30	.83	.83
Biorex 70 0.35 M KCl	12	35	19.8	0.56	.66	.20	6.75	5.6
DS DNA 0.35 M KCl	1.4	0.84	7.52	8.95	.38	.076	16	89.5
Oligonucleotide Affinity	0.4	0.0065	3	461	.40	.03	52	4,610

* One unit of activity is defined as that amount of protein which is sufficient to bind 22 pmoles of probe under standard gel shift conditions (see Methods).

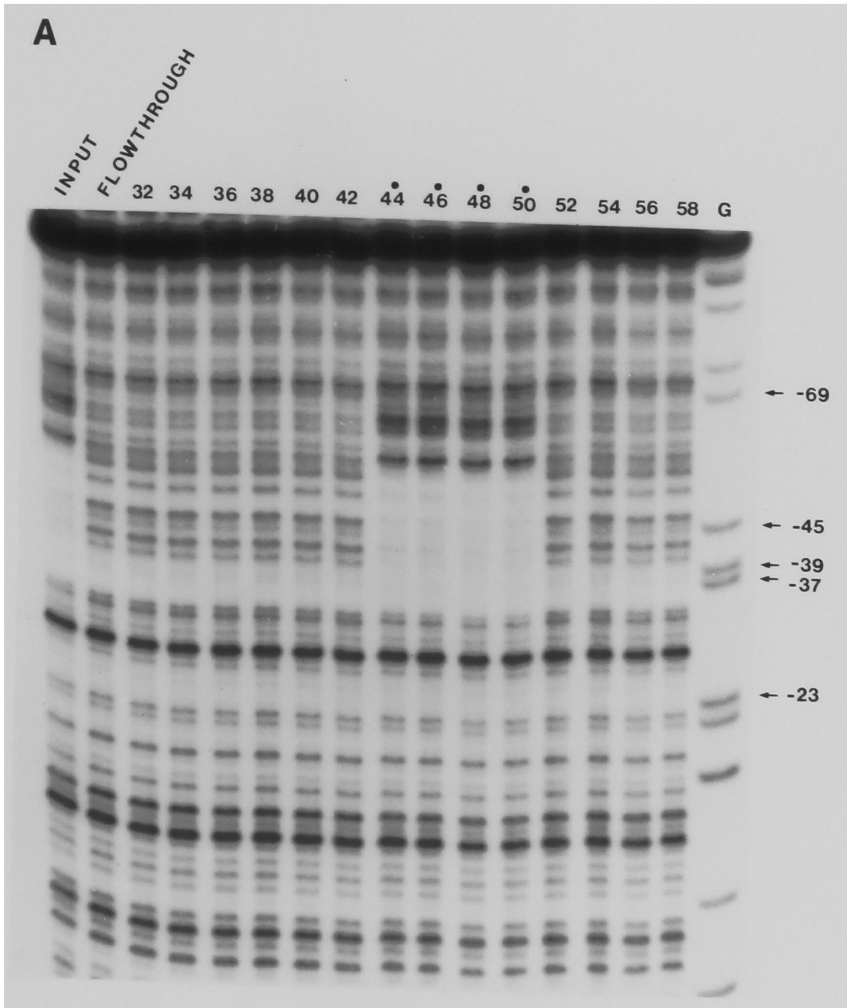
FIGURE 6. Analysis of Chromatographic Fractions From the Specific Oligonucleotide Affinity Column.

Material eluted from the non-specific DNA column was applied to the specific DNA affinity column which had been constructed by coupling an oligonucleotide which contained the H2b octamer sequence to CnBr-Sepharose 4B. The column was then washed and eluted with a linear gradient of KCl from 70 to 700 mM. Analysis of the eluted fractions is described below.

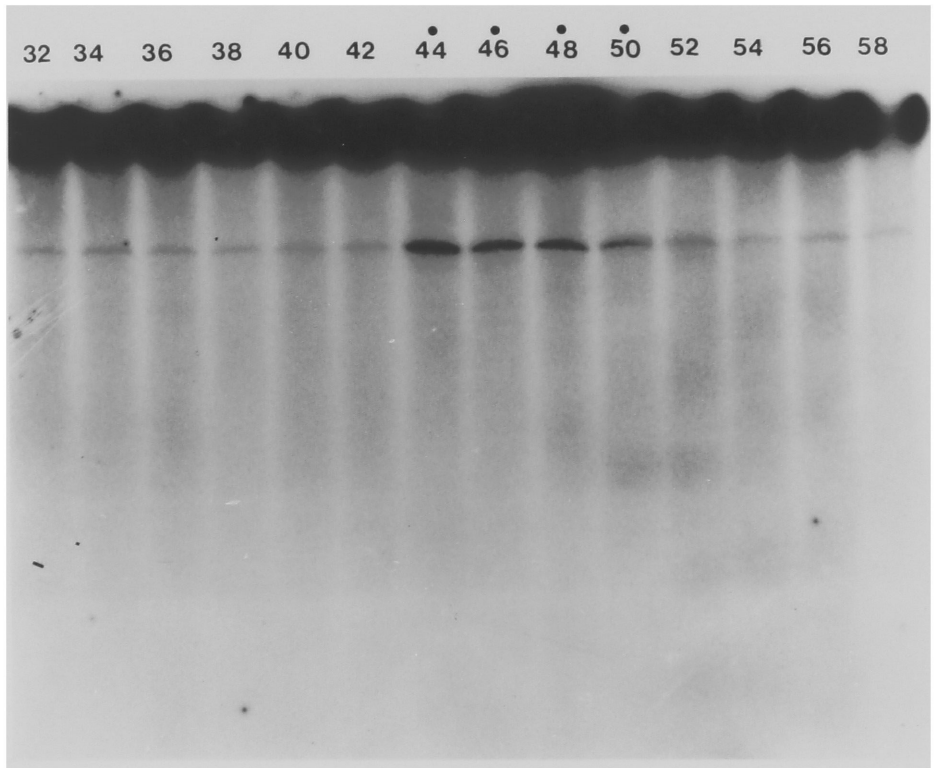
A) DNase I Protection Assay: One ng of a single end labeled probe spanning the promoter from -110 to +28 was incubated with 1 ul of the indicated fractions in buffer containing 20 mM Hepes (7.9), 2 mM $MgCl_2$, and 5 ug/ml salmon sperm DNA. Fraction numbering began with loading, and the flowthrough lane represents a pool of fractions 6 through 20. Fractions 32 through 58 span the gradient from 70 to 600 mM. A Maxam-Gilbert G reaction was run in the rightmost lane. The positions of relevant G residues relative to the cap site are indicated. The region of protection extends from -35 to -55.

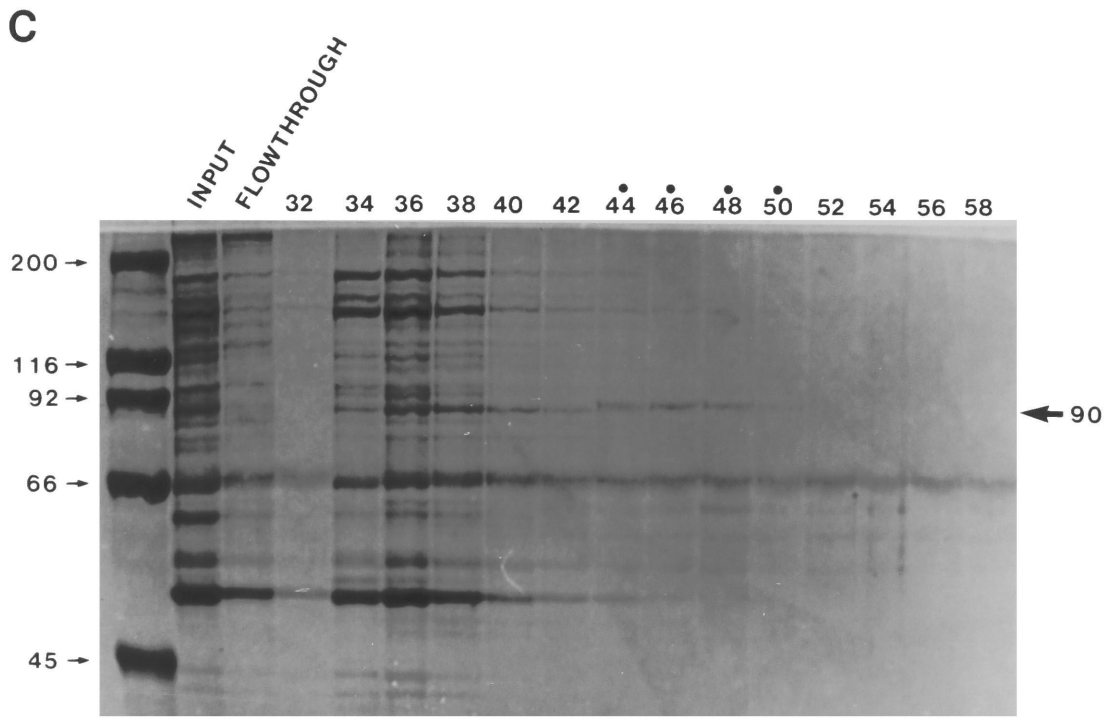
B) In vitro Transcription Assay: The reactions were carried out as described in Methods, with the phosphocellulose 0.5 M KCl and 0.8 M KCl fractions being used instead of nuclear extract. In addition, 1 ul of each of the indicated fractions was added. The template used was the -59 construct.

C) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of the Chromatographic Fractions: Five ul of each of the indicated fractions were loaded on a 7% SDS-polyacrylamide gel, after electrophoresis the gel was silver stained. The appearance of a 90 kDa band in fractions 44 to 50 is noted by an arrow. The sizes of the molecular weight markers are indicated. The artifactual band at 66 kDa is routinely observed with this silver staining protocol.


A
INPUT
FLOWTHROUGH
32
34
36
38
40
42
44
46
48
50
52
54
56
58
G
-69
-45
-39
-37
-23

B



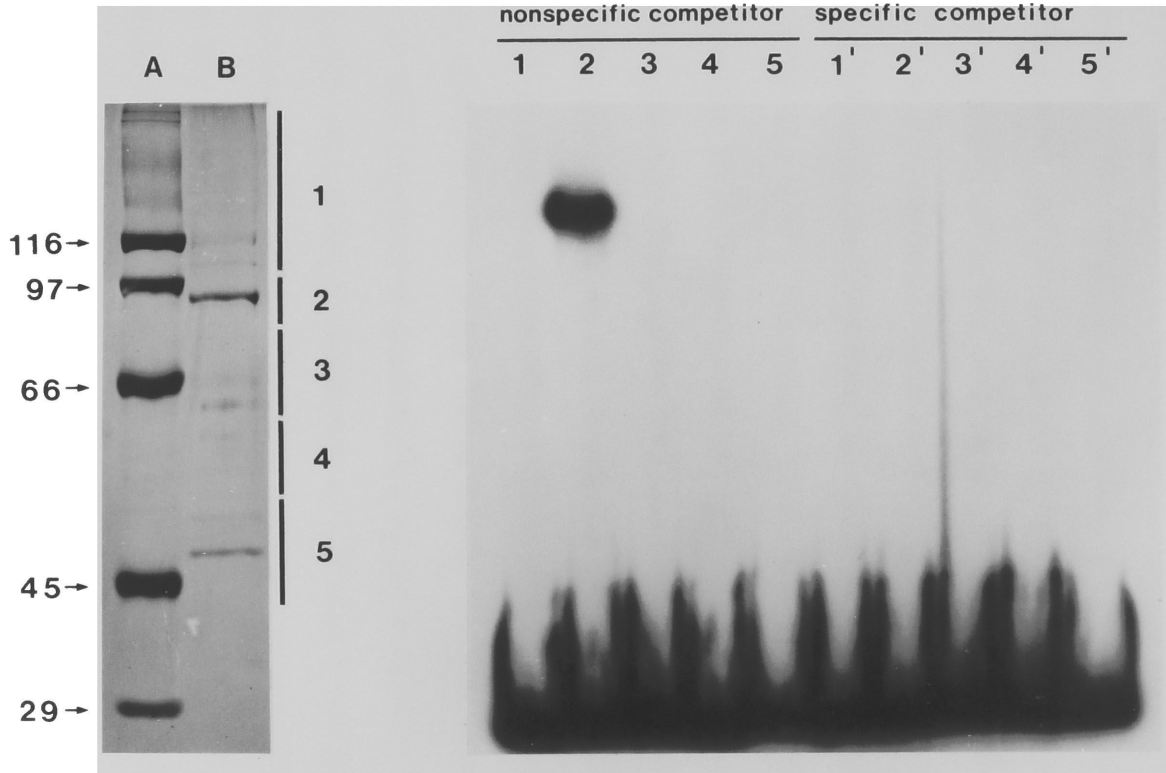

C
INPUT
FLOWTHROUGH
32
34
36
38
40
42
44
46
48
50
52
54
56
58
200
116
92
66
45
90

which indicated that the stimulatory activity was also present in fractions 44-50. Panel C shows a silver stained SDS gel in which proteins in the same fractions were analyzed. Inspection of this gel revealed a 90 kDa band which appeared to co-elute with both the DNA binding and the transcription stimulatory activities. It is also apparent that the other bands in these fractions resulted from trailing of the proteins from preceeding fractions and not from a strict co-elution with the binding and functional activities (see also figure legend). Furthermore, quantitation of the DNA footprinting experiment indicated that the 90 kDa band was the only protein present in a quantity sufficient to account for stochiometric binding to the H2b probe.

In order to identify unambiguously the 90 kDa protein as the relevant polypeptide we attempted to renature it after SDS-PAGE (Hager and Burgess, 1980). In the experiment shown in Figure 7, DNA affinity purified material was electrophoresed through a 7% polyacrylamide gel along side of two reference lanes. Lanes A and B of Figure 7 reflect, respectively, analysis of molecular weight markers and an aliquot of the purified material. After electrophoresis the gel was cut into five sections, the boundaries of which are marked next to Lane B. Proteins were eluted by diffusion from these crushed gel slices and precipitated with acetone. After the pellets were resuspended and denatured in 8 M GuHCl the protein was allowed to renature upon 50 fold dilution of the GuHCl. The samples were then analyzed by a standard gel retention assay (Fried and Crothers, 1981, Garner and Revzin, 1981), also shown in Figure 7. As is evident, the only binding activity recovered was contained in the 85-95 kDa region (slice 2). This renatured protein was further judged to be the *bona fide* factor on the basis of the following. It generated a complex which had a relative mobility equal to that generated by the input material (data not shown) and which could be specifically competed by an oligonucleotide containing the octamer binding site (lanes 1'-5').

FIGURE 7. Renaturation of Binding Activity After SDS PAGE.

Lane A contained a mixture of molecular weight markers, lane B contained 5 ul of the pooled affinity purified material (fractions 44 to 50, see previous figure). Approximately 150 ng of purified OTF-1 was loaded in the sample lane. The regions of the sample lane that were excised are indicated (1-5) next to lane B. After renaturation the material was used in a gel shift assay containing 3 ng of end labelled probe (-59 to -10), 100 ng of salmon sperm DNA and 100 ng of either a nonspecific oligonucleotide (lanes 1-5) or a oligonucleotide of sequence from -58 to -38 (lanes 1'-5')(see Methods).


A
B
116
97
66
45
29
1
2
3
4
5
nonspecific competitor
specific competitor
1
2
3
4
5
1'
2'
3'
4'
5'

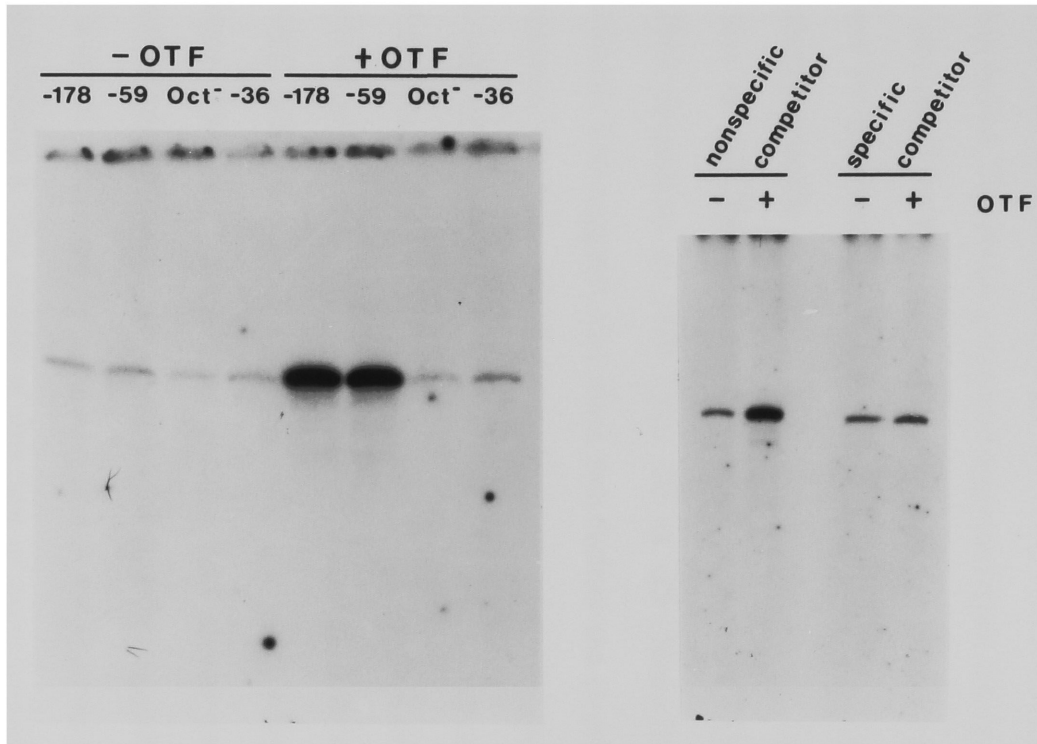
Transcription Activity of the Affinity Purified Octamer-binding Factor

To delineate the promoter sequence and DNA binding requirements for transcription stimulation, we have used the final affinity purified preparation in transcription reconstitution experiments. These experiments employed the templates described above as well as a fourth template (-36) in which the entire region upstream of the TATA box was deleted. Transcriptional activity was monitored in an *in vitro* reconstituted system consisting of the phosphocellulose 0.5 M KCl and 0.8 M KCl fractions (containing the basic transcription factors), FPLC purified ATP, CTP and UTP, 3'O-Methyl GTP, RNase T1 and α-^{32}P CTP (Sawadago and Roeder, 1985). Transcription through the G-minus cassette gave rise to a discrete 370 nucleotide RNA species that was visualized by autoradiography following electrophoresis through an 8% denaturing polyacrylamide gel. Figure 8 shows transcription of these templates either without (lanes 1-4) or with (lanes 5-8) addition of the octamer-binding factor. These results indicate that the octamer-binding factor was able to stimulate transcription equally well from either the wild type or -59 template. In contrast, it was unable to stimulate transcription from the template containing a double point mutation in the core octamer sequence (Oct$^-$), nor did it have any effect on transcription mediated solely by the TATA element (-36). Furthermore, we observed no stimulation of either the histone H4 or adenovirus major late promoters in similar complementation assays (data not shown).

Also shown in this figure (right panel) is an oligonucleotide competition experiment which indicates that the DNA binding activity was required for transcription stimulation. Thus, the data show that addition of a twenty fold molar excess of an oligonucleotide containing an octamer binding site completely

FIGURE 8. In vitro Transcription With the Affinity Purified Material.

Transcriptions were performed essentially as described in Figure 6, except that 3 ng of the pooled affinity purified material was added to all reactions labelled +OTF. The templates used are indicated. In the competition assays a 40 fold molar excess of either a nonspecific or specific oligonucleotide (see Methods) was added to the reaction cocktail with OTF approximately 5 minutes before addition of the phosphocellulose fractions and subsequent incubation at 30^{o} C.


– OTF
+ OTF
-178 -59 Oct⁻ -36 -178 -59 Oct⁻ -36
nonspecific competitor
specific competitor
– + – +
OTF

inhibited the stimulatory effect of the added factor, whereas addition of a non-specific oligonucleotide had no effect.

Determination of the Specific Binding Site of OTF-1

Figure 9 shows the results of a footprinting experiment which was performed in order to delineate more precisely the binding site of the purified transcription factor. The factor protected approximately 20 bp of DNA on the upper and lower strands of the H2b promoter, although the protected regions were slightly offset. It can be observed that the overlapping protected region coincides with the defined subtype specific sequence. It is interesting to note that this binding site overlaps that of the TATA binding general transcription factor TFIID, which protects DNA sequences on H2b from -38 to -20 (Masami Horikoshi, personal communication). This suggests that these two factors are extremely closely apposed on the promoter. We have noted the appearance of a minor DNase hypersensitive site at the edge of each footprint with arrows. In order to map the sequence which comprised the actual close contact points of OTF-1 we performed this assay with a different cleavage reagent, methiumpropyl-EDTA·Fe(II) (MPE) (Hertzberg and Dervan, 1982). MPE is a small synthetic molecule which intercalates in the DNA minor groove through its methidium residue and cleaves DNA through a free radical mechanism. This is an extremely useful cleavage agent as it has high affinity for DNA, it is not influenced by sequence, and is not sterically hindered (by bound protein) as is DNase I. The results of this type of assay are shown in Figure 10. In this experiment a single-end labelled fragment was cleaved in either the absence or presence of affinity purified OTF-1. The labelled strand corresponds to the lower strand of the promoter. This experiment maps the close contact points on the lower strand to the ATAAACGTA bases of the H2b consensus sequence.

FIGURE 9. DNase I Protection Assay.

Footprinting reactions were performed as described in Figure 6. Either the upper or lower strands of the 138 bp probe were uniquely end labeled with the Klenow fragment of *E. coli* DNA polymerase. Samples were electrophoresed in tall 8% polyacrylamide/50% urea gels and Maxam-Gilbert G reactions were run in adjacent lanes. The regions of protection are indicated, and the relevant region of the promoter is displayed. Minor, but consistently observed, hypersensitive sites are indicated with arrows.

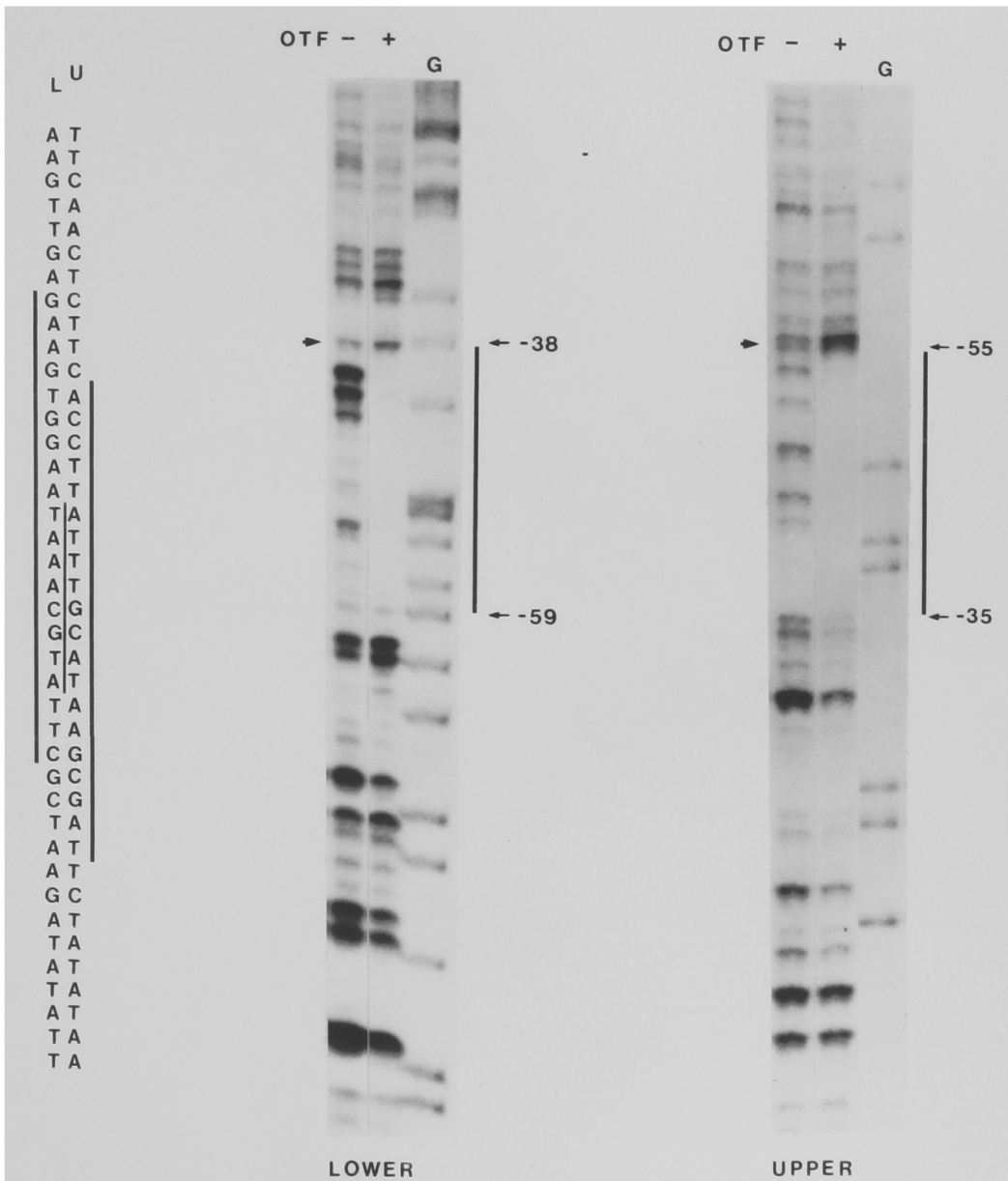
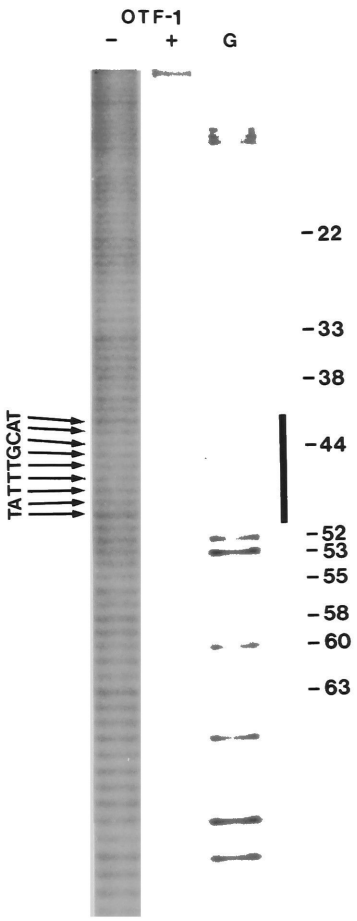

OTF – +
G
L U
-38
-59
OTF – +
G
-55
-35
LOWER
UPPER

FIGURE 10. MPE Protection Assay.

The lower strand of the 138 bp probe was uniquely end labelled and manipulated as described for the DNase I protection assay. The only modification was the substitution of MPE for DNase I. The probe was subjected to MPE treatment either without (lane 1) or with (lane 2) the addition of purified OTF-1. The positions of the G residues are indicated, as well as the protected sequence.


OTF-1
–
+
G
TATTTGCAT
-22
-33
-38
-44
-52
-53
-55
-58
-60
-63

The purification of this factor, and the development of a differentially regulated *in vitro* transcription system will now allow an investigation of key mechanistic questions regarding the cell-cycle mediated regulation of histone gene transcription. One question concerns the detailed mechanism by which the factor increases the transcription of the H2b gene. We consider it unlikely that the factor nucleates the formation of a completely new transcription complex at the G1/S transition because of the *in vivo* evidence of a pre-existing basal complex (LaBella et al., 1988). At this point we cannot distinguish between modulation of the DNA binding properties of this factor or modulation of its transcriptional stimulatory activity *per se*. Thus, although the basal activity outside of S phase does not require binding of the factor (LaBella et al., 1988), it may well be that the factor is capable of binding in an inactive state. A second major question concerns the mechanism by which OTF-1 and the other subtype transcription factors are coordinately activated.

CHAPTER 2

Characterization of OTF-1 at Different Stages in the Cell Cycle.

With this factor in hand it was of immediate interest to derive the biochemical tools that would allow us to investigate its fine structure and function with the aim of investigating some of the questions discussed above. These tools included a monospecific antisera and sufficient amino acid sequence to allow the isolation of cDNA clones for the factor. At this point I will discuss the development and use of the antisera, and reserve discussion of the isolation of cDNA clones.

Monospecfic mouse antisera was developed by a recently described technique. The strategy employed was to electrophorese the purified material through an SDS-polyacrylamide gel and transfer the resolved proteins to nitrocellulose paper. The transferred protein was detected by staining the nitrocellulose with Fast Green and the appropriate band was cut out. This nitrocellulose piece was then dissolved in DMSO and injected interperitonially into mice. Each mouse was injected with 300-500 nanograms of protein, and then injected twice more at one month intervals. The specificity of the sera was assayed by western blot analysis of purified material and nuclear extract (Figure 11, panels A & B). As is evident the sera specifically recognizes a 90 kDa species. That this was in fact OTF-1 is suggested by the fact that the sera recognizes OTF-1 independently purified from Namalwa cells (see below). Incubation of this sera with nuclear extract shows that there is little non-specific binding. The technique allowed us to develop sera of very high titer (usually 1/10,000).

The initial and fundamental question we were interested in pursuing regarded the mechanism of the regulation of the transcriptional stimulatory activity of OTF-1. Our initial experiments were aimed at determining if the binding activity

FIGURE 11. Western Blot Analysis.

Samples of highly purified OTF-1 (HeLa){2}, OTF-1 (Namalwa){3}, and OTF-2 (Namalwa){4} (30 ng each) were electrophoresed through a 10% SDS-polyacrylamide gel and transferred to nitrocellulose as described in Materials and Methods. Transferred material was incubated overnight at 4^{o} C with mouse anti-sera diluted 1/1000 in IMDM. After washing the membrane in PBST it was incubated for 2 hrs. with monoclonal goat anti-mouse IgG antisera conjugated to alkaline phophatase. The membrane was washed again, and the visualization procedure was performed according to manufacturer's suggestion (Promega-Biotec). Lane 1 is a similar analysis of Hela nuclear extract (12 ug). The molecular weights of the standards are as follows; 180,000, 116,000, 84,000, 58,000, 48,500, 36,500, and 26,600. .


1 2 3 4 M

or total mass of OTF-1 changed during the cell cycle. We have used this sera to quantitate the mass of OTF-1 present in nuclear extracts prepared from cells synchronized in either S or G2 by western blot analysis. Equivalent amounts of extract were electrophoresed in SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and then incubated with the mouse antisera. Addition of a second anti-mouse monoclonal antibody conjugated to alkaline phophatase allowed detection of the bound primary antibody. As is evident there is no significant change in the amount of OTF-1 present in these two extracts (Figure 12). To determine the relative amounts of binding activity present in the two extracts a standard electrophoretic mobility shift assay was performed, as is shown in Figure 13. It is clear that there is no significant difference in the binding activty present in the two extracts. From this data we conclude that the functional activity of the factor is not a reflection of changes in mass or binding activity.

At this point it was of interest to determine if the transcription stimulation of OTF-1 was dependent on ancillary factors which may be present in the crude fractions used in the transcription reconstitution assay. We reasoned that if OTF-1 could stimulate transcription in the crude G2 extract this would argue that the purified factor had intrinsic stimulatory activity and, furthermore, eliminate the idea of negative regulation in G2. This add back experiment (Figure 14) demonstrates that it is possible to stimulate transcription in this G2 extract. Addition of the purified factor to an extract prepared from cells synchronized in S phase reveals no stimulation of transcription. This result allows us to make several conclusions. First, even though both extracts contain an equivalent amount of binding activity, the G2 extract is not saturated in functional transcription stimulatory activity while the S extract is. (Protein titration was performed to ensure that the amount of template was not limiting in either extract). Second, this shows as well that there is no activity in the G2 extract which eliminates the function of the

FIGURE 12. Western Blot Analysis of Extracts Prepared From Synchronized Cells.

Samples of nuclear extract prepared from cells synchronized either in S or G2 were electrophoresed through a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Western Blot analysis was performed as described in the previous legend. Equal amounts, in terms of cell equivalents, were analyzed in the ratios indicated (i.e., 1, 2x, 3x). Synchronization and preparation of the extracts is described in Methods. Transferred material was incubated overnight at 4^{o} C with mouse anti-sera diluted 1/1000 in IMDM. After washing the membrane in PBST it was incubated for 2 hrs. with monoclonal goat anti-mouse IgG antisera conjugated to alkaline phophatase. The membrane was washed again, and the visualization procedure was performed according to manufacturer's suggestion (Promega-Biotec). Position and sizes of molecular weight standards are indicated.

G2 S

1 2 3 1 2 3

- 180

- 116

- 84

- 58

- 48

- 36

- 26

FIGURE 13. Electrophoretic Mobility Shift Assay of Extracts Prepared From Synchronized Cells.

Standard electrophoretic mobility shift assay was performed using a probe corresponding to sequences from -65 to -19 on the H2b promoter. The probe was incubated with samples of nuclear extract {prepared from cells synchronized in either S or G2} in the presence of 500 ng of dI-dC carrier DNA. The same amounts and ratios of protein as in the previous figure were used, except every sample was diluted 10 fold. The specific protien/DNA complex is indicated by an arrow.

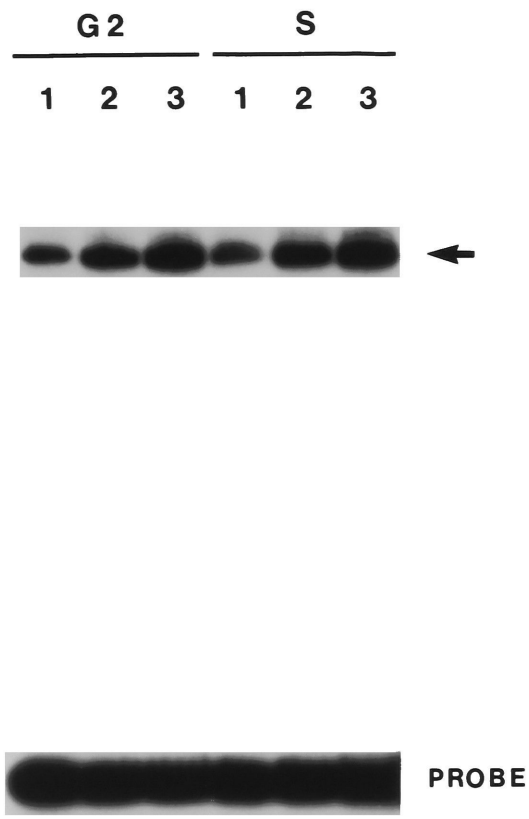
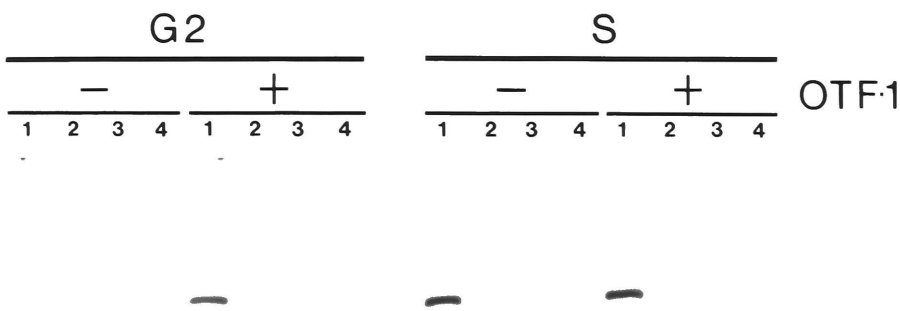

G2
S
1 2 3 1 2 3
PROBE

FIGURE 14. Transcription Stimulation by OTF-1 in Extracts Prepared From Synchronized Cells.

In vitro transcription were performed as described in Figure 4. Equal amounts of extract, in terms of cell equivalents, were used. This amount of activity was not saturating for the amount of template as determined by protein titration (data not shown). Addition of purified OTF-1 (5 ng) specifically stimulated transcription in the G2 extract. The template used were 1) wild type, 2) a 5' deletion to -59, 3) Oct$^-$, a double point mutation in the octamer sequence, 4) a 5' deletion to -36.


G2
S
−
+
−
+
1 2 3 4 1 2 3 4
1 2 3 4 1 2 3 4
OTF-1

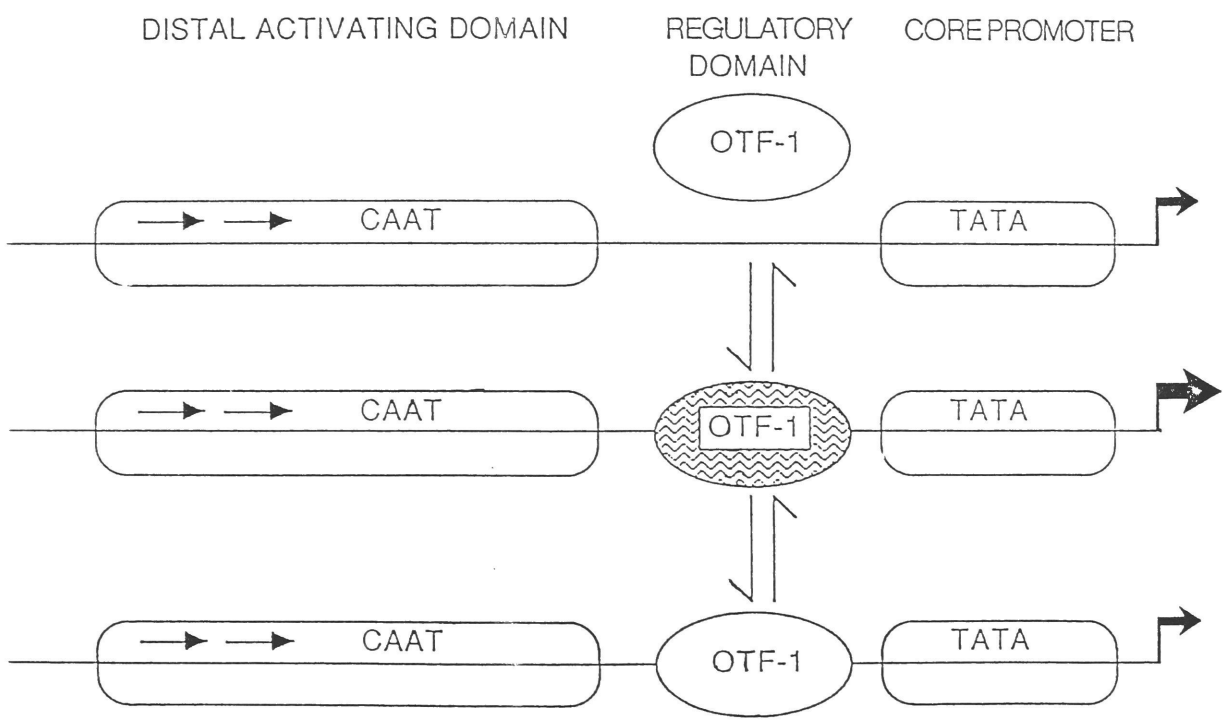
octamer factor. Given that this factor can stimulate transcription in a background of G2 extract it would seem that no other S phase specific ancillary factors are required for OTF-1 function. Naturally, definitive conclusions regarding the role of ancillary factors will have to await transcription reconstitution experiments using highly purified general factors. Interestingly, the -59 deletion is not stimulated as much as the wild type promoter, suggesting that OTF-1 can interact with the distal binding factors. This difference was not observed in reconstitution assays as they did not contain the upstream binding factors. It should be noted that these experiments only address the question of ancillary factors in regard to the histone H2b gene. We are currently aware of the implication of OTF-1 in function of HSV early genes as a component of a complex that involves at least Vmw65 as a functional cofactor (Preston et al., 1988, O'Hare and Goding, 1988, T. Gerster, personal communication).

Model of Transcriptional Activation of the H2b Promoter by OTF-1

From this data we have developed a model which describes the induction of transcription of the histone genes during the G1/S transition (presented in Figure 15). First, given the experimental methodology of the *in vivo* analysis we can make two conclusions. We can conclude that there is no effect of chromosomal location on the regulation of gene expression because transient transfection assays were performed. In addition, since this promoter was analyzed when cells were blocked at the G1/S boundary it is clear that transcription activation does not occur in the absence of DNA synthesis. This is in agreement with previous analyses, but stands in contrast to the observations regarding histone transcription in yeast, where histone transcription appears to be induced late in G1 (Hereford et al., 1982, Matsumoto et al., 1987).

FIGURE 15. Model of Transcriptional Activation of Histone H2b Promoter by OTF-1.

Sequence elements shown are those specifically analyzed in the H2b promoter, but the domain structure proposed emphasizes the functional equivalence of these domains in the promoters controlling transcription of the various histone gene subtypes. Constitutive transcription (thin arrow) is mediated by two separable promoter domains: the core promoter comprised of the TATA motif, and the distal activating domain comprised of the indicated sequence elements. The regulatory domain consists of the OTF-1 binding site and can function in the absence of the distal activating domain. Specifically, we propose that the S phase specific increase in transcription (thick arrow) is due to stimulation of the complex by "active" OTF-1 which is bound to the H2b subtype specific consensus sequence. The model does not distinguish between functional activation of OTF-1 either as a free monomer or while it is bound to the promoter. Furthermore, the model does not address the possibility that "active" OTF-1 may also exist in significant amounts in as a free monomer.


DISTAL ACTIVATING DOMAIN
REGULATORY DOMAIN
CORE PROMOTER
OTF-1
CAAT
TATA
CAAT
OTF-1
TATA
CAAT
OTF-1
TATA

In terms of the promoter structure itself, we can divide it into three functional domains. These include the core domain, comprising the TATA motif, the regulatory domain, composed of the H2b subtype specific sequence, and the distal activating domain, composed of the CAAT motif and the direct repeats. From the fact that deletion of the upstream sequences causes decreased transcription at both stages of the cell cycle, we conclude that the upstream factors comprise a basal complex that is active throughout the cell cycle. Therefore we conclude that it is unlikely that OTF-1 nucleates the formation of a new transcription complex upon entry into S phase. In fact, we can conclude from both the *in vivo* and *in vitro* data that OTF-1 is able to stimulate transcription in the absence of the upstream factors. Further, we can conclude that this stimulation is dependent upon binding to the octamer sequence. The fact that OTF-1 is unchanged in mass during the course of the cell cycle suggests that functional regulation is achieved by a post-translational mechanism. Interestingly, we have not detected any modulation of binding activity which could account for transcriptional regulation.

The idea that function (i.e. transcription activation) and DNA binding are uncoupled is unusual but not unprecedented. Examples of this include the binding activity of the heat shock transcription factor and GAL4 in yeast (Johnston et al., 1987, Sorger et al., 1987) and also the serum response factor in higher eukaryotes (Treisman, 1986). Those examples stand in contrast to cases where differential binding was detected by *in vitro* assay (Sen and Baltimore, 1986a, Seguin and Hamer, 1987). However, it is entirely possible that there may be an *in vivo* restriction on binding that we do not detect with our *in vitro* assay. This will have to be investigated by *in vivo* footprinting experiments. As such, it is not possible at this point to distinguish between the two possible models presented in Figure 15. However, the idea that specific modifications mediate functional activity while

binding is unregulated makes sense in light of the octamer motif being implicated in a variety of differently regulated functions (discussed below).

Perhaps the most striking conclusion is that the coordinate activation of the histone gene family must be due to the activation of multiple, independent subtype specific factors. This follows from the fact that OTF-1 does not interact with the histone H4 promoter, nor do the H4 promoter factors interact with the H2b promoter (Dailey et al., 1986, Fletcher et al., 1987). Further support for this idea is derived from the recent demonstration that the transcription induction of a chicken histone H1 gene transfected into synchronized HeLa cells is dependent on an upstream H1 subtype specific sequence (Dalton and Wells, 1988). Thus, the mechanism for activation of the histone gene transcription factors is pleiotropic and could be important for the regulated expression of other S phase induced genes.

Investigation of Differential Functional Activity of OTF-1

An additional question of functional regulation relates to the the fact that distinct elements containing the octamer motif have been implicated in tissue specfic promoter function (Bergman et al., 1984, Falkner and Zachau, 1984, Parslow et al., 1984, Mason et al., 1985, Mizushima-Sugano and Roeder, 1986), in both ubiquitous and tissue specific enhancer function (Banerji et al., 1983, Mattaj et al., 1985, Krol et al., 1985, Sen and Baltimore, 1986, Bohmann et al., 1987, Parslow et al., 1987), and in DNA replication (Pruijn et al., 1986, Wides et al., 1987, Rosenfeld et al., 1987). This differential function could result from 1) multiple octamer binding factors, each with a specific functional activity, or 2) interaction of a common octamer factor with different factors in each complex. Furthermore, in the latter case this interaction could be mediated either by a single or by multiple functional

domains resident in the octamer protein. It is not unlikely that all of these possibilities are, in fact, true.

For instance, observations in support of a distinct octamer-binding activity specific to lymphoid cells was provided by electrophoretic mobility shift assays (Staudt et al., 1986, Landolfi et al., 1986), although no functional activity was demonstrated. This factor has now been purified to homogeneity in our lab and shown to function selectively in activating immunoglobulin genes *in vitro* (Scheidereit et al., 1987). It was of interest to determine if these two factors were structurally related, and a western blot analysis was performed to determine if the factors were immunologically related. The results of this type of assay are presented in Figure 11. It is clear that the polyclonal mouse antisera raised against OTF-1 purified from Hela cells recognizes OTF-1 purified from Namalwa cells, but it does not react with purified OTF-2. This suggests that the two factors are not highly related in primary structure, although the formal possibility exists that OTF-2 corresponds to a sequence in OTF-1 which is very non-antigenic.

Although the ubiquitous (see next ¶) octamer-binding factor (OTF-1) and the B cell specific factor (OTF-2) appear to function selectively in stimulating transcription from the H2b and immunoglobulin kappa promoters (Mizushima-Sugano and Roeder, 1986, A. Heguy and C. Fletcher, unpublished observations) each factor appears to bind in an indistinguishable fashion by gel-shift and footprinting assays, to either of the octamer-containing genes (cf Scheidereit et al., 1987). Furthermore, the ubiquitous factor has been shown to bind not only to the H2b promoter but also, in a competitive fashion, to the octamer-containing elements of the U2 gene, the SV40 enhancer and the adenovirus replication origin (Sive and Roeder, 1986, E. O'Neill and C. Fletcher, unpublished observations, see below). Apart from suggesting that binding events *in vitro* may not always be relevant to *in vivo* function, these observations raise an additional significant

mechanistic question - namely, how the factors mediate selective function in the absence of selective binding. This might be explained, in part, by promoter-dependent differences in the ability of a given factor to interact functionally with either one or more of the common factors (e.g. the TATA factor) or with additional gene specific factors (discussed in Scheidereit et al., 1987). This might be due, for example, to variations in the position or orientation of the octamer element.

Is Function of OTF-1 Modulated by Sequences Flanking the Binding Site?

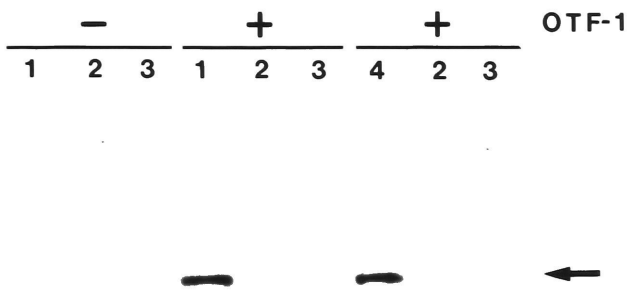
As for the second case, there is as yet no evidence for more than one octamer-binding factor in non-B cells. Cells in which only OTF-1 type octamer mobility-shift complexes have been detected include EL4, YAC-1, P 815, HeLa, Cos, 293, 3T3, MEL, rabbit reticulocyte, and *Xenopus laevis* oocytes. In the case of a single multifunctional octamer-binding factor, the requirement for the octamer sequence in several (presumably) non cell-cycle regulated genes (Tso et al., 1985, Parslow et al., 1987, Tom Maniatis, personal communication) suggests that the function of this protein is not restricted to S phase and that there might be cell-stage specific modifications important for S versus non-S function. As a preliminary step in investigation of the promoter geometry constraints on functional activity, and as a means of addressing the question of the extended conservation of sequence comprising the H2b subtype specific motif (CYTNATTTGCATAC, Wells, 1986), I constructed a promoter mutation in which the H2b flanking sequences were replaced by sequences derived from the u heavy chain enhancer (GGTAATTTGCATAA). This construct is detailed in Figure 16. This template was then used in a transcription reconstitution assay, the results of which are also presented in Figure 16. From this data one can conclude that the *in vitro* functional activity of OTF-1 is not significantly modulated by the surrounding sequence in the

FIGURE 16. In Vitro Transcription Assay Using a Template With a u Heavy Chain Enhancer Octamer Sequence Substituted for the H2b Consensus.

In vitro transcription assays were performed with the phosphocellulose 0.5 M and 0.8 M KCl fractions, with or without purified OTF-1, and the following templates. Template 1 is the wild type H2b promoter, template 2 is the octamer double point mutant, template 3 is a 5' deletion to -36, and template 4 is a multiple point substitution around the octamer sequence. The substituted sequence, which corresponds to the u heavy chain enhancer sequence, is shown below (top line). The lower line is the H2b wild type sequence; differences are indicated with asterisks.

```
TTCCACCACCTGGGTAATTTGCATTTCTGAT
   *  ********  *         ****
TTCAACTCTTCACCTTATTTGCATAAGCGAT
```

It is apparent that OTF-1 stimulates transcription from both templates equivalently.



1. WT
2. OCT⁻
3. −36
4. pHμ

promoter. Extensive mutation and binding analysis does suggest a role for the T two bases upstream and the purine following the final T (Ed O'Neill, personal communication). This situation is different, therefore, from the mechanism of transactivation by Vmw65, which appears to involve the flanking sequences which make up the TATTGARAT motif (O'Hare and Goding, 1988, Preston et al., 1988, T. Gerster, personal communication). Analysis of constructs in which the spacing and orientation of the octamer motif have been altered is being performed currently.

Is OTF-1 Able to Function On Other Genetic Elements?

The identification of an octamer dependent DNA replication stimulatory activity (NFIII/ORP-C)(Pruijn et al., 1986, Wides et al., 1987, Rosenfeld et al., 1987), and its purification and characterization (O'Neill et al., 1988) demonstrated *in vitro* stimulation of DNA replication by an octamer binding factor. Preliminary comparative studies indicated that the H2b transcription factor and adenovirus DNA replication factor are identical in size and chromatographic behaviour (C. Fletcher and E. O'Neill, unpublished observations, see below). It was clear that a thorough comparative analysis of the two systems would provide an opportunity to substantiate the theory that this factor possesses a promiscuous DNA binding activity, while its functional activity is determined by context, geometry or modifications. Thus, it was essential to determine if OTF-1 was able to stimulate DNA replication. If that were shown to be true, one would then be able to compare the stimulatory mechanisms and regulatory circuits. To this end, I initiated a collaboration with Ed O'Neill in Dr. Kelley's lab at The Johns Hopkins School of Medicine. The results of that collaboration are described in the following section.

CHAPTER 3

Introduction

The biochemical and genetic description of the initiation of transcription of RNA and replication of DNA has shown these two events to be dissimilar in many aspects. It is apparent, however, that both of these events require the function of sequence specific DNA-binding proteins (Dynan and Tjian, 1985, Ptashne, 1986, Kelly and McMaken, 1987). The identification of functional sequence elements within the adenovirus origin of replication (Ad Ori) (Wides, et al., 1987) which are similar to elements within promoters of transcription suggests that the cognate binding factors may have functional roles in both of these events. The isolation and characterization of these proteins is the first step in understanding their mechanism of action.

Fractionation of the components required for adenovirus *in vitro* DNA replication has allowed the identification of several proteins which can participate in the replication of DNA within HeLa cells (Kelly et al., 1988). Three of these proteins are viral in origin: the 80 kDa pre-terminal protein (pTP), the 140 kDa DNA polymerase (Ad Pol), and the 72 kDa DNA binding protein (DBP). The other proteins involved with adenovirus DNA replication are cellular in origin: a topoisomerase, NF-II (Nagata et al., 1983), and two sequence specific DNA binding proteins, NF-I (Nagata et al., 1982, Rosenfeld and Kelly, 1986) and NF-III (Pruijn et al., 1986, Rosenfeld et al., 1987). NF-III has recently been purified and found to be a single polypeptide of MW 92,000 (O'Neill and Kelly, 1988).

Similarly, *in vitro* reconstitution of transcription has demonstrated that accurate initiation by RNA polymerase II requires a number of different soluble factors. General transcription factors, such as TFII B, E and D, are required for the

transcription of all genes of this class (Matsui et al., 1980, Samuels et al., 1982, Sawadago and Roeder, 1985). In contrast, promoter-specific factors may act on a wide variety of promoters, or they may interact with only limited set of genes (reviewed in Dynan and Tjian, 1985, Maniatis et al., 1987). It has been demonstrated that several of these highly purified factors have a sequence specific DNA binding activity and that stimulation of transcription is dependent on binding to the cognate sequence element (Wiederrecht et al., 1987, Jones et al., 1987, Fletcher et al., 1987, Scheidereit et al., 1987).

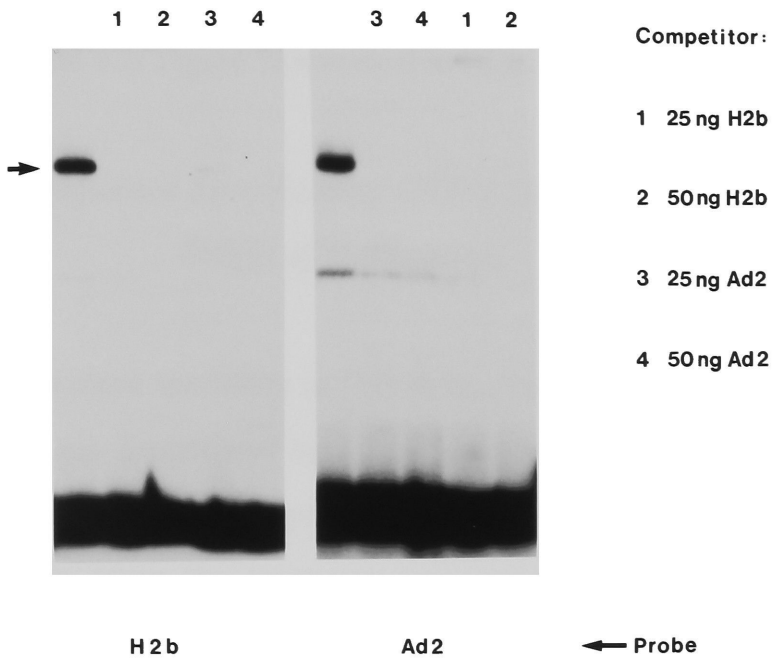
Are OTF-1 and NF-III Identical?

The suggestion that OTF-1 and NF-III may be identical was prompted by the observation that the octamer sequence is similar to a sequence within the adenovirus origin of replication that is recognized by NF-III (Rosenfeld et al., 1987). Recently it has been shown that NF-III can recognize the octamer sequence contained within the human histone H2b promoter (O'Neill and Kelly, 1988). A electrophoretic mobility shift assay using a semi-purified fraction of OTF-1 suggested that OTF-1 could recognize the adenovirus origin (see Figure 17). The observation that NF-III and OTF-1 have similar if not identical molecular weights strengthened the possibility that NF-III and OTF-1 are the same polypeptide. We present data below which demonstrates that OTF-1 is capable of recognizing the adenovirus origin of replication and stimulating *in vitro* DNA replication in a manner indistinguishable from NF-III.

In previous characterizations of OTF-1 and NF-III, elution and renaturation of specific DNA binding activity from SDS-polyacrylamide gels has proven that both of these proteins have a molecular weight of around 90,000 Da. To ascertain whether the slight difference in the original calculations of apparent

FIGURE 17. Electrophoretic Mobility Shift Assay With an Adenovirus Probe.

A standard electrophoretic mobility shift assay was performed as described in Figure 7. In this case, however, a partially purified sample of OTF-1 was used (Biorex-70 step). The H2b probe is described in Figure 7, the Ad-2 probe is described in Figure 19. The amounts of competitor are indicated. The arrow indicates the specific protein/DNA complex. It is apparent that both complexes are competed by each sequence, suggesting that a single octamer dependent binding activity is present in the crude fraction.


1 2 3 4 3 4 1 2
Competitor:
1 25 ng H2b
2 50 ng H2b
3 25 ng Ad2
4 50 ng Ad2
H2b
Ad2
Probe

molecular weight was real, we simultaneously analyzed samples of OTF-1 and NF-III by SDS-PAGE (Fig. 18). While the NF-III used in this experiment is from a late step in purification, it is not the only polypeptide species in the sample. It is clear, however, that the species previously identified as OTF-1 and NF-III have identical molecular weight. It should be noted that the lanes were loaded with amounts of material which were equivalent in terms of specific DNA binding activity. The fact that we observe equivalent mass of the 90 kDa proteins suggests that these two species have similar affinity for the specific binding element. The preparation of OTF-1 analyzed in lane b of Figure 18 was used in all subsequent experiments.

Determination of Sequence Specificity of OTF-1 Binding on the Adenovirus Origin of Replication

The sequence specificity of OTF-1 binding activity on the adenovirus origin of replication was determined by a DNase I protection assay. As shown in Figure 19, one observes a gap in the partial digestion ladder which maps over the octamer-like sequence when the fragments are analyzed by polyacrylamide gel electrophoresis. The location and extent of protection by OTF-1 is identical to that of NF-III (O'Neill and Kelly, 1988), and spans a region from nucleotide 35 to 52 in the Ad Ori. To confirm that the purified preparation of OTF-1 contains a single binding activity that recognizes both the Ad Ori and the H2b promoter, we assayed the ability of various DNA fragments to compete for the binding of OTF-1 to the Ad Ori. Constant amounts of purified OTF-1 and an end-labelled Ad Ori fragment were incubated in the presence or absence of unlabelled competitor DNA fragments. The degree of binding of OTF-1 to the labelled Ad Ori fragment was monitored by the standard electrophoretic mobility shift assay. The quantity of

FIGURE 18. SDS Polyacrylamide Gel Electrophoresis of OTF-1 And NF-III.

Samples of purified OTF-1 and NF-III each containing 43 units of Ad Ori specific binding activity were electrophoresed through an 8% SDS-polyacrylamide gel. After electophoresis the polypeptides were visualized by silver staining. The molecular weight standards (M_r) were myosin (205,000), b-galactosidase (116,000), phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000). The 90,000 Da polypeptide is indicated with an arrow. A schematic depiction of the NF-III purification protocol is included.

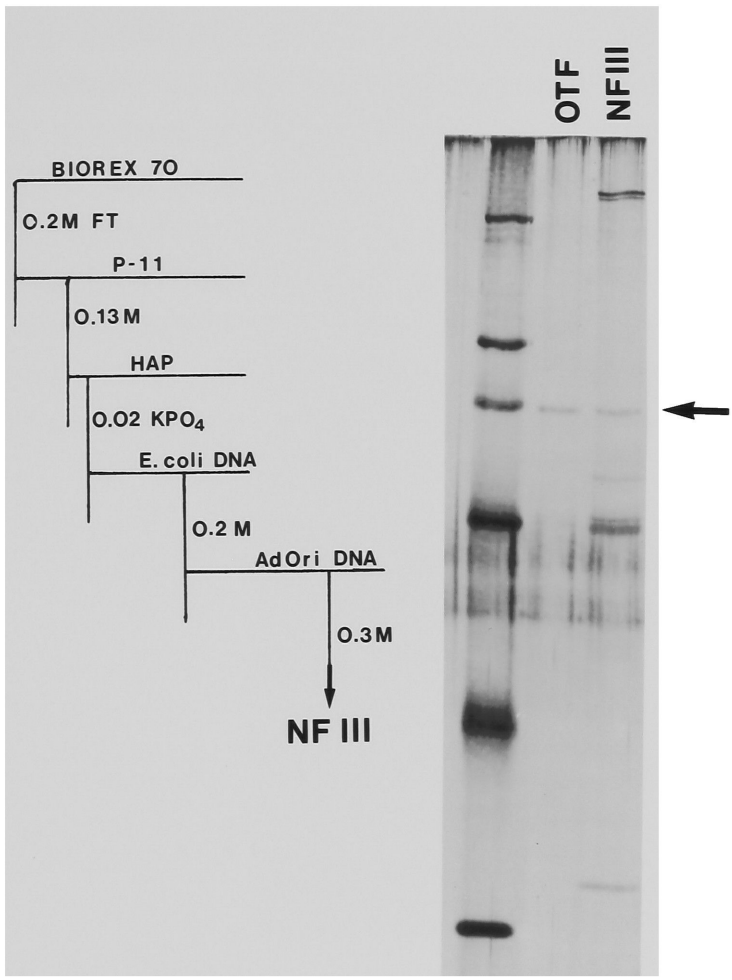
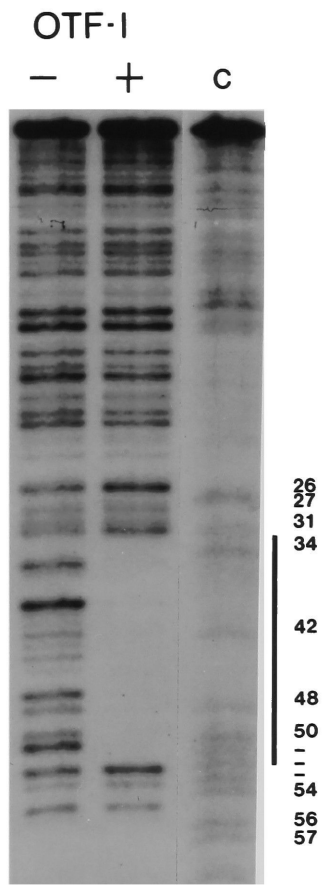

BIOREX 70
0.2M FT
P-11
0.13M
HAP
0.02 KPO4
E. coli DNA
0.2 M
AdOri DNA
0.3M
NF III
OTF
NFIII

FIGURE 19. DNase I Protection Assay of OTF-1 Bound to the Adenovirus Origin of Replication.

The plasmid pUdl 67 was linearized with Bam HI, end-labelled with polynucleotide kinase, and then restricted with Pvu II. The 157 bp fragment containing the adenovirus origin of replication was isolated by electroelution and precipitation following electrophoresis on an 8% polyacrylamide gel. This fragment was manipulated as follows and then electrophoresed as in Figure 10. Lane 1, the DNA fragment subjected to DNase I cleavage, Lane 2, the DNA fragment subjected to DNase I cleavage in the presence of 22 Ad Ori specific DNA binding units, Lane 3, the DNA fragment subjected to a sequencing reaction specific for C residues. The nucleotide positions of the C residues are indicated (for sequence, see next figure).


OTF-I
−
+
C
26
27
31
34
42
48
50
54
56
57

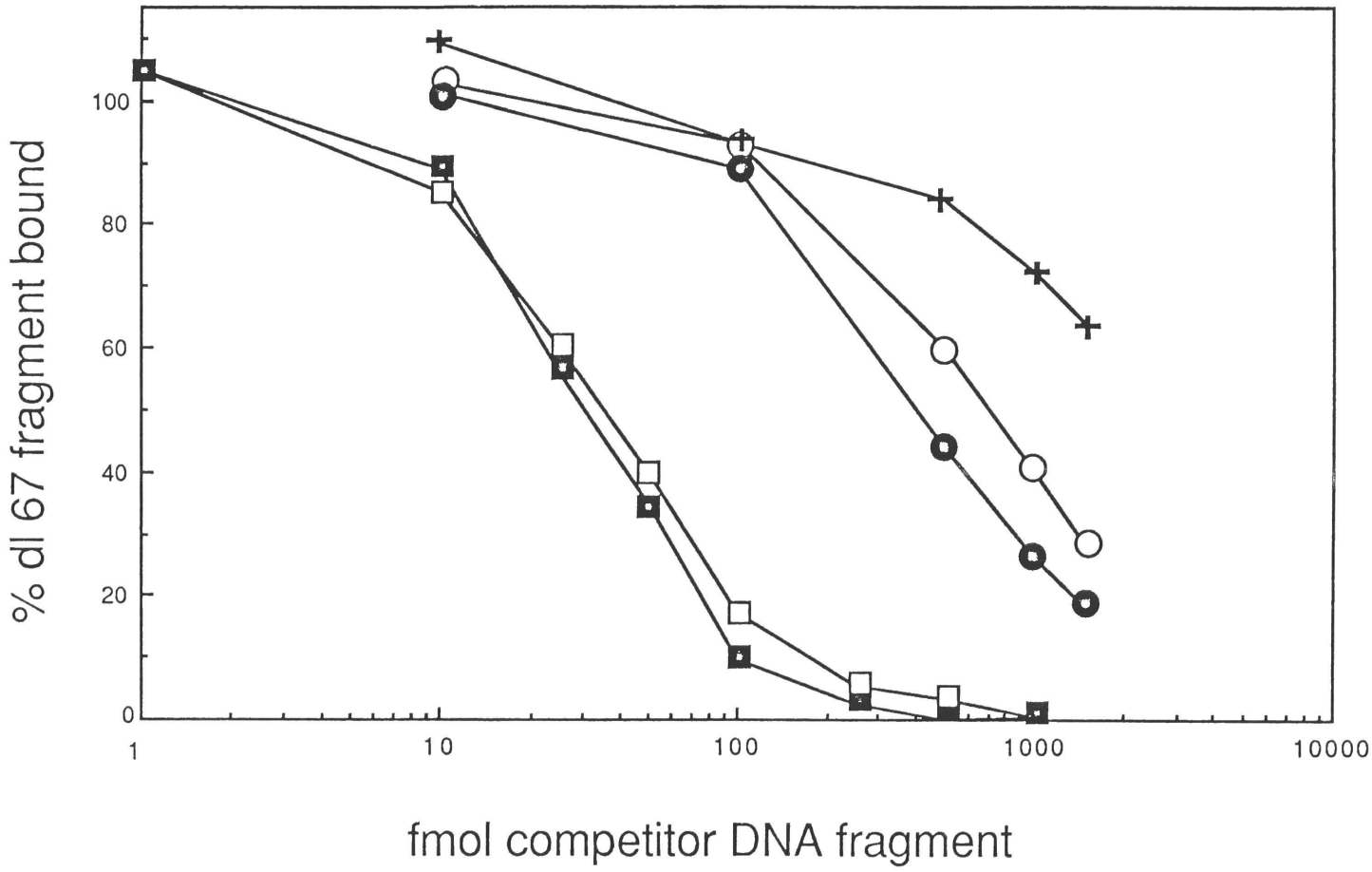
competitior DNA required to reduce the amount of Ad Ori fragment bound to protein (shifted species) by a defined amount (50%) is taken to be indicative of the relative affinity of OTF-1 for the competitor DNA. The results of the experiment are presented graphically in Figure 20. It is evident that the Ad Ori fragment and H2b fragment are equally effective in competing for the OTF-1 which is bound to the labelled Ad Ori fragment. DNA fragments containing base substitution mutations within the consensus octamer sequence in the Ad Ori or the H2b promoter bind OTF-1 with significantly lower affinity relative to either wild type fragment. From this data we conclude that the preparation of OTF-1 contains a single binding activity with high affinity for both the H2b promoter and the adenovirus origin of replication and that this activity specifically recognizes the octamer consensus sequence. Moreover, the fact that the competition curves of NF-III and OTF-1 are identical demonstrates that both of the preparations have equivalent affinity for the test fragments (data not shown).

DNA Replication Activity of OTF-1

The ability of OTF-1 to activate initiation of adenovirus DNA replication was tested in a cell free replication system. The initial event in the replication process is the formation of an ester linkage between the α phosphate of dCMP and the b-OH of a serine residue in the virus encoded 80 kDa pre-terminal protein (pTP) (Kelly et al., 1988). The synthesis of a new viral DNA strand takes place by extension from the free 3' hydroxyl group present in the pTP-dCMP initiation complex. The initiation reaction can be monitored *in vitro* by measuring the incorporation of α-^{32}P·dCTP into the SDS-resistant pTP-dCMP complexes. The minimal proteins required for this reaction are the viral preterminal protein and DNA polymerase and the cellular derived proteins NF-I and NF-III (Pruijn et al.,

FIGURE 20. Competition for Binding of OTF-1 to the Adenovirus Origin of Replication.

Relative affinities of potential octamer sequences for purified OTF-1 were determined by competition analysis using the gel electrophoresis DNA binding assay. The radioactive marker fragment containing the entire adenovirus origin of replication (bp 1-67) was derived from pUdl 67 (see previous legend). Competitor fragments were: []--[] the 157 bp Bam HI/Pvu II fragment derived from pUdl 67 (wild type Ad Ori); O--O the 157 bp Bam HI/Pvu II fragment derived from pUpm 46 (Ad Ori with an A to T transversion at base 46 in the NF-III binding site; []--[] the 170 bp Hind III fragment derived from p16.3 C_2AT (wild type H2b promoter); 0--0 the 170 bp Hind III fragment derived from pOMA (mutant H2b promoter with a double base substitution generating the sequence ATTCAACT); +--+ the 90 bp. Bam HI/Pvu II fragment derived from pUC 9. Varying quantities of competitor DNA fragments were mixed with 5 femtomoles of labelled marker fragment. The DNA was then incubated with 1.1 Ad Ori specific DNA binding units of OTF-1 for 30 minutes. Following electrophoresis of the reaction mix the gel was dried and autoradiographed. The bands corresponding to bound and unbound DNA were cut out and quantitated by liquid scintillation counting. The amount of radioactive DNA bound by OTF-1 in the absence of competitor DNA was determined and the fraction of this DNA bound in the presence of competitor was calculated.



10 20 30 40 50 60

CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGT···

GTAGTAGTTATTATATGGAATAAAACCTAACTTCGGTTATACTATTACTCCCCCACCTCA···

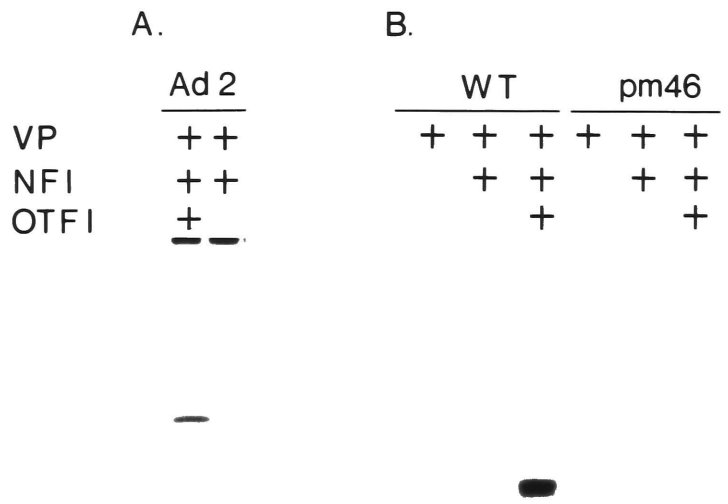
1986, O'Neill and Kelly, 1988). Figure 21 shows that OTF-1 is capable of replacing the required NF-III in the reaction. Furthemore, titrations of OTF-1 and NF-III that are normalized for equivalent amounts of DNA binding activity show equivalent stimulation of replication initiation (data not shown).

In Figure 21 we have used a reconstituted *in vitro* replication reaction using cloned plasmid templates in order to show that the NF-III binding site within the adenovirus origin of replication is required for OTF-1 to stimulate viral DNA replication. In this assay the products of the replication assay are distinguishable from unreplicated DNA by their reduced mobility in SDS agarose gels. The reduction in mobility is a result of the covalent attachment of the pre-terminal protein (serving as a primer for DNA replication) to the newly replicated DNA molecule (Tamanoi and Stillman, 1983, Wides, et al., 1987). The most efficient reconstituted system requires all of the protein components described above as well as the virus encoded 72 kDa DNA binding protein (Rosenfeld, et al., 1987). The appearance of the reduced mobility species is taken as a direct measure of the replication efficiency of each reaction. When the replication template contains a wild type Ad Ori the reaction is dependent on addition of NF-I and is stimulated by OTF-1 (Fig. 21, a-c). A base substitution within the Ad Ori at position 46 reduces the affinity of OTF-1/NF-III for the Ad Ori by approximately 20 fold (see Fig. 20). When a template containing this mutation is used in the reconstituted replication assay OTF-1 no longer has any effect on the reaction (Fig. 21, d-f). Note that the point mutation has no effect on the ability of NF-I to stimulate the replication of this template. Furthermore, in no case is the plasmid fragment which does not contain an Ad Ori replicated (Fig. 21, a-f).

FIGURE 21. In Vitro DNA Replication Assay.

A) Initiation of adenovirus DNA replication is dependent on OTF-1. The standard *in vitro* initiation reaction was performed as described previously (O'Neill and Kelly, 1988). Reaction mixtures contained adenovirus DNA-protein complex as template and α-^{32}P·dCTP as the only deoxynucleotide triphosphate. After incubation, the Ad template is digested with mung bean nuclease and the reaction mixture is electrophoresed in an SDS-polyacrylamide gel. Extent of initiation is monitored by the amount of label incorporated into the preterminal protein by covalent attachment of the α-^{32}P CTP. VP: the adenovirus encoded replication proteins, pTP and Ad Pol, purified as described (Rosenfeld et al., 1987) (1.2 ug). NF-1: Nuclear Factor-1, purified as described (Rosenfeld and Kelley, 1986). OTF-1: Octamer-binding Transcription Factor, purified as described (Fletcher, et al., 1987) (1.7 Ad Ori specific binding units). The appearence of the labelled band only in the first lane shows that initiation on the Ad template is dependent upon addition of OTF-1.

B) Stimulation of adenovirus DNA replication by OTF-1 is dependent on the presence of the NF-III binding site in the Ad Ori. Reconstitution of the replication of DNA containing the cloned adenovirus origin of DNA replication was performed as previously described (Wides et al., 1987) with the following modifications. The plasmids pUdl 67 or pUpm 46 were digested with Eco RI and Ava II and incubated with combinations of purified protein components: lanes 1 and 4; viral proteins (pTP, 3.9 ug, Ad Pol, 0.017 U, DBP, 0.12 ug), lanes 2 and 5; viral proteins and Nuclear Factor I (25 ng), lanes 3 and 6; viral proteins, Nuclear Factor-I and OTF-1 (13.6 Ad Ori specific DNA binding units). Each reaction contained 60 ng of DNA, dATP, dGTP, dTTP and α-^{32}P CTP. The largest restriction fragment contains the adenovirus terminus positioned at one end. The replication product (of lower mobility, visible in lanes 2, 3, 5 & 6) consists of a covalent complex between that DNA fragment and the viral preterminal protein. Extent of replication is measured by the incorporation of label into that fragment. The small fragment, and the unreplicated large fragment incorporate label non-specifically and are thus visible. It is apparent that addition of NF-I has a slight effect on replication, and that further stimulation by OTF-1 is dependent on an intact octamer binding site (lane 3 versus lane 6).


A.
B.
Ad 2
WT
pm46
VP
NFI
OTFI

Transcription Activity of NF-III

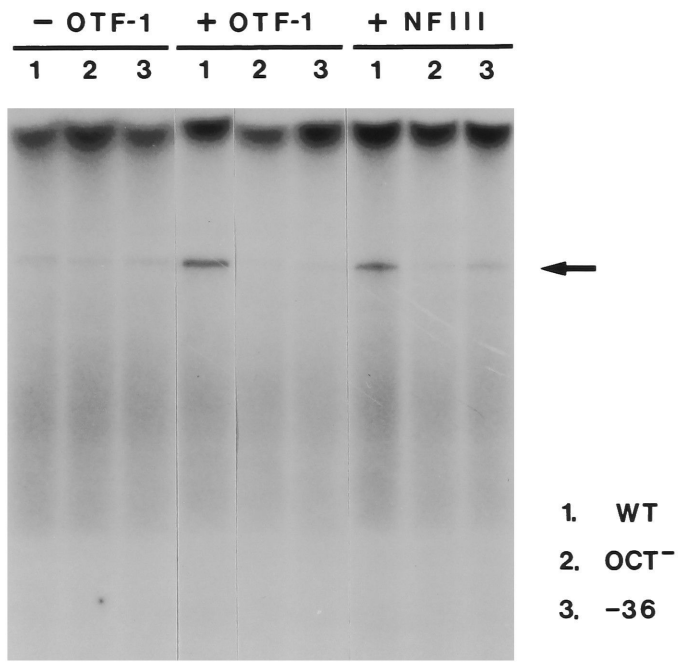
In addition, the preparation of NF-III was assayed for its ability to stimulate transcription from the H2b promoter. The results of that experiment are presented in Figure 22. The templates used in this assay are the wild type promoter, a double base sustitution in the octamer sequence, Oct-, and a 5' deletion to -36. In addition to the general transcription factors contained in the higher salt steps from the P-11 fractions, equivalent amounts of OTF-1 of NF-III based on Ad-2 binding activity were added to the reaction (lanes 4-9). As is evident, these preparations were both able to stimulate transcription equivalently in an octamer dependent manner.

Discussion

From the data presented we conclude that that the previously purified and characterized OTF-1 is functionally equivalent to NF-III. By binding competition and footprint analysis we have shown that the OTF-1 preparation contains a single binding activity that displays a high affinity for both the Ad2 and H2b octamer elements. Furthermore, OTF-1 stimulates adenovirus DNA replication as effectively as NF-III. Furthermore, NF-III is capable of stimulating transcription from the H2b promoter. Finally, when equivalent amounts of binding activity are analyzed by SDS-PAGE we observe in both preparations a ~90 kDa band of equivalent mass and apparent molecular weight. Given that OTF-1 has been identified as a single polypeptide of roughly 90 kDa we surmise that OTF-1 and NF-III are identical polypeptides. Interestingly, the octamer element in the

FIGURE 22. In Vitro Transcription Assay With OTF-1 and NF-III.

In vitro transcription assays were carried out with the phosphocellulose 0.5 M and 0.8 M KCl fractions without or with the addition of purified OTF-1 or NF-III. Equivalent amounts of OTF-1 and NF-III, based on Ad-2 specific binding activity, were added as indicated. The templates used are indicated over each lane, they are the H2b wild type, the octamer point mutant and the 5' deletion to -36, respectively. Position of the specific transcript is indicated by the arrow.


– OTF-1
+ OTF-1
+ NFIII
1 2 3 1 2 3 1 2 3
1. WT
2. OCT⁻
3. –36

Ad Ori differs by two bases from the H2b consensus. Extensive mutation and binding analysis suggests that the octamer consensus sequence is actually TYATTUUCATU (E.O. and C.B.,
YU
UY
unpublished observations).

This result raises many intriguing questions concerning the relationship between eukaryotic DNA replication and RNA transcription. While there are examples in viral systems of transcription and replication being coupled (Thomas and Mathews, 1980, Li et al., 1986, Luskey and Botchan, 1986, Enver et al., 1988), the curious overlap of transcription and replication elements in many viral systems has not been explained (reviewed in DePamphilis, 1988). Unfortunately, even less is known about the relationship of chromosomal replication and transcription. Given that at least one function of OTF-1 is the regulation of the S phase specific induction of transcription of a human histone H2b gene then if OTF-1 is involved in chromosomal DNA replication there may be a common mechanism which activates this factor for these two S phase functions. It is a tantilizing idea that this mechanism is responsible for the coupling of histone gene transcription and DNA replication. It not now possible, however, to determine if the mechanism which initiates replication at the Ad Ori is representative of a large class of eukaryotic origins of DNA replication or is an Ad Ori specific subversion of the cellular transcription system. In any case, the data presented above raises a significant question, i.e., does the functional activity of this factor operate through a common biochemical mechanism in these two systems?

It has been previously shown that another Ad replication factor, NF-I, is probably identical to the transcription factor CTF (Jones et al., 1987). The fact that there are at least two factors which function in both events suggests that there may be a fundamental, and perhaps common, mechanism by which sequence

specific DNA-binding proteins act. Such proteins may act by changing the structure of DNA upon binding, or through interaction with other factors. The stimulation of ColE1 replication by DNA melting (Dasgupta et al., 1987, Masukata et al., 1987) suggests that one mechanism of stimulation could be alteration of DNA topology. The second possibility is that OTF-1/NF-III could interact with different factors to stimulate either DNA replication or RNA transcription. In regard to this, the H2b promoter and the Ad Ori are quite different in terms of the orientation and spacing of the various sequence elements. Furthermore, it appears that OTF-1/NF-III does not function in the absence of NF-I. Therefore it is possible that these different functions could be ascribed to these differences. We are currently examining the structural constraints of the Ad Ori and the H2b promoter in functional terms. We anticipate that comparative analysis of these two model systems will be highly profitable in terms of understanding the biochemical function of these sequence specific DNA-binding factors.

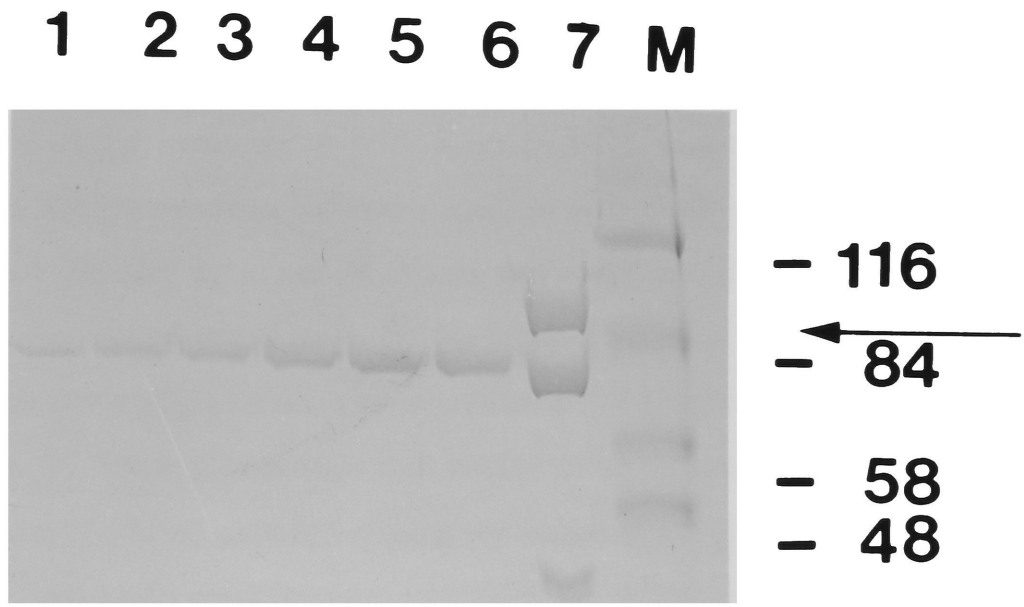
CHAPTER 4

Amino Terminal Sequence Analysis of Purified OTF-1

It is clear that analysis of the fine structure of OTF-1 would be greatly aided by 1) knowledge of the amino acid sequence of the protein and 2) development of system to define and investigate the functional domains of the protein. Naturally, achievment of these two goals would rely on isolation of a cDNA clone for OTF-1. The cloning of this factor was initiated in collaboration with Alessandra Pierani by pursuing the usual strategy of obtaining amino acid sequence information sufficient for the synthesis of oligonucleotide probes. The difficulty of obtaining sufficient amounts of pure material necessitated careful consideration of strategies for sample preparation. As the material eluted from the oligonucleotide affinity column was not entirely homogeneous, especially when the purification was performed on a large scale, we needed an additional final purification step. We considered HPLC gel filtration, electroelution following SDS-PAGE, and electrotransfer following SDS-PAGE to activated glass filters. Initial experiments indicated that none of these techniques would be useful to us, due either to poor resolution or poor recovery. Fortunately, we obtained a protocol for electrotransfer to a newly developed membrane, Immobilon (Paul Matsudira, personal communication). This technique allowed for rapid and reliable electrotransfer following SDS-PAGE to a membrane that required no manipulation prior to micro-sequencing. We quantitated the mass of OTF-1 in our eluted fraction by SDS-PAGE, Coomasie blue staining and scanning densitometry, as shown in Figure 23. A sample of 100 picomoles was applied to a preparative SDS-polyacrylamide gel and, following electrophoresis and transfer, the Immobilon membrane was stained and

FIGURE 23. Quantitation of OTF-1 Preparation Submitted for Amino Terminal Sequence Analysis.

Specific quantitation of mass amounts of OTF-1 in the preparation submitted for sequence analysis was performed as follows. Aliquots of the preparation were electrophoresed through a 7% SDS-polyacrylamide gel (lane 7, OTF-1 indicated by arrow), as were known amounts of BSA in adjacent lanes (lanes 1-6). BSA was quantitated by Bradford analysis and optical density measurement at 660 nm. Following electrophoresis the gel was stained with Coomassie Brilliant Blue, dried on cellulose and analyzed densitometrically. A representative gel is shown here.


1 2 3 4 5 6 7 M
116
84
58
48

the appropriate band excised. Two separate preparations of protein were sequenced, with the following sequence information being generated, VXSGNKAAVVLXMDVGFTMXNXIP (see Figure 24). Given that the mass of OTF-1 and the quantitation of the amino acid residue signals agreed, we were confident that we had obtained the appropriate sequence. We decided to evaluate several strategies for the preparation of oligonucleotide probes. These included synthesis of degenerate oligos, inosine substituted oligos, and "guessmers" or oligonucleotide probes whose sequences conform to observed rates of codon usage (Maruyama et al., 1986). The notable feature of this sequence in regard to codon degeneracy is the seven amino acid stretch between residues 13-19. In this region there are two methionines, which are encoded by a single codon, two other amino acids each encoded by a pair of codons and three amino acids encoded by four codons. The rate of codon usage for the latter amino acids reveals in each case a strong bias for a single codon. The sequences of two of the derived probes is shown in Figure 24. These probes were then evaluated by northern blot analysis. One of the guessmers (AP-4) gave a promising signal and this probe was used to screen a cDNA library under conditions determined by the following equation: $T_m = 69.3 + 0.41(G+C)\%$ (Maniatis et al., 1982).

Isolation of cDNA Clones

Following three rounds of screening we had isolated two promising phage. The insert fragments carried by the phage vector were excised by Eco RI digestion, purified by electrophoresis and elution from an agarose gel, and then subcloned into pBKS$^-$ (Stratagene). The inserts derived from the clones were sequenced using the chain termination method of Sanger (Sanger et al., 1977). The partial nucleotide sequence of the two clones is shown in Figure 25. A region in

FIGURE 24. Amino Acid Sequence, Possible Nucleotide Sequence and Oligonucleotide Probe Sequence.

This figure diagrams the experimentally derived amino acid sequence (top line, single letter code), the corresponding possible nucleotide sequence (next line), and the sequence of two synthetic oligonucleotide probes constructed on the basis of these results (labelled AP-4, AP-3). Sequence for the most degenerate codons was assigned based on known codon usage rates, the two slightly degenerate codon choices were evaluated empirically.


V S G N K A A V V L M D V G F T M A N I P
5' GTAXXXTCAGGAAACAAAGCAGCAGTAGTACTAXXXATGGACGTAGGATTCACAATGGCAAACXXXATACCA 3'
C C C T G C C C C C T C C T C T C C T C C T C C
G G G G G G G G G G G G G G G G T G
T T T T T T T T T T T T T T T T
AGC TTA
T G
3' TACCTGCACCCGAAGTGGTAC 5' AP-4
TACCTACACCCGAAATGGTAC AP-3

each clone which contains a 20/21 match with the probe sequence is boxed. It is evident that clone 36 can be translated to give a derived amino acid sequence with high homology to our known amino acid sequence, as shown in Figure 25 (correct residues marked with asterisks). In contrast, the sequence of clone 11 differs from 36 almost immediately upstream of the boxed region. The divergence of sequence of the clones was puzzling at first. We observed that not only did the large clone not encode some of the known amino acid sequence, but it also contained a stop codon just upstream of the junction. Our tentative conclusion is that the large clone is an artifact resulting from fusion by blunt ligation of two unrelated cDNA fragments during construction of the library. The smaller clone, on the other hand, contained our entire known amino acid sequence, which was in frame with an open reading frame that spanned the entire clone. The smaller clone was then employed as a probe to screen another cDNA library that was constructed by Moses Chao from mRNA isolated from a human metastatic melanoma (personal communication). Several clones were isolated from this library, the sequence and orientation of which are illustrated in Figure 26. Two of the clones (20a and 14d) were sequenced in their entirety. The other clones were only sequenced enough to allow for their mapping. The fact that we do not see any clones like 11 further supports the idea that 11 is a cloning artifact. As is noted in Figure 27 these clones contain an open reading frame which encodes a protein of 86 kDa. Although the derived amino acid immediately 5' to our first known residue is a methionine, we do not know if this is the initial residue because we have not mapped the clones relative to mRNA. Furthermore, we do not see any stop codons 5' of our known amino acid sequence, therefore we have chosen to begin translation at the 5' end of the cDNA clones. Interestingly, several clones contain polyA tracts begining at 2507. There is one clone (14d), however, which continues downstream from this point for another 138 nucleotides.

FIGURE 25. Clone Orientation and Sequence, Including Derived Amino Acid Sequence.

This figure diagrams the relative orientation of the two initial clones, 11 & 36. isolated from a HeLa cell cDNA library (Clonetech). Also shown is the nucleotide sequence of the indicated region. The derived amino acid sequence from the major open reading frame is indicated. Those residues which match the experimentally derived amino terminal sequence of OTF-1 are noted with an asterisk. The region corresponding to the oligonucleotide probe is boxed, the divergent sequence of clone 11 is overlined.

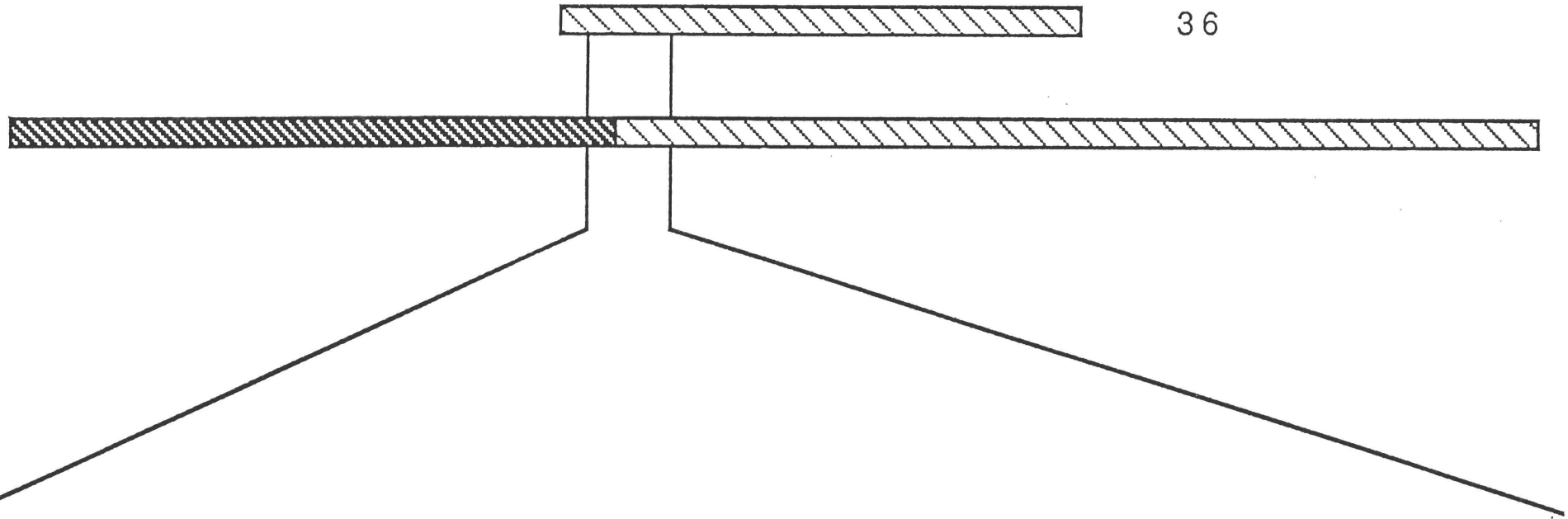

36
11
ATG GTG CGG TCG GGG AAT AAG GCA GCT GTT GTG CTG TGT ATG GAC GTG GGC TTT ACC ATG AGT AAC TCC ATT CCT
M V R S G N K A A V V L C M D V G F T M S N S I P
* * * * * * * * * * * * * * * * * * *
AGA GAA TTA CTT CAA ATT GAG TAA TTC AGA AAA ACG TGT ATG GAC GTG GGC TTT ACC ATG AGT AAC TCC ATT CCT
END F R L T C M D V G F T M S N S I P
* * * * * * * * * * * * * * * * * * *

FIGURE 26. Clone Maps and Sequencing Strategy for All the Analyzed Clones.

This figure diagrams the orientation of all the clones that have been extensively analyzed. Also indicated is the sequencing strategy for clones 14d and 20a, which were sequenced in their entirety. Indicated as well is the position of the derived amino acid sequence corresponding to the experimentally determined sequence. The vertical arrow at the right indicates the position of polyA stretches at the ends of some of the clones (stipled boxes). ( ) ends localized by sequence analysis, ( ) ends localized by restriction fragment length.

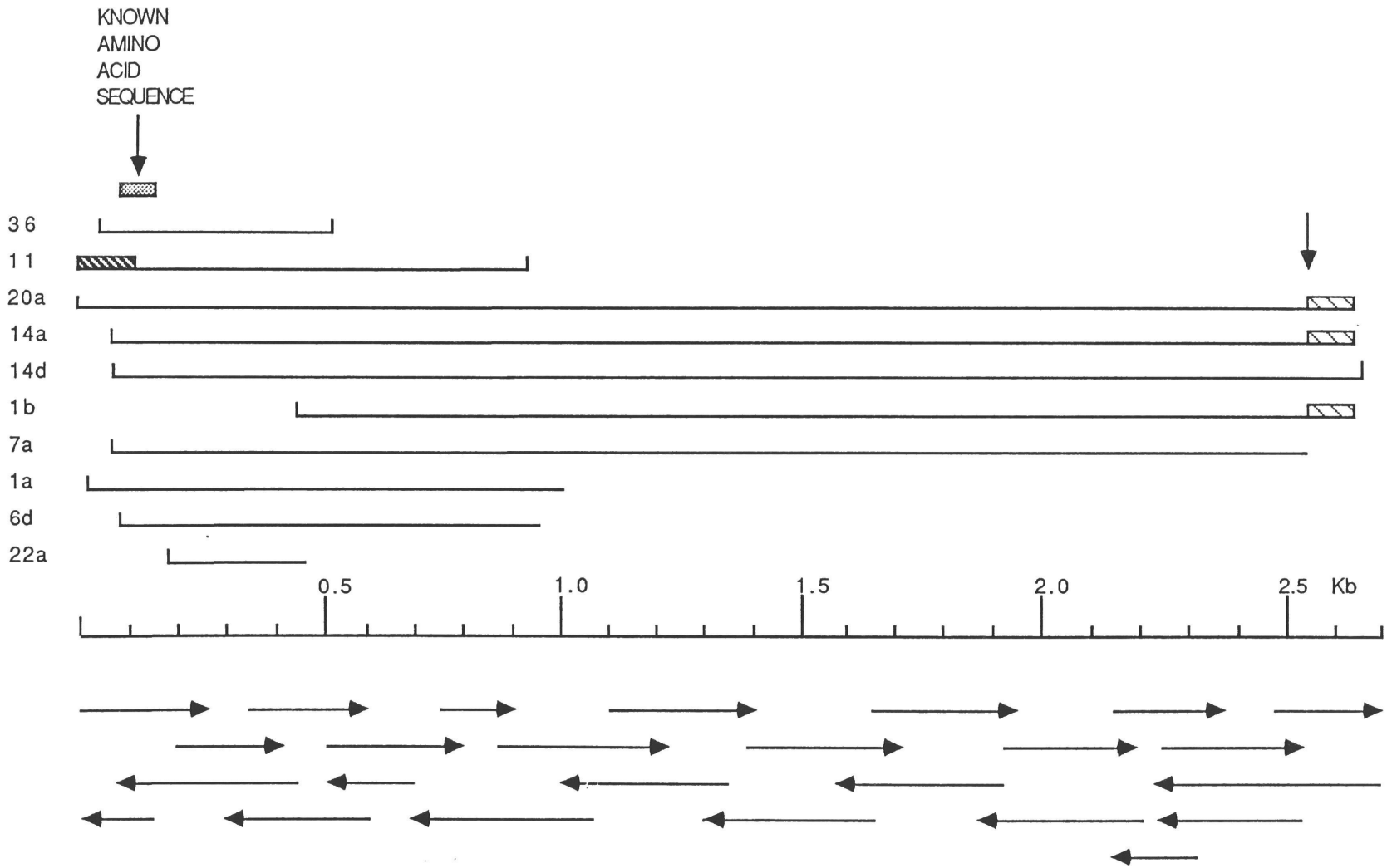

KNOWN
AMINO
ACID
SEQUENCE
36
11
20a
14a
14d
1b
7a
1a
6d
22a
0.5
1.0
1.5
2.0
2.5
Kb

FIGURE 27. Complete Nucleotide and Derived Amino Acid Sequence.

The complete nucleotide and amino acid sequence (derived from the largest open reading frame) is presented. Clone 20a corresponds to nucleotides 1-2507, after which the cDNA has a stretch of 46 A's. Clone 14d corresponds to nucleotides 65-2645. The top strand of nucleotides reads from 5' to 3', the derived amino acids are indicated on the second line.

```
        10         20         30         40         50         60
CCGGAAGCGAGTTGCGACACGGCAGGTTCCCGCCCGGAAGAAGCGACCAAAGCGCCTGAG
 R  K  R  V  A  T  R  Q  V  P  A  R  K  K  R  P  K  R  L  R

        70         80         90        100        110        120
GACCGGCAACATGGTGCGGTCGGGGAATAAGGCAGCTGTTGTGCTGTGTATGGACGTGGG
  T  G  N  M  V  R  S  G  N  K  A  A  V  V  L  C  M  D  V  G

       130        140        150        160        170        180
CTTTACCATGAGTAACTCCATTCCTGGTATAGAATCCCCATTTGAACAAGCAAAGAAGGT
  F  T  M  S  N  S  I  P  G  I  E  S  P  F  E  Q  A  K  K  V

       190        200        210        220        230        240
GATAACCATGTTTGTACAGCGACAGGTGTTTGCTGAGAACAAGGATGAGATTGCTTTAGT
  I  T  M  F  V  Q  R  Q  V  F  A  E  N  K  D  E  I  A  L  V

       250        260        270        280        290        300
CCTGTTTGGTACAGATGGCACTGACAATCCCCTTTCTGGTGGGGATCAGTATCAGAACAT
  L  F  G  T  D  G  T  D  N  P  L  S  G  G  D  Q  Y  Q  N  I

       310        320        330        340        350        360
CACAGTGCACAGACATCTGATGCTACCAGATTTTGATTTGCTGGAGGACATTGAAAGCAA
  T  V  H  R  H  L  M  L  P  D  F  D  L  L  E  D  I  E  S  K

       370        380        390        400        410        420
AATCCAACCAGGTTCTCAACAGGCTGACTTCCTGGATGCACTAATCGTGAGCATGGATGT
  I  Q  P  G  S  Q  Q  A  D  F  L  D  A  L  I  V  S  M  D  V

       430        440        450        460        470        480
GATTCAACATGAAACAATAGGAAAGAAGTTTGAGAAGAGGCATATTGAAATATTCACTGA
  I  Q  H  E  T  I  G  K  K  F  E  K  R  H  I  E  I  F  T  D

       490        500        510        520        530        540
CCTCAGCAGCCGATTCAGCAAAAGTCAGCTGGATATTATAATTCATAGCTTGAAGAAATG
  L  S  S  R  F  S  K  S  Q  L  D  I  I  I  H  S  L  K  K  C

       550        560        570        580        590        600
TGACATCTCCCTGCAATTCTTCTTGCCTTTCTCACTTGGCAAGGAAGATGGAAGTGGGGA
  D  I  S  L  Q  F  F  L  P  F  S  L  G  K  E  D  G  S  G  D

       610        620        630        640        650        660
CAGAGGAGATGGCCCCTTTCGCTTAGGTGGCCATGGGCCTTCCTTTCCACTAAAAGGAAT
  R  G  D  G  P  F  R  L  G  G  H  G  P  S  F  P  L  K  G  I

       670        680        690        700        710        720
TACCGAACAGCAAAAAGAAGGTCTTGAGATAGTGAAAATGGTGATGATATCTTTAGAAGG
  T  E  Q  Q  K  E  G  L  E  I  V  K  M  V  M  I  S  L  E  G

       730        740        750        760        770        780
TGAAGATGGGTTGGATGAAATTTATTCATTCAGTGAGAGTCTGAGAAAACTGTGCGTCTT
  E  D  G  L  D  E  I  Y  S  F  S  E  S  L  R  K  L  C  V  F
```

```
      790        800        810        820        830        840
CAAGAAAATTGAGAGGCATTCCATTCACTGGCCCTGCCGACTGACCATTGGCTCCAATTT
  K  K  I  E  R  H  S  I  H  W  P  C  R  L  T  I  G  S  N  L

      850        860        870        880        890        900
GTCTATAAGGATTGCAGCCTATAAATCGATTCTACAGGAGAGAGTTAAAAAGACTTGGAC
  S  I  R  I  A  A  Y  K  S  I  L  Q  E  R  V  K  K  T  W  T

      910        920        930        940        950        960
AGTTGTGGATGCAAAAACCCTAAAAAAAGAAGATATACAAAAAGAAACAGTTTATTGCTT
  V  V  D  A  K  T  L  K  K  E  D  I  Q  K  E  T  V  Y  C  L

      970        980        990       1000       1010       1020
AAATGATGATGATGAAACTGAAGTTTTAAAAGAGGATATTATTCAAGGGTTCCGCTATGG
  N  D  D  D  E  T  E  V  L  K  E  D  I  I  Q  G  F  R  Y  G

     1030       1040       1050       1060       1070       1080
AAGTGATATAGTTCCTTTCTCTAAAGTGGATGAGGAACAAATGAAATATAAATCGGAGGG
  S  D  I  V  P  F  S  K  V  D  E  E  Q  M  K  Y  K  S  E  G

     1090       1100       1110       1120       1130       1140
GAAGTGCTTCTCTGTTTTGGGATTTTGTAAATCTTCTCAGGTTCAGAGAAGATTCTTCAT
  K  C  F  S  V  L  G  F  C  K  S  S  Q  V  Q  R  R  F  F  M

     1150       1160       1170       1180       1190       1200
GGGAAATCAAGTTCTAAAGGTCTTTGCAGCAAGAGATGATGAGGCAGCTGCAGTTGCACT
  G  N  Q  V  L  K  V  F  A  A  R  D  D  E  A  A  A  V  A  L

     1210       1220       1230       1240       1250       1260
TTCCTCCCTGATTCATGCTTTGGATGACTTAGACATGGTGGCCATAGTTCGATACGCTTA
  S  S  L  I  H  A  L  D  D  L  D  M  V  A  I  V  R  Y  A  Y

     1270       1280       1290       1300       1310       1320
TGACAAAAGAGCTAATCCTCAAGTCGGCGTGGCTTTTCCTCATATCAAGCATAACTATGA
  D  K  R  A  N  P  Q  V  G  V  A  F  P  H  I  K  H  N  Y  E

     1330       1340       1350       1360       1370       1380
GTGTATTGTGTATGTGCAGCTGCCTTTCATGGAAGACTTGCGGCAATACATGTTTTCATC
  C  I  V  Y  V  Q  L  P  F  M  E  D  L  R  Q  Y  M  F  S  S

     1390       1400       1410       1420       1430       1440
CTTGAAAAACAGTAAGAAATATGCTCCCACCGAGGCACAGTTGAATGCTGTTGATGCTTT
  L  K  N  S  K  K  Y  A  P  T  E  A  Q  L  N  A  V  D  A  L

     1450       1460       1470       1480       1490       1500
GATTGACTCCATGAGCTTGGCAAAGAAAGATGAGAAGACAGACACCCTTGAAGACTTGTT
  I  D  S  M  S  L  A  K  K  D  E  K  T  D  T  L  E  D  L  F

     1510       1520       1530       1540       1550       1560
TCCAACCACCAAAATCCCAAATCCTCGATTTCAGAGATTATTTCAGTGTCTGCTGCACAG
  P  T  T  K  I  P  N  P  R  F  Q  R  L  F  Q  C  L  L  H  R
```

```
      1570       1580       1590       1600       1610       1620
AGCTTTACATCCCCGGGAGCCTCTACCCCCAATTCAGCAGCATATTTGGAATATGCTGAA
  A   L   H   P   R   E   P   L   P   P   I   Q   Q   H   I   W   N   M   L   N

      1630       1640       1650       1660       1670       1680
TCCTCCCGCTGAGGTGACAACGAAAAGTCAGATTCCTCTCTCTAAAATAAAGACCCTTTT
  P   P   A   E   V   T   T   K   S   Q   I   P   L   S   K   I   K   T   L   F

      1690       1700       1710       1720       1730       1740
TCCTCTGATTGAAGCCAAGAAAAAGGATCAAGTGACTGCTCAGGAAATTTTCCAAGACAA
  P   L   I   E   A   K   K   K   D   Q   V   T   A   Q   E   I   F   Q   D   N

      1750       1760       1770       1780       1790       1800
CCATGAAGATGGACCTACAGCTAAAAAATTAAAGACTGAGCAAGGGGGAGCCCACTTCAG
  H   E   D   G   P   T   A   K   K   L   K   T   E   Q   G   G   A   H   F   S

      1810       1820       1830       1840       1850       1860
CGTCTCCAGTCTGGCTGAAGGCAGTGTCACCTCTGTTGGAAGTGTGAATCCTGCTGAAAA
  V   S   S   L   A   E   G   S   V   T   S   V   G   S   V   N   P   A   E   N

      1870       1880       1890       1900       1910       1920
CTTCCGTGTTCTAGTGAAACAGAAGAAGGCCAGCTTTGAGGAAGCGAGTAACCAGCTCAT
  F   R   V   L   V   K   Q   K   K   A   S   F   E   E   A   S   N   Q   L   I

      1930       1940       1950       1960       1970       1980
AAATCACATCGAACAGTTTTTGGATACTAATGAAACACCGTATTTTATGAAGAGCATAGA
  N   H   I   E   Q   F   L   D   T   N   E   T   P   Y   F   M   K   S   I   D

      1990       2000       2010       2020       2030       2040
CTGCATCCGAGCCTTCCGGGAAGAAGCCATTAAGTTTTCAGAAGAGCAGCGCTTTAACAA
  C   I   R   A   F   R   E   E   A   I   K   F   S   E   E   Q   R   F   N   N

      2050       2060       2070       2080       2090       2100
CTTCCTGAAAGCCCTTCAAGAGAAAGTGGAAATTAAACAATTAAATCATTTCTGGGAAAT
  F   L   K   A   L   Q   E   K   V   E   I   K   Q   L   N   H   F   W   E   I

      2110       2120       2130       2140       2150       2160
TGTTGTCCAGGATGGAATTACTCTGATCACCAAAGAGGAAGCCTCTGGAAGTTCTGTCAC
  V   V   Q   D   G   I   T   L   I   T   K   E   E   A   S   G   S   S   V   T

      2170       2180       2190       2200       2210       2220
AGCTGAGGAAGCCAAAAAGTTTCTGGCCCCCAAAGACAAACCAAGTGGAGACACAGCAGC
  A   E   E   A   K   K   F   L   A   P   K   D   K   P   S   G   D   T   A   A

      2230       2240       2250       2260       2270       2280
TGTATTTGAAGAAGGTGGTGATGTGGACGATTTATTGGACATGATATAGGTCGTGGATGT
  V   F   E   E   G   G   D   V   D   D   L   L   D   M   I

      2290       2300       2310       2320       2330       2340
ATGGGGAATCTAAGAGAGCTGCCATCGCTGTGATGCTGGGAGTTCTAACAAAACAAGTTG
```

2350 2360 2370 2380 2390 2400
GATGCGGCCATTCAAGGGGAGCCAAAATCTCAAGAAATTCCCAGCAGGTTACCTGGAGGC

2410 2420 2430 2440 2450 2460
GGATCATCTAATTCTCTGTGGAATGAATACACACATATATATTACAAGGGATAATTTAGA

2470 2480 2490 2500 2510 2520
CCCCATACAAGTTTATAAAGAGTCATTGTTATTTTCTGGTTGGTGTATTATTTTTTCTGT

2530 2540 2550 2560 2570 2580
GGTCTTACTGATCTTTGTATATTACATACATGCTTTGAAGTTTCTGGAAAGTAGATCTTT

2590 2600 2610 2620 2630 2640
TCTTGACCTAGTATATCAGTGACAGTTGCAGCCCTTGTGATGTGATTAGTGTCTCATGTG

GAACC

This suggests that there may be different polyA sites, and this conjecture is in agreement with the presence of two mRNAs as detected by northern blot analysis (discussed below).

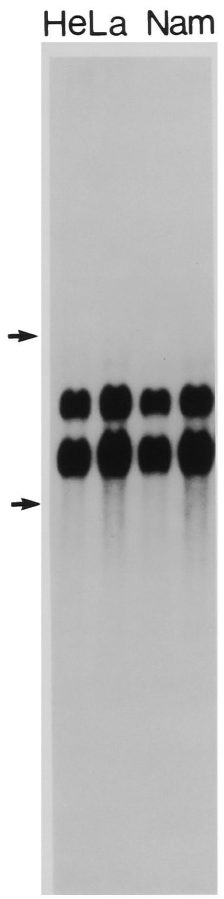
We have initiated experiments aimed at proving definitively that this cDNA encodes OTF-1. Our evidence so far is the following. Isolation and identification by three independent investigators of OTF-1 as a 90 kDa protein suggests that this is the correct polypeptide. Secondly, quantitation of binding activity suggest that the major proportion of the band must be active. Additionally, given that completely distinct purifications result in preparations which have equivalent mass of OTF-1 based on specific binding activity suggests that this band is homogeneous. Finally, N-terminal amino acid analysis agrees in molar amounts with mass quantitation of OTF-1 and gives a single sequence. We have subcloned the cDNA into a bacterial expression vector and hope to obtain specific functional activity from lysates of bacteria harboring this plasmid. We have proceeded with more extensive characterization of the cDNA on the assumption that it is the *bona fide* clone.

Characterization of Clone 20a

Our initial experiment was to perform northern blot analysis of oligo-dT selected RNA from asynchronously growing Hela and Namalwa cells. This RNA was probed with radiolabelled 20a insert DNA. As is shown in Figure 28 this revealed the presence of two RNAs which hybridized strongly to the probe. The estimated size of the two RNAs are 2.7 and 4.0 kb. If the smaller RNA corresponds to p20a this would suggest that the clone is very close to full length. We are currently performing S1 analysis to determine if the size difference of the two RNAs is due to differences at the 3' end, as is suggested by the cloning data. It is apparent

FIGURE 28. Northern Blot Analysis of Oligo-dT Selected RNA Isolated From HeLa and Namalwa Cells.

Oligo-dT selected RNA (2 and 4 ug) isolated from HeLa and Namalwa cells was electrophoresed through formaldehyde/agarose gels and transferred to nitrocellulose. The membrane-bound RNA was then hybridized with a radiolabelled probe derived from purified 20a cDNA fragment. The pairs of lanes are two different amounts of RNA, the arrows indicate the positions of 18S and 28S RNA. The size of the detected bands are approximately 2.7 and 4.0 kb.


HeLa
Nam

that we do not detect any Namalwa specific RNA, suggesting that OTF-1 and OTF-2 are not highly similar, at least at the nucleotide level. Preliminary experiments have not detected any change in RNA levels in the course of the cell cycle.

There are several interesting features of the protein sequence that are worth noting (see Figure 29). There are multiple phosphorylation site consensus sequences, including one for casein dependent kinase, one for cAMP dependent kinase and one for tyrosine kinase (W. Todd Miller, personal communication). There is also a consensus sequence (Gly X Gly X X Gly X_{15-20} Lys) located between amino acids 197 and 218, which is found in the ATP binding domain of many kinases (Hunter, 1987). This sequence lacks, however, a series of other motifs which are diagnostic for kinase activity (Arg·Asp·Leu, Asp·Phe·Gly, Ala·Pro·Glu). We have initiated experiments aimed at determining if these sites are relevant to function of OTF-1.

We have not observed any homology to other known transcription factors, including the GRFs, TFIIIA, Sp1, and bacterial factors, such as cro and lambda repressor. We have also looked for sequences derived from CnBr peptides of OTF-2 (representing 12% of the total protein sequence of OTF-2), and have not found them contained in our derived sequence. Nor have we detected any of the known DNA binding motifs, such as have been found in bacterial activators, homeo-box proteins, or Zn finger containing proteins (Pabo and Sauer, 1985, Laughon and Scott, 1985, Berg, 1986). It has been recently observed by Steve McKnight that there is a series leucines repeated every seven amino acids contained within the DNA binding domain of C/EPB and a region of similar sequence in c-myc (personal communication). As these investigators have observed this repeat in several other nuclear oncogenes and transcription factors they have proposed that this motif represents a structural feature of a new class of DNA-binding proteins. We have observed a similar leucine repeat between amino acids 163 and 190 and are

FIGURE 29. Derived Amino Acid Sequence

The derived amino acid sequence of the largest open reading frame is presented. Highlights indicate consensus sequences for kinase phosphorylation sites (overline), and an ATP binding motif (double underline). Also indicated is a sequence of repeated leucines (underline). {S·K·K, Casein kinase, D·E·I·Y, Tyrosine kinase, and K·K·A·S, cAMP kinase}.

```
                                    10                                  20
R  K  R  V  A  T  R  Q  V  P  A  R  K  K  R  P  K  R  L  R
                                    30                                  40
T  G  N  M  V  R  S  G  N  K  A  A  V  V  L  C  M  D  V  G
                                    50                                  60
F  T  M  S  N  S  I  P  G  I  E  S  P  F  E  Q  A  K  K  V
                                    70                                  80
I  T  M  F  V  Q  R  Q  V  F  A  E  N  K  D  E  I  A  L  V
                                    90                                 100
L  F  G  T  D  G  T  D  N  P  L  S  G  G  D  Q  Y  Q  N  I
                                   110                                 120
T  V  H  R  H  L  M  L  P  D  F  D  L  L  E  D  I  E  S  K
                                   130                                 140
I  Q  P  G  S  Q  Q  A  D  F  L  D  A  L  I  V  S  M  D  V
                                   150                                 160
I  Q  H  E  T  I  G  K  K  F  E  K  R  H  I  E  I  F  T  D
                                   170                                 180
L  S  S  R  F  S  K  S  Q  L  D  I  I  I  H  S  L  K  K  C
                                   190                                 200
D  I  S  L  Q  F  F  L  P  F  S  L  G  K  E  D  G  S  G  D
                                   210                                 220
R  G  D  G  P  F  R  L  G  G  H  G  P  S  F  P  L  K  G  I
                                   230                                 240
T  E  Q  Q  K  E  G  L  E  I  V  K  M  V  M  I  S  L  E  G
                                   250                                 260
E  D  G  L  D  E  I  Y  S  F  S  E  S  L  R  K  L  C  V  F
                                   270                                 280
K  K  I  E  R  H  S  I  H  W  P  C  R  L  T  I  G  S  N  L
                                   290                                 300
S  I  R  I  A  A  Y  K  S  I  L  Q  E  R  V  K  K  T  W  T
                                   310                                 320
V  V  D  A  K  T  L  K  K  E  D  I  Q  K  E  T  V  Y  C  L
                                   330                                 340
N  D  D  D  E  T  E  V  L  K  E  D  I  I  Q  G  F  R  Y  G
```

S D I V P F S K V 350 D E E Q M K Y K S E 360 G

K C F S V L G F C 370 K S S Q V Q R R F F 380 M

G N Q V L K V F A 390 A R D D E A A A V A 400 L

S S L I H A L D D 410 L D M V A I V R Y A 420 Y

D K R A N P Q V G 430 V A F P H I K H N Y 440 E

C I V Y V Q L P F 450 M E D L R Q Y M F S 460 S

L K N S K K Y A P 470 T E A Q L N A V D A 480 L

I D S M S L A K K 490 D E K T D T L E D L 500 F

P T T K I P N P R 510 F Q R L F Q C L L H 520 R

A L H P R E P L P 530 P I Q Q H I W N M L 540 N

P P A E V T T K S 550 Q I P L S K I K T L 560 F

P L I E A K K K D 570 Q V T A Q E I F Q D 580 N

H E D G P T A K K 590 L K T E Q G G A H F 600 S

V S S L A E G S V 610 T S V G S V N P A E 620 N

F R V L V K Q K K 630 A S F E E A S N Q L 640 I

N H I E Q F L D T 650 N E T P Y F M K S I 660 D

C I R A F R E E A 670 I K F S E E Q R F N 680 N

690 700

F L K A L Q E K V E I K Q L N H F W E I

710 720

V V Q D G I T L I T K E E A S G S S V T

730 740

A E E A K K F L A P K D K P S G D T A A

750

V F E E G G D V D D L L D M I

intrigued by the possibility that this region may fit the leucine repeat model. One way of ascertaining if it is a functional motif would be to determine if we could map DNA binding activity to an adjacent region as this motif has been associated with DNA binding regions in other proteins. It is also possible that if this motif is important for protein-protein interaction then it may play a role in transactivation by Vmw65, for instance. The manipulation and expression of the cDNA should allow us to make a detailed analysis of the structure and function of this factor. We hope to be able to use either purified factor derived from bacteria in *in vitro* transcription and replication assays, or factor produced in eukaryotic cells lacking OTF-1 (by transfection) in hopes of being able to define and dissect the relevant functional domains.

SUMMARY and PERSPECTIVES

Conclusions

A central question of histone gene regulation concerns the pleiotropic induction of transcription at the G1/S boundary. This extremely rapid and dynamic regulation of transcription has been shown to operate through promoter proximal DNA sequences. Recent *in vivo* analyses have identified the subtype specific sequences of human H2b and chicken H1 as necessary and sufficient for this regulation (LaBella et al., 1988, Dalton and Wells, 1988). This text describes the purification and characterization of a transcription factor which specifically binds the H2b subtype element and activates transcription from this promoter *in vitro*. Functional characterization of this factor has demonstrated that it has an intrinsic stimulatory activity, which is dependent upon binding to the H2b subtype specific consensus element. Furthermore, distal binding factors are not required as stimulation of transcription requires only the TATA motif and general transcription factors. Investigation of binding and function has demonstrated that activity of the factor *per se* is dependent on the octamer sequence (ATTTGCAT) only.

Although this factor may productively interact with distal binding factors, the detection of basal transcription activity throughout the cell cycle (by *in vivo* analysis) suggests that this factor does not induce transcription of the H2b gene by nucleation of a completely new transcription complex. Functional analysis, by *in vitro* transcription, has shown that the stimulatory activity is absent in nuclear extracts prepared from cells synchronized in G2. It is possible to stimulate transcription in this extract by addition of purified OTF-1, suggesting that function does not require any other S phase specific factors. Interestingly, we have not detected any change in the mass or binding activity of this factor during the cell

cycle. Therefore we suggest that activation of S phase specific function is due either to *in vivo* constraints on binding, or to covalent (or other) modifications of OTF-1.

This data has substantially refined previous observations regarding regulation of histone gene expression. We present a model which suggests that regulation of the histone gene family is due to the action of multiple, independent subtype specific factors. This suggestion is based on two observations. The first is the that OTF-1 does not interact with the H4 promoter, nor do H4 factors interact with the H2b promoter. The second is that there is a striking conservation of sequence and position of the various subtype specific elements. Thus, the model emphasizes the general similarities in histone promoter structure in defining three domains of these promoters. These include the core promoter (TATA motif and cap site), the distal activating domain, and the regulatory domain (proximal subtype specific element).

Another feature of histone gene regulation is that it appears to be tightly coupled (in higher eukaryotes) to DNA synthesis. We have demonstrated that OTF-1 is able to stimulate initiation of adenovirus DNA replication in a manner that is indistinguishable from NF-III. Not only do these factors display the same DNA binding specificity and affinity, but they have equivalent molecular weights. We therefore conclude that OTF-1 and NF-III are identical. Our observation that OTF-1 is able to activate replication of adenovirus DNA suggests the possibility that this factor could be active in chromosomal DNA replication as well. It is an exciting possibility, then, that a common mechanism of regulation of activation of OTF-1 could be responsible for the biochemical coupling of histone gene transcription and DNA synthesis. At the very least, this observation raises fundamental questions concerning the mechanism of action of sequence specific DNA-binding proteins.

The opportunity to investigate these two reasonably well defined systems in a comparative fashion should yield valuable insight into the regulation of gene expression. This type of analysis should be greatly facilitated by the opportunity to manipulate the factor through mutagenesis of a cDNA clone. Our preliminary data suggests that we have isolated a clone for OTF-1; our immediate aim is to prove this definitively. Assuming that we do have the clone, we have the tools necessary for investigation the fine structure and primary sequence of OTF-1 as they relate to these different stimulatory functions.

Finally, we have an opportunity to investigate the regulation of cellular proliferation by virtue of the fact that we have identified and isolated an end substrate for the biochemical cascade that culminates in the entry of cells into S phase. Development of immunological tools will facilitate the identification of the activity which regulates OTF-1. Our long term prospect then is to be able to define the regulatory circuits which govern the commitment to cellular reproduction.

MATERIALS AND METHODS

Construction of fusion genes. A subset of the 5' deletion and point mutants in the H2b promoter described by Sive et al., 1986 was employed in this study. The parental wild type promoter fragment, which was derived from the 3' deletion 16-3, spanned the promoter from a Pst I site at -178 to a Hind III linker at -19. Ligation of a Hind III linker to the 5' Alu site of the G minus cassette (Sawadago and Roeder, 1985) and subsequent cleavage at the 3' Bam HI site allowed these fragments to be ligated into Pst I/Bam HI cut pUC 12. A Hind III/Hae II (-21) fragment from a -59 5' deletion mutant was then ligated to a Hae II/Bam HI cassette fragment derived from the initial construct. In addition, a Pst I (-178)/Hae II(-21) fragment from the octamer double point mutant (OM-A) was ligated to the Hae II/Bam HI cassette fragment to construct the Oct- mutant. A Hinf I(-36)/Bam HI cassette fragment was also subcloned to make a TATA only mutant. A substitution of the H2b consensus sequence was constructed by ligating synthetic oligonucleotides to the Hinf I/Bam HI cassette fragment. The u heavy chain enhancer sequence oligonucleotides consisted of the following sequence

AATTCCACCACCTGGGTAATTTGCATTTCTG . This Eco RI/BamHI
GGTGGTGGACCCATTAAACGTAAAGACTAA

cassette fragment was then subcloned with a Hind III/Eco RI fragment derived from OMF which spanned the promoter from -178 to -68 in order to reconstruct the entire promoter.

Preparation of nuclear extracts, and in vitro transcription. Nuclear extracts were prepared essentially as described by Heintz and Roeder, 1984. Cell synchronization was performed essentially as described in Heintz et al., 1983. *In vitro* transcriptions were performed in buffer containing 10 ug/ml template DNA, 3mM $MgCl_2$, 12 mM Tris (7.9), 0.1 M Hepes (N-2-hydroxyethylpiperazine-N'-2-

ethanesulfonic acid) (7.9), 60 mM KCl, 200 uM each of FPLC purified ATP and UTP, 0.25 uM CTP, 2 units of RNase T1, 4 mM 3'O methyl GTP, 12% glycerol, and 5 uCi of a-^{32}P CTP. After incubation for 30 minutes at 30^{o} C the reactions were processed as described in Dignam et al., 1983. In the case of reconstitution assays, 60 ug of protein from the phosphocellulose 0.5 M KCl and 0.8 M KCl fractions were used instead of nuclear extract. The latter were isolated by step elution of the phophocellulose-bound material described below.

In Vitro DNA Replication Assay. *In vitro* DNA replication and initiation reactions were performed essentially as described (Wides et al., 1987, Rosenfeld and Kelly, 1986 [respectively]). The standard *in vitro* replication reaction mixture (25 ul) contained 30 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2% dimethyl sulfoxide, 2 mM ATP, 25 uM α-^{32}P dCTP (18 Ci/mmol), 25 uM each dATP, dGTP, dTTP, 20 ug of plasmid DNA per ml, and purified proteins as described in the figure legend. Reactions were terminated by the addition of SDS to 1% and EDTA to 25mM. The reaction products were collected by ethanol precipitation, dissolved in 1% SDS-10mM EDTA and analyzed by electrophoresis through a 1.4% agarose gel. The standard replication initiation reaction (25 ul) contained 25 mM HEPES, 5 mM $MgCl_2$, 1 mM dithiothreitol, 4 mM ATP, 40 ug/ml aphidicolin, 0.15 uM α-^{32}P dCTP (3,000 Ci/mm), 50 ng Ad DNA-protein complex, and purified proteins as indicated in the figure legend. Reactions were terminated by incubation at 70^{o} C for 5 min, and subsequently digested with micrococcal nuclease (15 U) at 37^{o} C for 30 min. Protein was precipitated by addition of one third volume of 2mg/ml deoxycholate/50% TCA and collected by centrifugation. The samples were resuspended in SDS-PAGE sample buffer and electrophoresed through an 8% SDS-polyacrylamide gel.

Chromatography. The initial 75 ml phosphocellulose (Whatman) and 35 ml DEAE Fast Flow Sepharose (Pharmacia) columns were equilibrated in buffer BC-100 (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsufonyl floride, 100 mM KCl) and run in tandem at one and two column volumes per hour, respectively. After 150 mls of nuclear extract (average 10 mg/ml, from 3 x 10^{10} cells) was loaded, the flowthrough was collected and dialyzed against the same buffer containing 50 mM Hepes (pH 6.9) in place of Tris (HC-100). The dialyzed material was then loaded at four column volumes per hour on a 15 ml Biorex 70 column equilibrated in HC-100. Bound material was eluted with 350 mM KCl (in HC buffer). Eluted protein was then dialyzed against BC-60. After loading on a 1.5 ml double-stranded DNA column at four column volumes per hour, the bound protein was eluted with 350 mM KCl (in BC buffer). Again the eluted step was dialyzed against BC-60 and subsequently loaded on the 200 ul oligonucleotide column in the same manner. After washing with several column volumes of BC-60 the oligonucleotide column was eluted with a ten column volume gradient of KCl from 70 to 700 mM. The DNA columns were prepared essentially as described by the manufacturer (Pharmacia), with approximately 250 ug of DNA coupled per ml of resin. The nonspecific column consisted of sonicated salmon sperm DNA (modal size 1 kb). The specific DNA column consisted of the oligonucleotide sequence shown below (Renaturation), except that the bottom strand had an additional ten nucleotides of nonspecific sequence to facilitate coupling. Large scale preparation of OTF-1 consisted of increasing volumes and column sizes three fold. In addition, after dialysis to 100 mM KCl, the material eluted from the non-specific DNA column was passed through a non-specific oligonucleotide column at 4 column volumes per hour. The flowthrough was collected, diluted 1:1 in BC-0, and applied to the specific oligonucleotide

column. The oligonucleotide column was the washed briefly with BC-60, then eluted with BC-225, and finally eluted with BC-650. Protein concentrations were determined by the method of Bradford, 1976. Activity recovery was quantitated by gel shift analysis. Aliquots of each step were incubated as described in Sive and Roeder, 1986; after electrophoresis and autoradiography the films were scanned with a Beckman densitometer. In addition, binding reactions (performed exactly as described by Sawadago and Roeder 1985a, except 500 ng of salmon sperm DNA was used as cold competitor) were filtered through nitrocellulose filters and were counted in scintillation fluid. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Laemmli, 1970. Gels were silver stained with Rapid Ag Stain according to manufacturer's instructions (ICN).

DNase I Protection. The probe used in the H2b footprinting reactions extended from -100 to +28. It was subcloned in pUC 12 to facilitate single end labeling. Either the upper or lower strand was labeled with the Klenow fragment of DNA Polymerase I. One ng of the probe was incubated in 20 mM Hepes, pH 7.9, 2mM $MgCl_2$, and 5 ug/ml salmon sperm DNA with the chromatographic fractions. After a 5 minute incubation at 20^{o} C, one ul of DNase I at 10 ug/ml was added and the reaction incubated at 30^{o} C for 30 seconds. The reaction was stopped by the addition of 80 ul of stop buffer (7 mM EDTA, 0.35 M sodium acetate, pH 5.2, 0.1% SDS and 500 ug/ml yeast tRNA). The DNA was isolated by phenol/chloroform and chloroform extractions followed by ethanol precipitation. The DNA was then analyzed on an 8% polyacrylamide/50% urea gel. A "G" reaction on each probe was performed by the method of Maxam and Gilbert, 1980. MPE protection assays were performed in the same manner except MPE was substituted for DNase I. For the MPE reactions, 2 ul of a 100 uM MPE-200 uM ferrous ammonium sulfate solution

was added and the reaction time was 2 minutes at 30° C. Preparation and manipulation of the Ad 2 probe was performed as described in O'Neill and Kelly, 1988.

Renaturation. Renaturation of activity was performed essentially as described by Hager and Burgess, 1980. The gel retention assay was performed as described by Sive and Roeder, 1986, using 10 ul of the 1 ml of renatured material and 3 ng of probe. The buffer included 100 ng of salmon sperm DNA and 100 ng of either a nonspecific double-stranded oligonucleotide GGGTGAGACTCCTCTTGC or a specific double-stranded oligonucleotide CTTCACCTTATTTGCATAAGC. All oligonucleotides were synthesized on an ABI 380B and purified by lyophilization, ethanol precipitation and gel filtration through G-50.

Electrophoretic Mobility Shift Assay With the Adenovirus Probe. Standard reactions (12.5 ul) contained 25 mM HEPES, 1 mM dithiothreitol, 0.5 mM EDTA, 0.05 % Nonidet P-40, 50 ng of poly dI-dC, 2.5 fmol probe, 0.4 mg BSA. Samples were electrophoresed through 2% agarose. Electrophoresis buffer contained 12mM Tris-acetate, 1 mM EDTA, and 0.01% NP-40, pH 7.5. Following electrophoresis gels were dried on DEAE paper under vacuum. Specific conditions for competition analysis are described in the figure legend.

Electrophoretic Transfer of Protein Following SDS-Polyacrylamide Gel Electrophoresis. After electrophoresis gels were briefly equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). The gel was then assembled into a sandwich as described by Towbin et al., 1979. Transfer was performed either for two hours at 70 V or overnight at 20 V (4° C in both cases). Membranes were then manipulated as described in figure legends. Transfer to

Immobilon was performed as follows. Gels were equilibrated in transfer buffer for 15 minutes (10 mM CAPS [3-{cyclohexylamino}-1-propanesulfonic acid], 10% methanol, pH 11). Blotting was performed for 30 minutes at 0.5 A. Following transfer the membrane was washed with deionized water, stained with 0.1% Coomassie Blue in 50% methanol for 2 minutes and destained with 50% methanol, 10% acetic acid. N-terminal amino acid sequence analysis was performed at the Rockefeller University Protein Sequencing Facility on an Applied Biosystem 470 with on-line PTH amino acid analysis.

Library Screening, Subcloning, Sequencing, and Northern Blot Analysis. Library plating, phage propagation and DNA preparation was performed essentially as described in Maniatis et al., 1982. Plaque lifts were processed as follows. Following the lift, filters were air dried for 5 minutes, assembled between sheets of Whatman 3MM paper and autoclaved at 100^{o} C under reduced steam pressure for 2 minutes, quick dried for 15 s., and the autoclave was then rapidly depressurized. The filters were then removed from the autoclave and baked at 80^{o} C for 2 hrs. Filters were probed in batch as described in Maniatis et al., 1982. Insert DNA was excised by Eco RI digestion and subcloned into pBKS^{-} by the usual techniques. Sequence analysis of double-stranded plasmids was performed using the US Biochemicals Sequenase Kit according to manufacturers instructions. Sequence analysis of insert DNA in lambda gt10 phage was performed in a similar manner (alkaline denaturation) using a 1:1 ratio of ^{23}P kinased oligonucleotide primer to template. Most of the sequence was obtained by the strategy of synthesizing specific oligonucleotide primers according to the suggestions of Barnes, 1987. Sequence compilation and analysis was performed using *Microgenie*. Northern blot analysis

was performed according to Maniatis et al., 1982. Insert DNA was radiolabelled by extension from random primers according to manufacturer's instructions (Boehringer Mannheim).

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