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A partial human ribosomal DNA repeat unit transfected into a human fibroblast cell line functions as a nucleolar organizing region

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A PARTIAL HUMAN RIBOSOMAL DNA REPEAT UNIT TRANSFECTED INTO A HUMAN FIBROBLAST CELL LINE FUNCTIONS AS A NUCLEOLAR ORGANIZING REGION

BY

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Abstract

A PARTIAL HUMAN RIBOSOMAL DNA REPEAT UNIT TRANSFECTED INTO A HUMAN FIBROBLAST CELL LINE FUNCTIONS AS A NUCLEOLAR ORGANIZING REGION

by Kathi Anne McDowell

Advisor: Professor Ann S. Henderson

The role of rDNA in nucleolar formation was investigated by creating a "new" rDNA site within the human genome. Human rDNA, isolated from a cosmid library, was transfected into human fibroblast cells. One transfectant (ML1 DNA) contained the rDNA transcription unit and most of the flanking non-transcribed spacer DNA. A second (ML4 DNA) contained the transcription unit and most of the non-transcribed spacer, but lacked a promotor region. One copy of ML1 DNA inserted into the pericentromeric region of the p-arm of human chromosome 3; ML4 DNA was localized to the q-arm of chromosome 4. The insertion site containing the promotor was transcriptively active, but there was no detectable transcription at the site lacking a promotor. The insertion site containing the rDNA promotor was present in nucleoli and participated in resultant satellite associations. This study shows that the rDNA promotor region is required in nucleolar formation and that tandem repetition of rDNA is not an absolute requirement for participation in nucleolar activity.

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INTRODUCTION

A. Objectives

The aim of this research was to examine the role of rDNA in nucleolar formation in human cells. We wished to determine how many contiguous units of the rDNA gene were required and whether the site must be transcriptively active for nucleolar participation. The study was initiated by constructing "new", additional rDNA site in the human genome by transfection of human rDNA containing critical portions of the rDNA repeat unit. The successful creation of the additional rDNA site resulted in new information as to the number and type of rDNA sequences required in nucleolar formation and resultant satellite associations. This study showed that the rDNA promotor region is required in nucleolar formation and that tandem repetition of rDNA is not an absolute requirement for participation in nucleolar activity.

Ultimately the cell lines created in this study will be used to determine the relationship between satellite association and chromosomal anomalies involving rDNA. Satellite association in metaphase is presumed to represent a remnant of previous nucleolar activity. The close association of acrocentric chromosomes in nucleolar formation could theoretically cause chromosomal aberrations such as nondisjunction and Robertsonian-type translocations. It is postulated that presence of an additional rDNA site could perturb the normal association pattern in nucleolar organization. If this is the case, these studies have provided information that can be expanded in the future to determine the probability of a couple having a "special" child with a specific type of chromosomal abnormality, such as those

associated with Down syndrome.

B. General Background

Mature ribosomal RNA (rRNA) molecules of 5.8, 18 and 28S form a portion of the eukaryotic ribosome. The genes have been mapped to the five pairs of acrocentric chromosomes in people (Henderson *et al.*, 1972). These chromosomes have an exceptionally high rate of chromosomal aberrations, including nondisjunction and Robertsonian translocation. Nondisjunction occurs when homologous chromosomes fail to separate during first meiosis or sister chromatids during second meiosis. It is estimated that 95-97% of Down Syndrome cases are caused by nondisjunction of acrocentric chromosome 21 (reviewed in Schmickel *et al.*, 1985). The majority (88%) of nondisjunctional Down Syndrome (trisomy 21) occur in the oocyte (Warren *et al.*, 1987). Nondisjunction during meiosis I accounts for 77.1% of maternal nondisjunction, whereas meiosis II is principally responsible for paternal nondisjunction at a rate of 77.8% (Antonarakis *et al.*, 1992). One out of every 200 conceptions produces a zygote which is trisomic for chromosome 21 with 20% developing to term (Kajii *et al.*, 1973). At an early developmental stage there is a loss of the extra chromosome 21 in some of these embryos producing a diploid/trisomic individual. These mosaics account for 1-2% of people with Down syndrome (Niikawa and Kajii, 1984).

It has been speculated that a decrease in crossover rate within chromosome 21 may either foster nondisjunction or be the result of a mechanism that promotes nondisjunction. The recombination frequency for chromosome 21 has been studied with respect to both sex and age. There is a high rate of recombination at both the

centromeric region and telomeric region which decreases with age. In females, the rate of decline involving centromeric crossovers is less than in males (Tanzi *et al.*,1992).

Robertsonian translocations usually involve acrocentric chromosomes. They occur when two chromosomes are joined at the centromere to form a contiguous chromosome. If breakage and union of two chromosomes occur in the p-arm adjacent to the centromere, a dicentric chromosome can form (John and Freeman, 1975). Robertsonian translocations account for 3-5% of the Down syndrome cases, with less than one percent being due to reciprocal translocations (reviewed in Schmickel *et al.*, 1985). The incidence of Down syndrome due to translocations is about 5.2% per year. (Pulliam and Huether, 1986). Approximately 95% of these cases are due to Robertsonian translocations. The two most common Robertsonian translocations are t(14,21) and t(21,21) which occur at frequencies of 45.7% and 40.0%, respectively.

There are several characteristics of acrocentric chromosomes that make them prone to nondisjunction and Robertsonian translocations, each related to the presence of rDNA on the secondary constriction regions in the p-arm of human acrocentric chromosomes (Henderson *et al.*, 1972). The DNA exists as tandem repeats of 30-50 units per acrocentric chromosome. Wellaur and Dawid (1979) determined that the average size of a repeat unit is approximately 44 kb. Among 22 repeat units, one was as small as 29 kb and another was as large as 54 kb. These findings were based upon results obtained from R-loop mapping of DNA fragments generated by restriction enzyme analysis.

The rDNA unit consists of an externally transcribed spacer region (ETS), the 18S gene, two internally transcribed spacer regions (ITS), the 5.8S gene, the 28S gene, and a nontranscribed spacer region (NTS) (Figure 1). A 45S precursor rRNA molecule is the initial result of transcription [which occurs from telomere towards the centromere (Worton *et al.*, 1988)]. The 45S rRNA processed into 28S, 18S, and 5.8S rRNA (Wellauer and Dawid, 1973) (Figure 2). The 5.8S gene maps to the same chromosomal location, and is in the same proportion as the 18S and 28S genes, as expected (Henderson *et al.*, 1980). Its position within the repeat unit was determined by Erickson *et al.* (1981).

The promotor region, ETS, 18S gene and ITS are highly evolutionarily conserved. There are other regions which are more variable. For example, a highly variable region is located 400-800 bp upstream of the ETS (Wilson *et al.*, 1982). Although most 28S genes are highly conserved, throughout evolution one portion of the 28S gene has changed at a rate 75-fold faster than a more highly conserved region (Schmickel, 1987). There are two 28S sequence variants found within human populations which are located 800 bp from a known *Eco*R1 site (Gonzalez *et al.*, 1985, Arnheim *et al.*, 1980). The NTS section of the repeat unit is also variable in both length and sequence (Arnheim and Southern, 1977; Krystal and Arnheim, 1978; Wellauer and Dawid, 1979). The heterogeneity is specific, with four different length classes 0.8 kb apart (Krystal and Arnheim, 1978). It is speculated that the heterogeneity is due to both homologous and nonhomologous exchange of rDNA among the acrocentric chromosomes (Arnheim and Southern, 1977; Arnheim *et al.*, 1980; Krystal *et al.*, 1981; Erickson and Schmickel, 1985). One

Figure 1. Schematic diagram of an acrocentric chromosome. Acrocentric chromosomes have a very short p-arm in relationship to the q-arm. In people, there are 10 acrocentric chromosomes (chromosomal pairs 13, 14, 15, 21 and 22). Sandwiched between the centromere and the telomere (satellite region) is the nucleolar organizing region (dashes). This region houses 30-50 copies of tandemly repeated units of rDNA. One repeat unit is divided into 4 sections based upon the *Eco*RI restriction sites. These regions are referred to as the A, B, C and D regions. The C and D regions contain a nontranscribed spacer region (NTS). An externally transcribed spacer region (ETS) starts at a polymerase I promotor located approximately 500 bp into the B region (Miesfeld and Arnheim, 1982). Transcription proceeds through the 18S gene which transverses both the B and A regions. The same transcript will include the 5.8S gene product which is flanked by both internally transcribe spacer regions (TS). Transcription continues through the 28S gene located in the A region and is terminated in the D region of the next repeat unit.

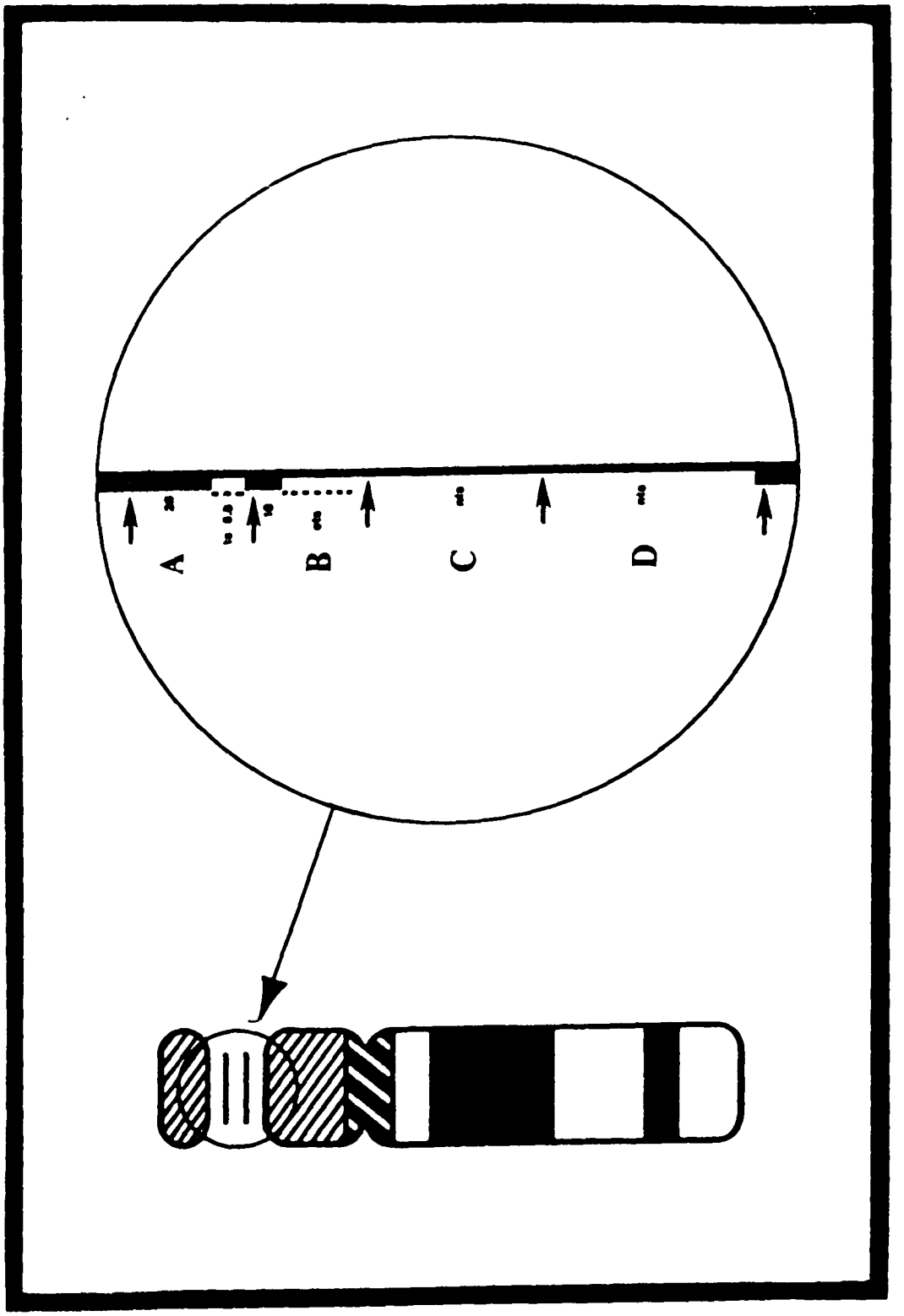
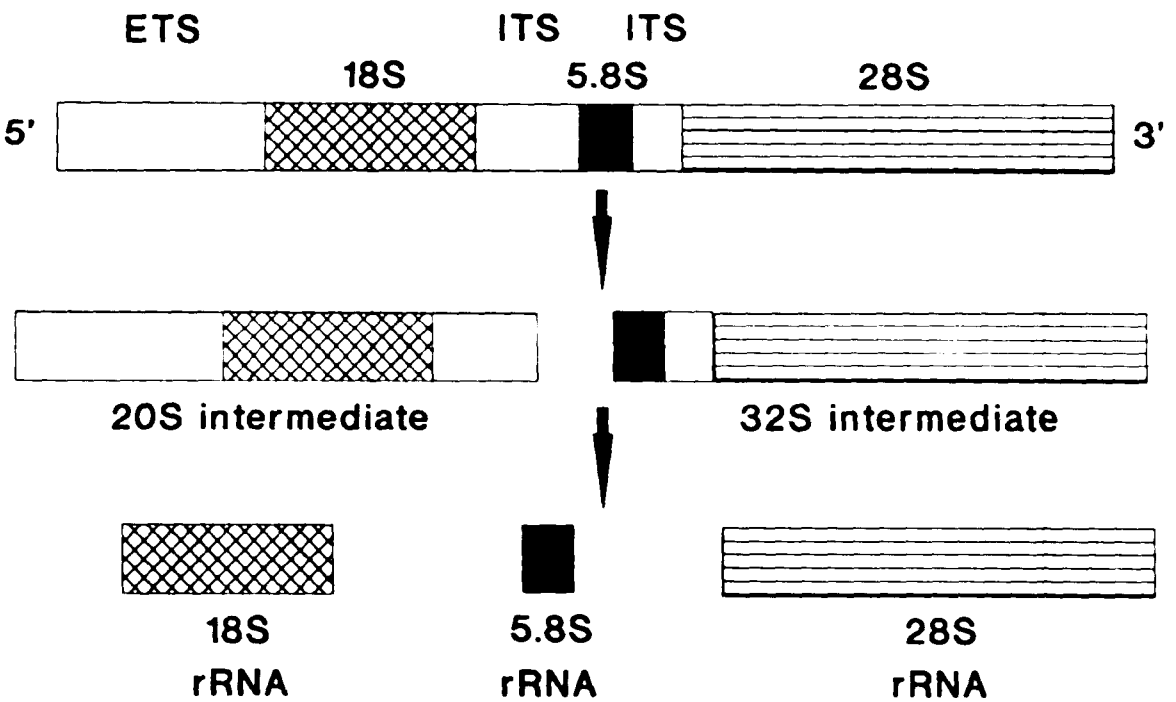

A
B
C
D

Figure 2. Ribosomal RNA processing.

The first stable intermediate in rRNA processing is the 45S precursor molecule. This molecule is then processed into two other stable intermediaries (20S and 32S). The 20S transcript is further processed into the 18S mature rRNA. The 32S transcript is processed into 5.8S and 28S rRNA (redrawn from Stryer, 1988).


ETS
ITS
ITS
18S
5.8S
28S
5'
3'
20S intermediate
32S intermediate
18S
rRNA
5.8S
rRNA
28S
rRNA

possible mechanism by which this can occur is by duplicative transposition or the duplication of the sequence with subsequent transposition to another chromosome (Reviewed in Dover, 1982).

Ribosomal DNA is transcribed by RNA polymerase I (pol I). The human rDNA transcription initiation site is located within the B region. The precise location was determined to be 480 bp from the *Eco*RI site which separates the B and C regions of the repeat unit (Miesfeld and Arnheim, 1982). Polymerase I recognizes a promotor which is composed of a core element and an upstream control element (UCE). The core element is situated at the region of transcription initiation and comprises nucleotides -45 to +20. Mutations in this region affect transcription from 3 to almost a 1000-fold (Jones *et al.*,1988). Duplication of the core element has an inhibitory effect on rDNA transcription (Haltiner *et al.*,1986). Nucleotide sequence from -26 to +7 of the core element is an absolute requirement for transcription (Learned *et al.*,1983). The UCE is located at nucleotides -234 to -107. This region is composed of several areas which influence transcription efficiency (Jones *et al.*,1988). The relative distance between the UCE and the core element is crucial for maximal transcription efficiency and both elements must be in the same orientation for transcription to occur (Haltiner *et al.*,1986).

Two transcription factors are required for pol I to bind to the core element and UCE. These transcription factors are upstream binding factor 1 (UBF1) and SL1. UBF1 has been localized to the nucleolus and is specific for rDNA transcription (Jantzen *et al.*,1990). The first step in the transcription of rRNA is recognition of both the core element and the UCE by UBF1. SL1, which is species-

specific, binds to UBF1 allowing pol I to attach and begin species-specific transcription (Bell *et al.*,1988; Learned *et al.*, 1985, 1986).

There are at least four functional termination sites and two inactive termination sites located between 360 bp and 1110 bp downstream from the end of the 28S gene (Safrany *et al.*,1989; Pfleiderer *et al.*,1990). The majority (90%) of rRNA transcripts terminate at the first termination site. There is also a termination site located 200 bp upstream from the transcription initiation site (Parker and Bond, 1989; Pfleiderer *et al.*,1990). Ribosomal RNA transcription is terminated when the growing rRNA chain reaches a specific 10-nucleotide DNA sequence (Pfleiderer *et al.*,1990). There are two DNA binding proteins found in human cells which interact with both this sequence and pol I (Bartsch *et al.*,1987; Pfleiderer *et al.*, 1990).

The upstream terminator and the downstream tandem termination sites are important role to the regulation of rRNA transcription. Nashimoto and Mishima (1988) proposed a model by which the terminator-binding proteins interact with the terminator sites to regulate rRNA transcription. They refer to the two terminator-binding factors as f_{T1} and f_{T2}. F_{T1} stimulates termination, whereas f_{T2} stimulates transcription initiation. If the two DNA binding proteins are not present in any termination region, then pol I continues transcription into the next repeat unit. The RNA polymerase is encumbered by the existing transcript which may impede further transcription. If both factors are present, then pol I remains attached to the DNA and f_{T2} causes the growing nascent transcript to be released from pol I. In the latter case, transcription is reinitiated when pol I reaches the next promotor. If only

f_{T1} is present on a given terminator, then transcription is terminated and pol I enters the free pool of enzymes. When this occurs at the upstream termination site, pol I is in close vicinity to the promotor and can bind to the promotor to reinitiate transcription (Nashimoto and Mishima, 1988). The interaction between pol I and termination factors can rapidly influence the rate of rRNA synthesis.

Associated with rDNA genes are the Nucleolar Organizing Regions (NORs) upon which the nucleoli form (Ohno *et al.*, 1961). The nucleolus consists of: (a) fibrillar center(s) which contain the NOR(s); (b) a dense fibrillar component which may be the site for rRNA synthesis, and (c) a granular component which contains RNP particles and is the site for preribosome synthesis (reviewed in Goessens *et al.*, 1987; Schwarzacher and Wachtler, 1987). In cultured cells, pol I is evenly distributed throughout the fibrillar centers as the precursor rRNA molecule is actively transcribed (Scheer and Raska, 1987). The genes are located both in the periphery of the fibrillar centers and in outside regions (Devictor *et al.*, 1987).

DNA begins to coil into recognizable chromosomes during prophase I of meiosis. At this stage, chromatin fibers from as many as three synapsed homologous chromosomes can be observed emanating from one fibrillar center (Mirre *et al.*, 1980). In fetal oocytes, each NOR passes through several fibrillar centers. In adult spermatocytes, only one fibrillar center containing one or more NORS is formed (Mirre *et al.*, 1980; Stahl *et al.*, 1983).

The formation of and variations in Robertsonian translocations are accounted for in a model proposed by Stahl and his collaborators (Mirre *et al.*, 1980; Stahl *et al.*,1983). According to the model, the satellite region of two or more

acrocentric chromosomes attach to the nuclear envelope. The NORs form fibrillar centers and nucleoli directly behind the satellite region. One nucleolus forms when several NORs are in close proximity. A Robertsonian translocation is produced when breakage and mismatched reunion of the chromosomes occurs. The varying lengths of the p-arms of nonhomologous acrocentrics would then lead to variation in the morphology of the translocations.

The etiology of nondisjunction may involve the process of prophase 1 *per se*. There is an average of seven active nucleoli at leptotene. The number decreases as prophase continues as the result of nucleolar fusion. The chromosomes begin to separate shortly after pachytene. Those acrocentric chromosomes associated within a common nucleolus are therefore prone to nondisjunction (Jullian *et al.*, 1987). The problem is compounded in humans since an oocyte may remain in prophase I for decades. The enzymatic machinery for dissolving the connections (nucleolar material) may become partially defective during the long time period which would result in chromosomes remaining connected (Mirre *et al.*, 1980). It should be noted that other mechanisms for nondisjunction can occur, *e.g.*, failure of proper pairing of homologues during synapsis (Warren *et al.*, 1987).

A third characteristic of acrocentric chromosomes which can lead to nondisjunction and Robertsonian translocations involves a related phenomena by which these chromosomes form satellite associations (SA). These associations are probably a remnant of improper nucleolar dissolution. The acrocentric chromosomes appear to be joined at the satellite regions in 60% of peripheral

blood leukocytes (Ferguson-Smith and Handmaker, 1961). Subvisible chromosomal interconnections are found in approximately 17% of metaphase plates and contain rDNA (Henderson *et al.*, 1973). It should be noted that the detectable presence of rDNA may not be mandatory (Warburton and Henderson, 1979), but there is a positive correlation between the extent to which SAs are observed and the amount of rDNA present within the cell (Warburton *et al.*, 1976). For example, a double satellited acrocentric chromosome containing a higher than usual quantity of rDNA undergoes SA more frequently than normal chromosomes (Henderson and Atwood, 1976). The terminal orientation of the rDNA is not an essential factor for SA. SAs have been observed in the Rhesus monkey, where the rDNA region is subterminal (Henderson *et al.*, 1974). SAs involving a dicentric chromosome have also been observed. This chromosome appears to be two complete acrocentric chromosomes fused at the terminal regions of the p-arms, thereby restricting the rDNA and satellite regions to the center of the chromosome (Warburton *et al.*, 1973). SAs tend to occur in organisms whose rDNA is located proximally to the centromere, but centromeric proximity is not a requirement. For example, in the cow, the NOR is located at the telomeric region of the q-arm of four chromosomal pairs which undergo extensive satellite associations (Mayr-Wohlfart *et al.*,1987).

Down Syndrome individuals have a higher rDNA copy number on each rDNA-bearing chromosome and show a positive correlation between amount of rDNA and SA. The parents lack any correlation between the quantity of rDNA and the amount of SAs present (Gross and Henderson, in preparation).

In summary, the five pairs of acrocentric chromosomes are hypothesized to be more susceptible to nondisjunction and Robertsonian translocation due to the presence of rDNA. The rDNA regions not only come in contact with each other in the nucleoli, but undergo recombination. The chromosomes tend to keep the attachments (SAs) formed in the nucleolus during meiosis or mitosis. Any combination of these events has the potential to interfere with proper separation of both homologous and nonhomologous chromosomes leading to nondisjunction and Robertsonian translocation.

C. Rationale for Research

Few studies have been done to determine the basic factors involved in nucleolar organization. This study was performed to further investigate the relationship of rDNA to nucleolar organization and satellite association. The purpose was to demarcate rDNA sequences responsible for nucleolar organization among the human rDNA-bearing chromosomes, *i.e.*, to determine the molecular basis for associations between rDNA-bearing chromosomes. The specific problems investigated were to determine the number of copies of rDNA which are critical for associative interactions between chromosomal regions carrying rDNA and if a special rDNA sequence, an entire repeat unit or multiple repeat units are required.

Four previous studies transfected a portion of rDNA from one species into cells of another closely related species. In three of the transfectants, transcription occurred, but at a lower level than expected based upon the amount of rDNA which was introduced into the recipient cells. Two of the three studies involved transfection of part of the mouse transcriptional unit containing the promotor and

various portions of mouse rDNA into CHO (Chinese hamster ovary) cells (Raziuddin *et al.*, 1989; Dhar *et al.*, 1985). The third involved transfection of mouse rDNA into rat cells (Vance *et al.*, 1985). In the fourth experiment, 1.2 kb of human rDNA including the promotor region was transfected into CHO cells. In this case, the rDNA was apparently transcribed by RNA polymerase II, not pol I. The reason pol I may have failed to transcribe this rDNA is that CHO cells can not utilize their species-specific machinery required for human rDNA transcription (Dhar *et al.*, 1987). These experiments demonstrate that it is possible to obtain transcription from transfected rDNA, but do not address issues concerning nucleolar formation nor the functionality of the nucleolus.

A pioneering experiment involved transfection of *Drosophila* rDNA into *Drosophila* embryos using P-element mediated transformation (Karpen *et al.*,1988). The experiment demonstrated the feasibility of constructing an rDNA site in a region other than a nucleolar organizer and obtaining transcriptional activation. In *Drosophila*, the rDNA insertion site behaved as a nucleolar organizing region, and was analyzed in depth in salivary gland chromosomes. The inserted rDNA contained a complete transcription unit plus flanking 5' and 3' spacer regions. The region surrounding the insertion site stained with an antibody specific for nucleoli, and the inserted rDNA was able to increase the viability of rDNA deficient mutants.

D. Experimental Design and Research Findings

The experiments performed in this project were designed to study the interaction between donor and endogenous rDNA in human cells in the formation of NORs and consequently satellite associations. Human ribosomal DNA was

isolated from a cosmid library and transfected into human cells. Two transfectants were studied. One contained a promotor; the other lacked a promotor region. The transfectant containing an intact promotor region formed a functional NOR. A functionally active site was defined on the basis of several criteria. First, it had to be present in the nucleolus. This was demonstrated by direct hybridization of associated cosmid DNA to nucleolar DNA at both a molecular and cytological level. Second, the newly created site should be transcriptively active. This was shown by positive silver staining at the new site. Third, the chromosome containing the transfected rDNA should participate in satellite associations. This was shown to be the case on the basis of cytological analysis.

The presence of the new rDNA site in nucleoli was determined by two methods. In one method, nucleoli were isolated from cell lysates under iso-osmotic conditions. Southern blot analysis was used to demonstrate that DNA isolated from nucleoli of transformed cells contained vector sequences and that the newly formed rDNA site was participating in nucleolar formation. In a second method, nucleoli were first identified by staining nuclei with a mixture of methyl green and pyronin Y, and hybridization *in situ* was used to directly examine the nucleoli for vector sequences.

Transcriptional activity was demonstrated by silver staining. Metaphase plates were silver stained, a technique which is specific for NORS which were actively transcribing (Schwarzacher *et al.*, 1978). The stain probably interacts with RNA polymerase I and/or other nucleolar proteins present in the rDNA regions. The results showed that the chromosome containing the transfected rDNA with a

promotor was transcriptively active.

Satellite association is a byproduct of the interactions which occurred among nucleolar organizing regions in the nucleolus. If the quantity of transfected rDNA is sufficient to form nucleoli, then these nonrandom associations could be observed by the close proximity between native rDNA sites and the insertion site. This was the case in the cell line with the rDNA construct containing the promotor.

Direct measurements of rRNA transcripts were also made. If the complete genes for 18S, 5.8S, and/or 28S rRNAs were present, then the transcripts would be indistinguishable from the endogenous rRNA molecules. This was the case in the cell lines studied. A search was made because of the possibility that transcription would not be terminated at the usual location, yielding a length polymorphism. The search for heterogeneity was made by hybridization with cosmid vector sequences on the assumption that read through could have occurred.

The experiments demonstrated that at least part of one repeat unit containing a promotor region is critical to nucleolar formation and nonrandom SA associations, and that the critical DNA to association is rDNA. The presence of the promotor (with the transcribed spacer) resulted in the formation of a new NOR with transcriptive activity, as indicated by its participation in satellite associations and nucleolar formation.

MATERIALS AND METHODS

A. Commonly Used Solutions; Abbreviations - All solutions are 1x unless indicated.

AMP: Ampicillin

Denhardts (50x): 1% ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin

DNA Lysis buffer: 10mM Tris, 10mM NaCl, 10 mM EDTA (pH 8.0), 5% SDS, 50µg/ml proteinase K.

E buffer (10x): 400mM Tris, 200mM sodium acetate, 1mM EDTA (pH 7.2)

G418: Geneticin

Loading buffer (10x): 0.25% bromophenol blue, 40% glycerol, 10x E buffer

LB AMP: 1% Bactotyptone (Difco), 0.5% Bactoyeast (Difco), 1% sodium chloride (Fisher), 50 µg Amp/ml with 1.5% Bactoagar (Difco) for plates.

MOPS: 20mM 3-[N-morpholino]propanesulfonic acid, 5mM sodium acetate, 1mM EDTA (pH 8.0) (Davis *et al.* 1986).

PBS (phosphate buffered saline): 150mM NaCl, 150mM Na phosphate (pH 7.2)

RE: Restriction enzyme

RE B Buffer: 10mM Tris, 5mM $MgCl_2$, 100mM NaCl, 1mM 2-mercaptoethanol (pH 8.0)

RE H Buffer: 10mM Tris, 10mM $MgCl_2$, 100mM NaCl, 1mM Dithioerythritol (pH 7.5)

RE L Buffer: 10mM Tris, 10mM $MgCl_2$, 1mM Dithioerythritol (pH 7.5)

RE M Buffer: 10mM Tris, 10mM $MgCl_2$, 50mM NaCl, 1mM Dithioerythritol (pH 7.5)

RNA lysis buffer: 0.1M Tris (pH 9.0), 0.1M NaCl, 20mM EDTA, 0.1% sarkosyl

RNase A: 10 mg RNase/ml 10mM Tris, 15mM sodium chloride (pH 7.5). Boil for 15 minutes and allow to cool. Store -20°C

Sevag's solution: 24:1 chloroform:isoamyl alcohol

SSC (20x): 3M sodium chloride, 0.3M sodium citrate (pH 7.0)

SSPE: 10mM Na_2HPO_4 (pH 7.2), 0.18M NaCl, 1mM EDTA

STE: 0.1M sodium chloride, 10mM Tris, 1mM EDTA (pH 8.0)

TBE(10x): 0.89M Tris, 0.89M boric acid, 0.1mM EDTA (pH 8.0)

TE: 10mM Tris, 1mM EDTA (pH 8.0)

tdH_20: triple distilled water

B. Commonly Used Formulae

DNA concentration: 1 optical density unit at $O.D._{260}$ ≈50μg DNA

RNA concentration: 1 optical density unit at $O.D._{260}$ ≈40μg RNA

X^2 analysis: $(\text{Observed minus expected})^2/\text{expected}$.

C. Cells and Culture Conditions

1. Derivation of Cell Lines

a. HAL cells. HAL cells are an immortal, temperature sensitive, SV40 transformed, pseudodiploid human fibroblast cell line (Radna *et al.*,1989). These cells were derived by a series of manipulations. The first step in the transformation was the transfection of HS74BM (a human bone marrow cell line) with a plasmid (pMK16) containing ori(-)SV40 encoding a heat-labile large T antigen. The cells were then mass cultured for 120 population doublings and treated with EMS. The cells were selected for thioguanine resistance, and subcloned.

b. Cell lines derived from HAL. The K-series cells were derived by transfecting HAL cells with rDNA containing cosmids. The T-series cell lines were derived from transfecting a plasmid containing Eco B into HAL cells (appendix).

c. HL-60 cells. HL-60 was derived from a single patient with acute promyelocytic leukemia (Rovera *et al.*, 1979). This line has distinct myeloid characteristics, but can be induced to differentiate into a number of cell types. The typical cell population consists of about 90-95% myeloblasts/promonocytes. About 5% of the cells spontaneously differentiate into myelocytes, metamyelocytes, and neutrophils.

2. Culture Conditions

All tissue culture is done under sterile conditions in a tissue culture hood.

a. HAL cells. Stock cultures of HAL cells and HAL derived cell lines are grown in flasks containing DMEM/F12 (Dulbecco's modified Eagle medium:nutrient mixture F-12 (Ham) 1:1 with 15mM HEPES buffer and L-glutamine) (Gibco) containing 15% FCS (fetal calf serum)(Sigma) and 1x antibiotic antimycotic solution (Sigma). The cells are cultured at 35°C in the presence of 5% carbon dioxide, 20% oxygen and 75% nitrogen. Media is replaced twice a week for actively growing cultures. HAL cells are an adherent cell line and therefore when the cells reach confluence (usually once a week) the media is replaced with 0.25% trypsin, 1mM EDTA (Gibco). The cells are suspended in the trypsin by gentle agitation for ≈5 minutes. Approximately 1/5 of these cells are placed in a flask containing fresh media. The trypsin is deactivated by the presence of fetal calf serum and by a dilution of more than 10-fold by media.

b. HAL-derived cell lines. Stock cultures of transfected cell lines use the same conditions as HAL cells with the addition of geneticin (G418)(Gibco) in the media. G418 is freshly dissolved to a final concentration of 300μg/ml. G418 is a

neomycin derivative which is toxic to human cells lacking a neo resistant marker. Media containing G418 is sterilized by filtration through a 0.22 micron cellulose acetate membrane using a disposable sterile filter system (Corning) of appropriate size. This filter apparatus is reused multiple times.

c. HL-60 cells. Stock cultures of HL-60 cells are cultured in flasks containing RPMI 1640 media with L-glutamine (Gibco) containing 10% FCS and 1x antibiotic antimycotic solution at 37°C. HL-60 cells are a nonadherent cell line. These cells are feed twice a week by decanting an appropriate volume of the cell suspension and adding fresh media.

3. Culture Conditions for Transfected Clones

a. Maintenance of cultures. The cells used were HAL or HAL-derived cell lines and the environmental conditions were the same as in subsection 2 of this section. 60mm tissue culture dishes, 24 well, 12 well and 6 well dishes are cultured in a self contained Tissue Culture Incubator (billups-rothenberg, inc. Modular Incubator Chamber-101 (MIC-101)). Humidity for the incubator is maintained by adding 3ml sterile tdH_20, containing 1x antibiotic solution, to 2-3 60mm dishes or by adding a small volume of this water to one or more wells of the multi-well dishes.

b. Expansion of transfected cloned cells. Clones are monitored twice a week. The media is completely replaced for rapidly growing healthy colonies when they fill approximately one fourth of the well. Half the media is replaced if the colonies are slowly growing and/or comprise less than one fourth the well. The media is not replaced if a colony is stagnant at low density. When a colony

reaches approximately 80% confluence it is trypsinized and placed in the next size microtiter dish. The cells are washed with a small aliquot of trypsin to dilute any remaining media and then a larger aliquot of trypsin is added and trypsinization is allowed to proceed until the cells become detached from the plate. The maximum amount of trypsin used is equivalent to one tenth the amount of media in the new well. This ensures that the trypsin will be rendered harmless by complete saturation from the proteins in the fetal calf serum. If a clone is stagnant at a high density it is trypsinized and plated in either the next size dish or the same size dish depending on its size. This allows the cells to be dispersed and stimulates them to enter into logarithmic growth. Fresh media is added to the well from which the clone was removed to allow regrowth of the culture. This serves as a backup for the transplanted cells and helps ensure that the clone will survive.

D. Hybridization: Southern Blots

1. DNA Purification

Genomic DNA is purified by modifying the method of Ausebel (1987). Cells are removed from the flask by digesting with trypsin-EDTA and washing with PBS, then lysis buffer. The cells are incubated in DNA lysis buffer overnight at 37^{0}C, rather than the conditions recommended by Ausebel (1987). The DNA is partitioned once in a phenol solution equilibrated with 0.1M Tris (pH 8.0). DNA is then extracted with phenol:Sevag's solution (50:50) and then Sevag's solution alone. The DNA is recovered by precipitation in 2 volumes of ethanol and dried. The DNA is dissolved in tdH_2O and adjusted to 0.1xSSC. RNase is added to a final concentration of 100 µg/ml and incubated for one hour at 37^{0}C. The solution

is then adjusted to 10mM EDTA (pH 8.0) (to chelate Mg^{2+} ions which serve as cofactors for DNases), 0.5% SDS and 50µg/ml proteinase K. The solution is incubated for 1.5 hours and the DNA is further purified by sequential extraction with equilibrated phenol, 50:50 phenol:Sevag's solution, and then Sevag's solution. The purified DNA is recovered by precipitation in ethanol.

a. Isolation of cosmid and plasmid DNA. Bacterial growth and amplification of the cosmid or plasmid DNA is performed in accordance with the method outlined in Maniatis (1982) with the following exceptions: (1) the initial overnight culture is 2 mls.; (2) 1 ml of overnight culture is used to inoculate 25ml LB AMP; (3) late log phase is determined by reaching a Klett count between 100 and 200, and (4) the final concentration of chloramphenicol is 85µg. Plasmid or cosmid DNA is isolated from bacteria as described by Qiagen (Qiagen plasmid maxi kit)

b. Purification of DNA from agarose gels. The desired band is excised from an agarose gel after electrophoresis. The DNA fragment is purified with Gene Clean (Bio 101, Inc.) as described by the manufacturers. Quantity and purity of the DNA is determined by agarose gel electrophoresis.

2. Restriction Enzyme Digestion of DNA

DNA is digested at 37°C overnight in the presence of the appropriate buffer supplied by the manufacturer (BMB). RE B Buffer is used for *Bam*HI and *Hind*III, RE L Buffer is used for *Kpn*I, RE M Buffer is used for *Bgl*II, *Eco*RI and *Sal*I. The concentration of enzyme used is based on the concentration of DNA present with a minimum of 1U enzyme/µg DNA. This concentration ensures sufficient enzyme for adequate restriction of the DNA.

3. Preparation of Gels

a. Electrophoresis of DNA. RE-digested DNA is mixed with 1/10th volume loading buffer and heated at 68°C for 10 minutes. DNA fragments are electrophoresed through a 0.8% agarose gel prepared with 0.5x TBE and 0.5x TBE is used as running buffer. If the purpose of the gel is to purify a DNA fragment then 1xE buffer is used. The gel is stained for one hour with ethidium bromide (7.5µg/ml) and the DNA bands are visualized.

b. Determination of restriction fragment lengths. The distance migrated by each fragment is measured from the bottom of the well to the bottom of the band. Lambda DNA digested with *Hind*III yields fragments which serve as molecular weight markers. The sizes of the unknown fragments are determined with computer-assistance. This is accomplished by comparing unknown fragment migration rate to a best fit curve obtained for lambda DNA.

c. Denaturation of DNA. This procedure is modified from Ausebel (1987) for transfer to nitrocellulose filters. The modifications are as follows: (1) incubation in 0.2N HCl twice for 15 minutes; (2) denaturation is for 1 hour without changing the buffer, and the neutralization buffer used is 0.25M Tris (pH 7.3), 0.75M NaCl. Incubation is for one hour.

d. DNA transfer (modified from Schleicher & Schuell, 1987).

Gels are rinsed with tdH_20 and inverted onto two layers of Whatmann 3MM paper which serve as a wick. 10x SSC passes from this wick through the gel. Placed on top of the gel is a sheet of Nytran (S&S) prewetted with tdH_20 followed by two pieces of 3MM paper, a stack of paper towels and a 5Kg weight. Evaporation is

reduced by wrapping the transfer apparatus and paper towels with saran wrap. Transfer is overnight. The filter is baked at 80°C for approximately 2 hours. The filter is stored desiccated at room temperature.

4. Hybridization Conditions

a. Radioactive labelling of DNA. DNA (0.125μg) is radioactively labelled using a Nick Translation kit (BMB). The reaction mixture consists of 20μM of each nonradiolabelled nucleotide (dATP, dTTP, dGTP), 1x reaction buffer (from a 10x stock solution), 50μCi (100pmoles) alpha-^{32}P-dCTP (specific activity = 3000Ci/mmole) (NEN)and DNA polymerase I/DNase I. The mixture is incubated for 60 minutes at 15°C. The volume of the reaction mixture is increased to 100μl with STE and loaded onto a Quick Spin Column (BMB) [G-50 Sephadex (fine)] previously centrifuged to remove the storage buffer (STE). The probe is collected by a brief (four minute) centrifugation in an International clinical centrifuge model CL at top speed (speed 7). Two aliquots (1μl each) are removed and mixed with 10ml Ecoscint (National Diagnostics) scintillation cocktail. The incorporated radioactivity is determined with a Packard tricarb model 4530 scintillation counter (efficiency of 88% for ^{32}P).

b. Hybridization and wash conditions. Prehybridizations and hybridizations are performed at 42°C with constant agitation in a Hybrishake water bath (Thomas Scientific). The nytran filter (Schleicher & Schuell) is prehybridized by incubating for a minimum of one hour at 42°C in 50% formamide (FisherBiotech Molecular Biology Grade), 10% Dextran Sulfate, 2x SSC, 5μM EDTA, 0.5%SDS, 2.5x Denhardts, 50μg/ml denatured ssDNA. The radiolabelled probe is denatured by heating in boiling water for 10 minutes and then immediately placed in ice for 10

minutes. The probe is added directly to the prehybridization solution and the filter is hybridized overnight.

Wash conditions: All washes are performed with agitation and are modified from Sambrook *et al.*(1989). The filter is removed from the prehybridization solution and washed to remove unhybridized probe. The washes are done in a series of three washes which involve increasing temperature and decreasing saline concentration. The wash conditions are as follows:

1) 2x SSC, 0.5% SDS twice at room temperature for fifteen minutes each wash.

2) 1x SSC, 0.5% SDS twice for fifteen minutes at 37°C.

3) 0.1x SSC, 0.5% SDS at 65°C for two hours.

Nytran filters are blotted on 3MM paper to remove excess fluid and placed on a second sheet of 3MM paper. The moist filters are wrapped in Saran wrap, placed in a light tight cassette and used to expose X-Ray film (Kodak X-omat AR film). The signal is amplified by using an intensifying screen (Cronex Lightning Plus, Dupont) placed on top of the X-ray film. The cassette is closed and covered with aluminum foil to ensure no light enters. The cassette is stored at -70°C. Various time exposures are achieved by replacing the X-ray film during progressive time increments. Probes can be removed from moist filters by incubating in 55% formamide/6xSSPE at 65°C for one hour and rinsed in 2x SSPE. The filters are then allowed to expose a sheet of X-ray film to ensure adequate probe removal.

E. Hybridization: Northern Blots

1. Isolation of RNA

The procedures are modified from Sambrook *et al.*(1989). Cells are washed

with PBS 3 times after trypsinization. The cells are lysed in an eppendorf tube with 0.8ml RNA lysis buffer without the addition of RNase inhibitors. No proteinase K digestion is done. Deproteinization of the RNAs was by extraction in phenol:Sevag's solution (2 times) followed by an extraction with Sevag's solution. RNA is selectively recovered by precipitation with 2.5 volumes 0.1M lithium chloride in ethanol at -70°C for 30 minutes. The pellet is washed once with 95% ethanol. The RNA enriched nucleic acid pellet is dissolved in td$H_2$0 and adjusted to 50mM Tris pH 8.0. RNase-free DNase (BMB) (40 units/100µl) is added and the DNA is digested for 30 minutes on ice with periodic agitation. Sodium acetate pH 5.2 is added to a final concentration of 0.25M and the solution is phase extracted one time with phenol:Sevag's solution and one time with Sevag's solution. Ethanol precipitation is carried out for 30 minutes. The pellet is washed one time in 95% ethanol and resuspended in TE (pH 8.0). The RNA is usually stored in the 95% ethanol wash but can be stored once resuspended in TE.

2. Preparation of Gels.

a. Electrophoresis of RNA (modified from Rosen and Villa-Komaroff, 1990; Matathias and Komro, 1989). Samples containing 5 µg of total RNA in 50% formamide, 0.62M formaldehyde, 1x MOPS, 0.5µg/µl ethidium bromide are heated at 68°C for 3 minutes. The sample is electrophoresed through a 1.2% agarose, 2.2M formaldehyde, 1x MOPS buffer gel after the addition of one tenth volume of loading buffer. Gels are soaked in td$H_2$0 for a few minutes prior to transfer.

b. Transfer of RNA (modified from Schleicher & Schuell Protocol, 1987). This method is essentially identical to the transfer of DNA to Nytran [see section

D (3d) of Materials and Methods]. There are two modifications to this procedure since formaldehyde is present in RNA gels: (i) the filter is washed in 10x SSC prior to being baked and ii) the filter is washed with 1x SSC, 0.1% SDS at 65 ^{0}C for one hour prior to hybridization.

F. Screening the Cosmid Library: Identification of rDNA

The cosmid library is screened as described in Ausebel *et al.*, 1987. Eco A and Eco B rDNA were used as probes.

G. Methods Used for Transfection

1. Determination of Cell Number.

HAL cells are trypsinized, pelleted and resuspended in media. An aliquot (0.1ml) is added to 14.9 ml of filtered phosphate buffered saline and the concentration is determined by a cell counter (Royco model 927 - TCL Tissue Cell Counter). The digital display multiplied by 3 x10^{4} (conversion factor at this dilution) indicates the number of cells per ml.

2. Transfection of HAL Cells (modified from Felgner *et al.*, 1987)

HAL cells are seeded at a concentration of 0.5x10^{6} cells/well of 60mm tissue culture dish and are cultured overnight in media containing 10% FCS. The cells are washed 3x in media without calf serum. The cells are incubated in 3ml media without serum containing lipofectin (30μg) and DNA (30μg). The transfection is terminated by adding media containing 20% FCS. The media is replaced by media with 10% FCS containing geneticin (G418) at 300μg/ml after 48 hours. G418 is a neomycin derivative which is toxic to human cells lacking a neo resistant marker. Transfected cells have this marker whereas nontransfected cells do not.

The media is changed twice a week. Resistant cells remain attached to the plate, but nontransfected cells die and are removed with the old media.

3. Isolation of Transfected Colonies

a. Cloning cylinders. Silicone based vacuum grease (Dow Corning high vacuum grease) is spread to approximately 1mm in thickness on the lower cover of a glass petri dish. Plastic cloning cylinders are placed in the vacuum grease and the petri dish is covered and sealed with tape. The petri dish is sterilized by autoclaving and stored horizontal until needed.

b. Isolation of colonies. Colonies are ready to be isolated approximately 16 days after transfection as judged by cell populations on nontransfected control plates containing geneticin. The plates are washed two times with fresh media to remove as many of the dead nontransfected HAL cells as possible. Fresh media is added to the plates to ensure the survivability of the clones while they are analyzed. Colonies are visualized as white clusters and are circled. The colonies are located and observed using phase contrast microscopy (Nikon TMS microscope). A plastic cloning cylinder is firmly placed over each chosen colony. The cloning cylinders are 0.6 or 0.8cm in diameter and are chosen depending on the size of the colony and the distance to other colonies. Care is taken to prevent the grease from covering the cells, since this presents difficulty in removing cells from the dish. Trypsin (30µl) is added to each cylinder. The trypsin is then removed with gentle stirring from one cylinder and placed in one well of a 24 well microtiter dish containing 0.5ml of media for a small clone and 1 ml of media for a large clone. Immediately after the trypsin is removed from one cylinder a second

aliquot of trypsin is added to that cylinder and the above procedure is repeated for the next cylinder. This process is repeated a second time and then the colonies are observed under the microscope to make sure the cells have been either removed or loosened.

H. Nucleolar Isolation

Exogenous rDNA presence in nucleoli is determined in isolated nucleolar preparations (modified from Beebee, 1986 and Higachi *et al.*, 1978). Cells are washed with PBS and resuspended in 10mM KCL, 1.5mM $MgCl_2$, 10mM Tris (pH 7.6). The cells are homogenized following the addition of Nonident P-40 (Sigma) at a final concentration of 0.2%. Nuclei are recovered by centrifugation at 2,000g for 10 minutes at 4°C. The nuclei were resuspended in 3ml 0.34M sucrose, 10mM magnesium chloride and washed by centrifugation through 0.88M sucrose at 2,000g for 10 minutes at 4°C. The nuclei are resuspended in 3ml 0.34M sucrose, 1mM magnesium chloride. Pores were made in the nuclear envelope by sonication (Sonics & Material Vibra Cell High intensity ultrasonic processor Model VC300). The nuclei were sonicated at a horn of 9.5 mm with an output control setting of one for 12 seconds. They are sonicated 3-4x with a brief cooling period on ice between sonications. The nucleoli are liberated from the damaged nuclear envelop by centrifugation through 0.88M sucrose at 2,000g for 20 minutes at 4°C. The nonnucleolar material stays in the upper sucrose solution. DNA is purified from both the nucleolar pellet and the nonnucleolar material by phase extraction. DNA is then analyzed by Southern hybridizations for the presence of exogenous rDNA sequences which contain pBR322.

I. Cytological Stains

1. Silver Staining

Silver staining of nucleolar organizing regions was used to determine transcriptional activity [procedure modified from Goodpasture and Bloom (1975) and outlined Verma and Babu (1989)]. The initial incubation was in 50% $AgNO_3$ for two minutes, instead of 10 minutes.

2. Nucleolar Staining (modified from Chayan *et al.*, 1973,)

Methyl green and pyronin Y were used to differentially stain nucleoli. If the nuclei were from freshly fixed cells, the slides were rinsed in 70% ethanol and incubated for 30 minutes in 0.2M acetate buffer pH 4.2. Acetate buffer is prepared by mixing 70% 0.2M acetic acid with 30% 0.2M sodium acetate. Slides containing interphase nuclei were stained with a solution containing 0.055% (w/v) methyl green, 0.004% pyronin Y in 0.2M acetate buffer pH 4.2 for 10 minutes. At a low pH, the methyl green is not incorporated as easily as the pyronin Y and therefore the maximum differentiation between the nucleoli and the nonnucleolar chromatin can be obtained by transferring the slide between 0.5% methyl green in 0.2M acetate buffer and the stain solution described above. Both stains are incorporated within the nucleic acids of the nuclei staining the nonnucleolar chromatin a green color and the nucleoli a rosy red color. Nucleoli on slides previously treated with trypsin to G-band chromosomes will not be differentially stained. Differentially stained nuclei can be photographed and subjected to cytological hybridization.

J. Hybridization *in situ*

1. Chromosome Identification

a. Preparation of slides. HAL cells are incubated at 35°C for 4 hours in media containing 15µl/ml of colcemid (Gibco). The cells are shaken vigorously to detach cells undergoing mitosis. Cells are pelleted and resuspended in 75mM KCl. This step can be omitted if the cells are for nucleoli staining. The cells are incubated at 35°C for 20 minutes. The cells are pelleted and the supernatant is decanted. The cells are fixed by slowly adding cold 3:1 methanol:acetic acid (freshly prepared) and then placed at 4°C for thirty minutes. The fixative is replaced with fresh fixative and the cells are placed at 4°C. The fixative is changed three times a week for two to three weeks. This step is not necessary for cells used in the preparation of slides used exclusively for nucleoli staining. Cells can be stored for extended periods at 4°C if fixative is replaced periodically.
Cells are pelleted and resuspended in fresh fixative prior to being dropped on slides.

Chromosomal spreads are prepared by dropping (4 drops/slide) the fixed cells a short distance (3-4 cms) onto a slide. The slide is quickly flicked to spread the chromosomes and then passed through a low flame. The flame permanently adheres the chromosomes and isolated nuclei to the slide. Slides can be used immediately for staining nucleoli but are stored desiccated at 37°C for a minimum of one week before G-banding.

b. G-banding of chromosomes (modified from Bignone *et al.*, 1983). All solutions are prepared fresh. Slides are immersed in 30% H_2O_2 (fisher) for 10 seconds to aide in the removal of cytoplasm. The slides are immediately washed in tdH_2O 2-3 times. Excess water is drained. The slides are placed in trypsin until

banding can be observed. Trypsin is prepared by diluting 2ml 10x trypsin (Gibco) with 18ml isoton pH 6.8 (Isoton 11 - balanced electrolyte solution (azide free)) (CMS). Excess trypsin is drained and the slide is washed 3 times in isoton solution containing 4% FCS (FCS inactivates the trypsin). The slide is briefly washed in isoton and then stained for an appropriate time interval in Giemsa. Giemsa is prepared by adding 2ml Gurrs giemsa (BDH chemicals LTD Poole England) to 50ml isoton. The slide is rinsed in tdH_2O followed by tap water and dried with compressed air. The quality of banding is determined by the use of a microscope. Incubation times in trypsin and giemsa are altered accordingly. Metaphase plates are photographed and their location recorded Slides are destained with 70% ethanol (2x) followed by 95% ethanol (1x) for 3-5 minutes each prior to use.

2. Isotopic Hybridization *in situ*

a. RNase procedure. Slides are placed in 2xSSC at 37°C with a final concentration of 100µg RNase A/ml. They are then washed with three changes of 2xSSC, 70% ethanol (2x) and 95% ethanol (1x) for 3-5 minutes each and stored desiccated at 37°C.

b. Preparation of radiolabelled probe. The DNA is nick translated with 100µCi ^{125}I dCTP (specific activity = 2200Ci/mmole) (NEN). The probe is ethanol precipitated in the presence of 3µg tRNA as carrier. The pellet is resuspended in 100µl tdH_2O. Denaturation of the probe is performed by boiling the solution for 10 minutes and rapidly placing the solution on ice for 10 minutes. The probe is diluted to give a final volume of 50 µl/slide with a final concentration of 0.01M Tris pH 7.6 50% formamide, 3xSSC.

c. Hybridization. Chromosomal DNA is denatured by heating the slides at 70°C in 95% formamide:1xSSC pH 7.0-7.2 for 1.5 hours. The slides are placed in cold 70% ethanol for 10 minutes to prevent renaturation of the DNA and washed for an additional two times in ice-cold 70% ethanol for 5 minutes each. The slides are washed with ice cold 95% ethanol (2x) and then at room temperature with 95% ethanol for 3 minutes each. The probe is placed on the slides and covered with a coverslip. Hybridization is overnight at 43°C in moisturizing chambers containing 50% formamide:3xSSC. The coverslips are removed by soaking in 50% formamide:3xSSC and the slides are washed in sequence once in 2xSSC at 45°C for five minutes; twice with 2x SSC at room temperature; once for one hour with 0.1M potassium iodide in 2xSSC; in 2xSSC for 2-3 hours with three to four changes of buffer (2 liter containers); a total of 2L 2xSSC for every 10 slides was used. The slides were then washed in 70% ethanol (3x), followed by 95% ethanol (2x) for 3-5 minutes each.

d. Statistical analysis. A statistical analysis of the data is used to determine the insertion site. The entire chromosomal complement of a cell is divided into 95 sections of approximately equal length. The number of grains over each chromosomal region was compared with the total number of grains present. The region containing a statistically significant number of grains (as determined by X^2 analysis) is accepted as the site of integration.

3. Non-isotopic Hybridization *in situ*

a. Preparation of probes. Non-isotopic probes are prepared using Oncor Non-isotopic probe labeling kit exactly as recommended by the manufacturer.

b. Method of hybridization. The protocol and reagents for the non-isotopic method of cytological hybridization are supplied by the manufacturer (Oncor) in "Oncorlight" chromosome *in situ* kit. The procedure is followed as outlined by the manufacturer using slight modifications. The slides are incubated for one hour in RNase (1x from stock RNase into 2xSSC, pH 7) at 37°C. The slides are then washed 4x in 40 ml of 2xSSC, pH 7, for two minutes each wash. They are dehydrated by washing in cold (-20°C) 70%, 80% and 95% ethanol, respectively.

Slides are prewarmed to 37°C in an incubator and then placed for two minutes in denaturation solution (70% formamide:2xSSC) at 70°C. The slides are subjected to a cold (-20°C) series of two minute ethanol washes (70%, 80%, 90% and 95%). Biotin-labelled DNA probe (50 µl in 50% formamide: 2xSSC) is added to each slide and a sealed coverslip is placed on each slide. The slides are incubated in moisturizing chambers (see section) containing ddH_2O at 42°C overnight. The postwash conditions are 50% formamide, 2xSSC for 12 minutes at 43°C with periodic agitation.

K. Photographic Procedures

1. Photography of Gels

Fluorescent bands are visualized with ultraviolet light using a Spectroline transilluminator model TR302 (302 nm) and positive photographed using Polaroid 667 film (ISO 3000).

2. Film Development

a. Technical pan film. Technical pan film (Kodak) is developed for 14 minutes in Microdol X developer at 22°C (Kodak) with constant agitation. The film

is washed 4 times with tap water to remove excess developer and then immersed in Rapid Fix solution A (Kodak) diluted 1:3 with water for 7 minutes. Rapid Fix is removed from the film by washing the film in running water for 5-8 minutes. The film is rinsed in Perma wash (5ml/L) for one minute to ensure that the film is properly fixed. Excess Perma wash is removed by washing the film in tap water for two minutes. The film is rinsed in Photoflo 200 (one capful per liter) to prevent water spots. Positive prints are produced using an Omega Pro Lab B66 enlarger.

b. X-ray film. X-ray film is developed in GBX developer (Kodak) for a few minutes, rinsed in tap water and fixed in Kodak rapid fix for 10 minutes. Excess rapid fix is removed by washing the filter for 20 minutes in tap water.

3. Autoradiographic Emulsion

The slides are dipped in melted (38°C) emulsion (Kodak NTB2) diluted with 1:1 td$H_2$0. The slides are allowed to dry for 30 minutes prior to being placed in light tight boxes containing drierite. The autoradiograms are exposed at 4°C. Periodically, a slide is developed in D-19 developer for 2.5 minutes. Development is stopped with 1% acetic acid and the slides are then fixed for four minutes with Rapid Fix (Kodak). Slides are stained in 1xE buffer: 10% giemsa for 18 minutes (if not G-banded) or for 2 hours (if G-banded). Slides are photographed using technical pan film.

RESULTS

A. Isolation and Identification of Human rDNA in a Cosmid Library

Ribosomal DNA for transfection studies was obtained from a human DNA cosmid library (Lau and Kan, 1983, 1984). The culture was diluted to yield 1000 colonies per plate, and 30 master plates were prepared. Each set of replica plates was subjected to colony hybridization using either the A or B region DNA as probes (Eco A and Eco B, respectively) (Figures 3 and 4). The Eco A region contains the 3' end of the 18S gene, the entire 5.8S gene, the majority of the 5' end of the 28S gene and both internally transcribed spacer regions. The B region contains the externally transcribed spacer and most of the 18S gene. There were 10 positive clones, each containing homology to the transcriptive regions of rDNA. The 10 clones were designated ML1 to ML10.

Clones containing rDNA were analyzed by Southern blot hybridizations to determine the portion of the repeat unit present. Lau and Kan (1983) constructed the cosmid library by partially digesting placental DNA with the restriction enzyme *Mbo*I and ligating this DNA into a unique *Bam*HI site of the cosmid pCV108 (Figure 5). The DNA of pCV108 contained two *Eco*RI sites which straddled the point of insertion, allowing the insert to be liberated from the 8 kb vector. Ribosomal DNA containing a complete repeat unit is expected to give four bands of 19 kb, 12 kb, 7.3 kb, and 6 kb following digestion with *Eco*RI (Figure 6). *Eco*RI digests of ten clones were analyzed by Southern hybridizations. Four cosmids ML1, ML3, ML4, and ML6, gave results most consistent with the expected and were chosen for further analysis.

Figure 3. Diagram of pAT-153 containing Eco A. The 7.3 kb *Eco*RI fragment of rDNA forms the A region of rDNA. Eco A DNA contains the 3' end of the 18S gene, the entire 5.8S gene, the majority of the 5' end of the 28S gene and both internally transcribed spacer regions (ITS). The arrow indicates 5' to 3' direction. This region was subcloned into the unique *Eco*RI (indicated by E in figure) site of plasmid pAT-153 by La Volpe *et al.* (1985). Eco A DNA is liberated from the plasmid prior to use with the restriction enzyme *Eco*RI.

ECO A

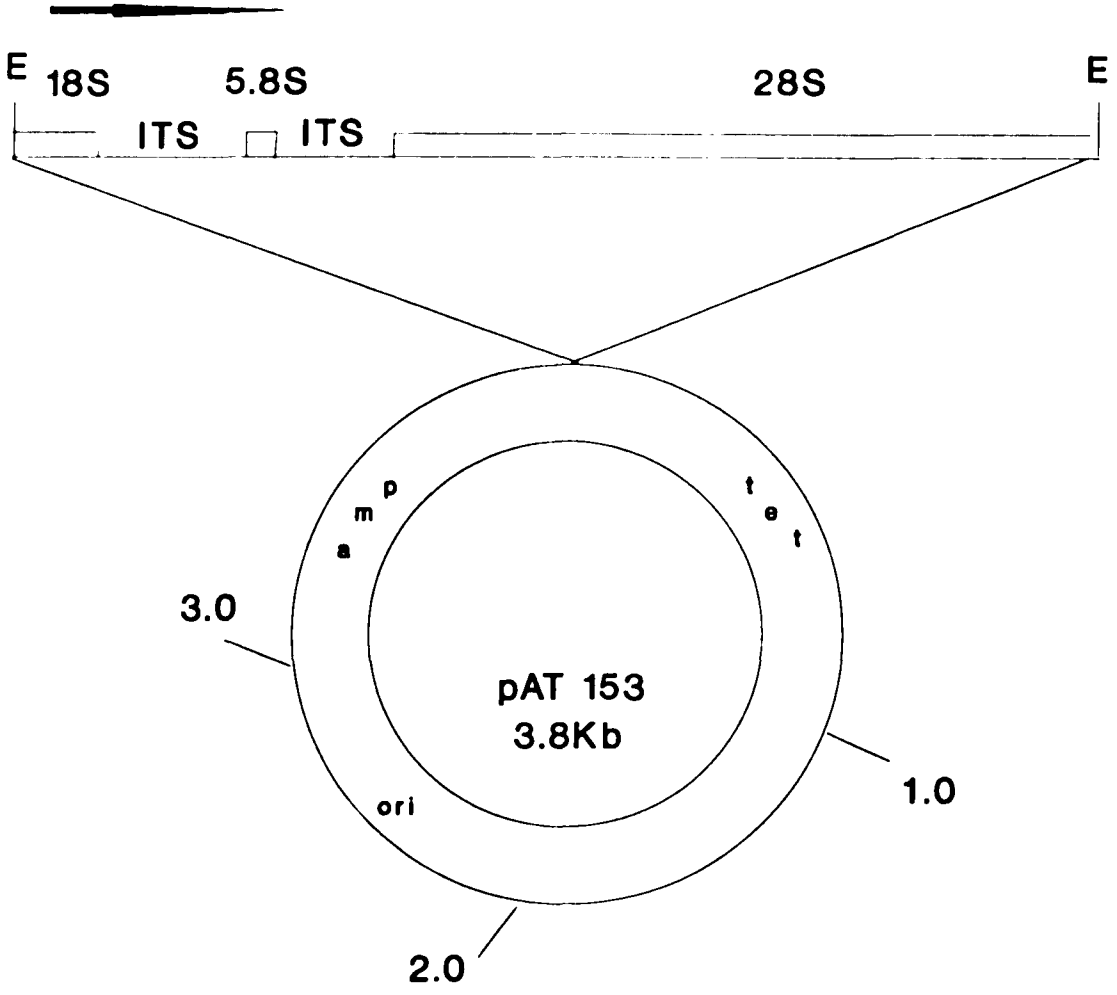


Figure 4. Diagram of pAT-153 containing Eco B. Eco B DNA consists of the 6 kb B region of rDNA obtained from an *Eco*RI digestion. Eco B DNA contains the externally transcribed spacer and most of the 18S gene. The arrow indicates 5' to 3' direction. This region was subcloned into the unique *Eco*RI site of plasmid pAT 153 (La Volpe *et al.*, 1985). *Eco*RI is used to remove Eco B DNA from the plasmid prior to use. *Sal*I digestion of this plasmid generated the fragment used as a probe for Northern hybridizations. Two other bands are produced by digestion with *Sal*I. These bands are not used as probes because both contain a portion of the vector. The restriction sites indicated in this figure are B(*Bam*HI), E(*Eco*RI), S(*Sal*I).

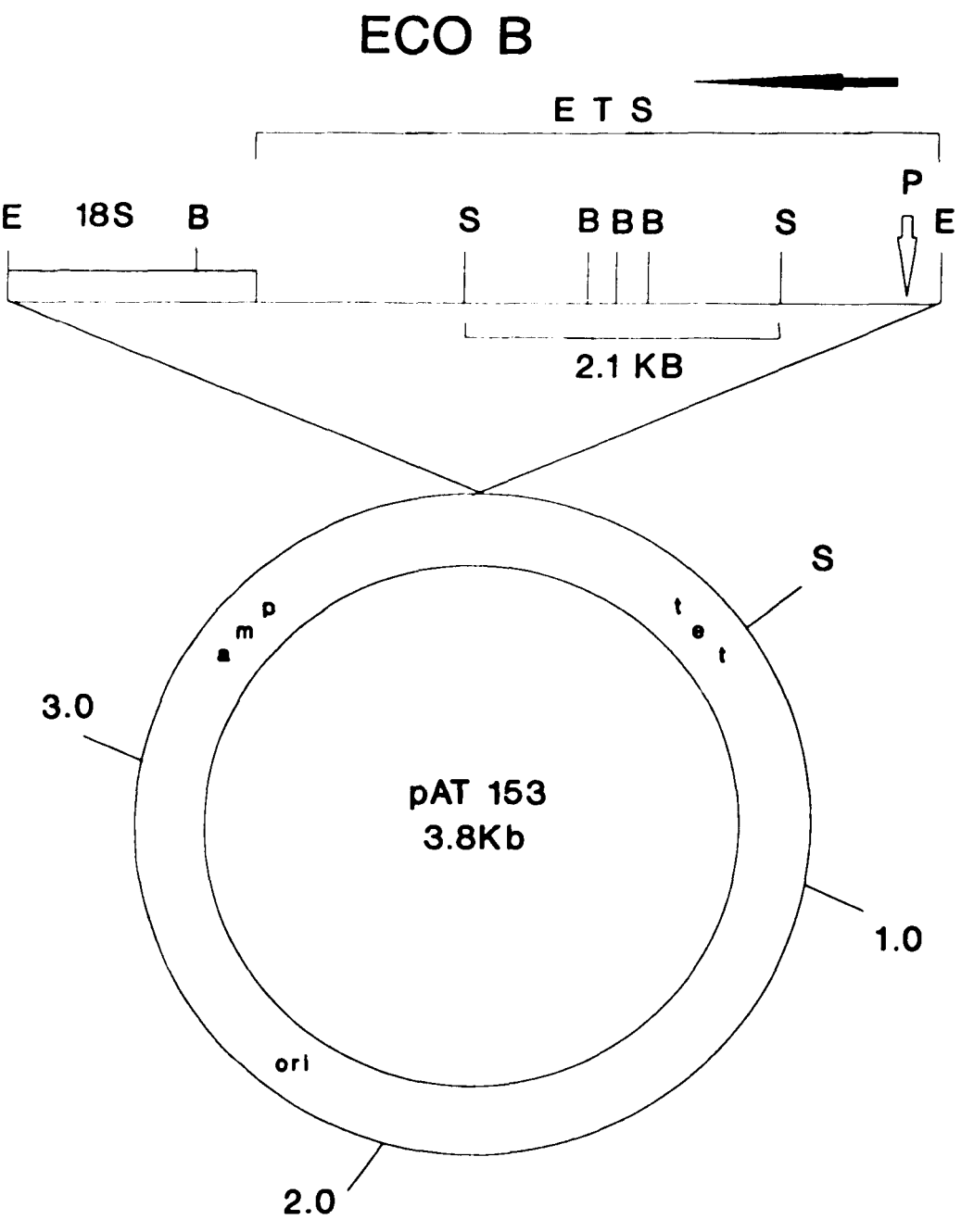

ECO B
E T S
P
E
18S
B
S
B B B
S
E
2.1 KB
S
amp
tet
3.0
pAT 153
3.8Kb
1.0
ori
2.0

Figure 5. Diagram of the cosmid vector. The vector (pCV108) used by Lau and Kan (1983) to create their library is a modification of plasmid pJB8. The modification converted a *Ba*II site into a *Bgl*II site. A *Bam*HI fragment (from plasmid pSV2neo) containing SV40 origin and neomycin markers was introduced into the *Bgl*II site of pJB8. Both *Bam*HI and *Bgl*II sites are destroyed in the ligation. Plasmid JB8 was synthesized by restricting plasmid HomerI at its unique *Eco*RI site, filling in the 3' ends and then blunt ligating the two ends together. This creates a plasmid with 2 *Eco*RI sites in close vacinity. HomerI was derived from pAT-153 by ligating a 1.78 kb *Bgl*II fragment containing the lambda cos site from bacteriophage Charon 4 into a unique *Bam*HI site. Plasmid pAT-153 consists of pBR327 which in turn comes from pBR322 minus 1089 bp (from 1430-2519). The human DNA sequences are inserted into pCV108 at the unique *Bam*HI site straddled by the two *Eco*RI sites. The origin of repliation, COS origin, neomycin marker and restriction sites for *Bam*HI, *Hind*III and *Mbo*I are indicated.

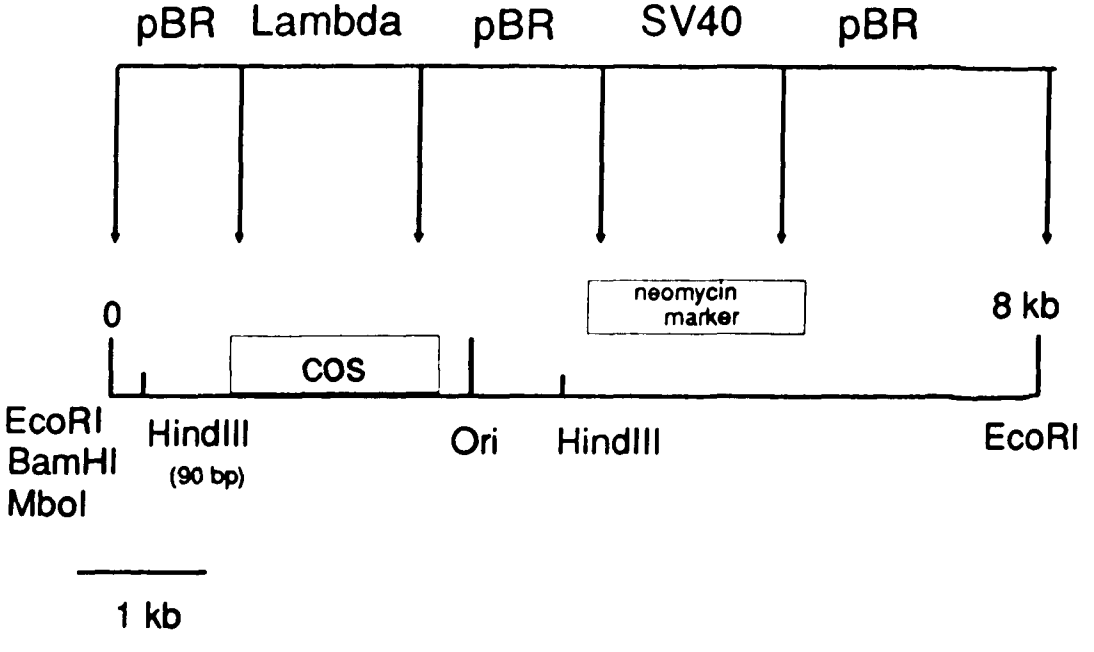
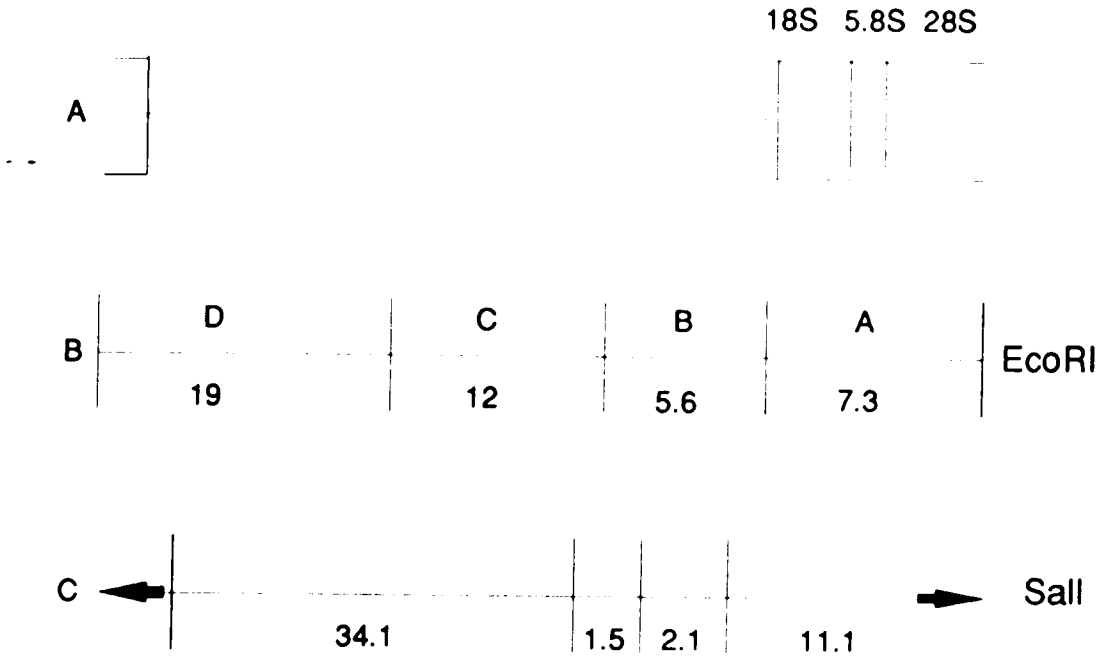

pBR
Lambda
pBR
SV40
pBR
0
neomycin marker
8 kb
cos
EcoRI
BamHI
MboI
HindIII
(90 bp)
Ori
HindIII
EcoRI
1 kb

Figure 6. The rDNA repeat unit. (A) rDNA repeat unit [the region of 18S, 5.8S, and 28S rRNA genes is boxed]; (B) and (C) the restriction sites for *Eco*RI and *Sal*I, respectively. The horizontal arrows at the ends of lines indicate that the fragment is continued in the next repeat unit and that there is not a restriction site at that spot. The *Eco*RI map is divided into four units A, B, C, and D. These units contain 7.3, 6, 12 and 19 kb, respectively, and compose one repeat unit. The B region contains the 5' portion of 18S rDNA. The A region contains the rest of the 18S, the 5.8S and most of the 28S genes. The remaining section of 28S rRNA gene is in the D region. The *Sal*I map is divided into four units. Three of these units overlap into the B region of rDNA and would therefore be homologous to the B region. These fragments are 1.5, 2.1 and 11.1 kb, respectively.


18S 5.8S 28S
A
B
D
C
B
A
EcoRI
19
12
5.6
7.3
C
34.1
1.5
2.1
11.1
SalI

The relevant results concerning the portion(s) of the transcriptional unit present in each cosmid are summarized in Table 1 (collaborative experiments with Michael Hadjiargyrou). The restriction enzymes employed were *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I, and *Sal*I, because their restriction sites within the rDNA repeat unit and vector are known. The probes used for Southern analysis were: (a) pBR322 DNA, which was homologous to the vector; (b) A and B region DNA which comprised the transcriptive region, and (c) Alu DNA sequences which were located within the nontranscribed spacer regions.

The results from *Eco*RI restriction enzyme and Southern analyses indicated that the cosmid vector and the *Eco*RI A region was intact in each of the 4 cosmids. In ML1, ML3 and ML6 the *Eco*RI B region was also intact, whereas ML4 was smaller by 1.8 kb. The restriction enzyme *Sal*I was used to determine precisely which portion(s) of the B region were deleted from ML4. Intact rDNA restricted with *Sal*I and probed with B region DNA resulted in three bands at 1.5 kb, 2.1 kb and 11.1 kb (see Figure 6). The 1.5 kb fragment has 1 kb homology to the B region and spans the *Eco*RI site between the B and C region. The 2.1 kb fragment is completely within the B region. The entire A region and approximately 300 bp of the D region comprise the 11.1 kb fragment. The results confirmed an intact transcriptional unit for ML1, ML3 and ML6. The ML4 restriction pattern was missing the 2.1 kb and 1.5 kb bands and had an additional band at 4.2 kb. The 4.2 kb band was homologous to pBR322, *i.e.*, it included vector sequences. These results showed that the vector was ligated within the B region of ML4 which deleted the first *Sal*I site.

Table 1

Southern analysis of cosmids ML1, ML3, ML4 and ML6

			Cosmid			
Enzyme	Probe	Expected	ML1	ML3	ML4	ML6
*Eco*RI	pBR322	8.0	+	+	+	+
	Eco A	7.3	+	+	+	+
	Eco B	6.0	+	+	4.2	+
*Sal*I	Eco B	11.1	+	+	+	+
		2.1	+	+	4.2[1]	+
		1.5	+	+	-	+

[1] The 4.2 kb band obtained from *Sal*I digestion was homologous to both Eco B and pBR322 and was the point at which the vector was ligated to rDNA in ML4.

In summary, ML4 DNA contained a 1.8 kb deletion within the 5' portion of the B region. The deletion included 1 kb of the 3' end of the 1.5 kb *Sal*I band and 0.8 kb of the 5' end of the 2.1 kb internal *Sal*I site. This was confirmed by the size of the fragment containing the vector-rDNA splice site. The fragment was 4.2 kb, or within the expected size range. The expected value was calculated by adding the distances from the unique *Sal*I site in the vector through the *Eco*RI site, the site of insertion at *Sal*I site within the B region. The results show that ML4 is missing the promotor region.

Schematic maps of cosmids ML1, ML3, ML4, and ML6 were made based on information obtained from RE and Southern analyses (Figures 7-10). ML1 (32 kb) had an intact transcriptional unit and contained portions of the C and D regions. ML3 (44.9 kb) also had an intact transcriptional unit and flanking nontranscribed spacer regions, but some rearrangements were present within the repeat unit. The rearrangements occurred within the NTS region and were demonstrated by the presence of additional *Eco*RI and *Hind*III sites. The additional restriction sites are presumed to be the result of mutations present prior to or during the construction of the cosmid library. It is known that regions of the NTS cloned into bacteriophage lambda generate extra restriction enzyme sites (Erikson and Schmickel, 1985). The orientation of the insert was opposite that of ML1.

ML4 was 36-37 kb (Figure 8). The D region and part of the C region near the D region junction were present but contained some rearrangements of the DNA. ML6 (28.6 kb) had the entire transcriptional unit and part of the flanking nontranscribed spacer.

Figure 7. Cosmid ML1. ML1 contains a portion of the rDNA repeat unit ligated into pCV108 (vector). This cosmid contains the entire transcriptional unit which has its origin within the B region of rDNA. The transcription initiation site is indicated by the arrow (P=Promotor). Transcription proceeds from the B region through the A region to the first termination site located in the D region. The transcriptional unit is flanked by portions of both the C and D regions. This cosmid is 32 kb in size. The restriction sites indicated are *Bam*HI(B), *Bgl*II(G), *Eco*RI(E), *Hind*III(H), *Kpn*I(K) and *Sal*I(S).

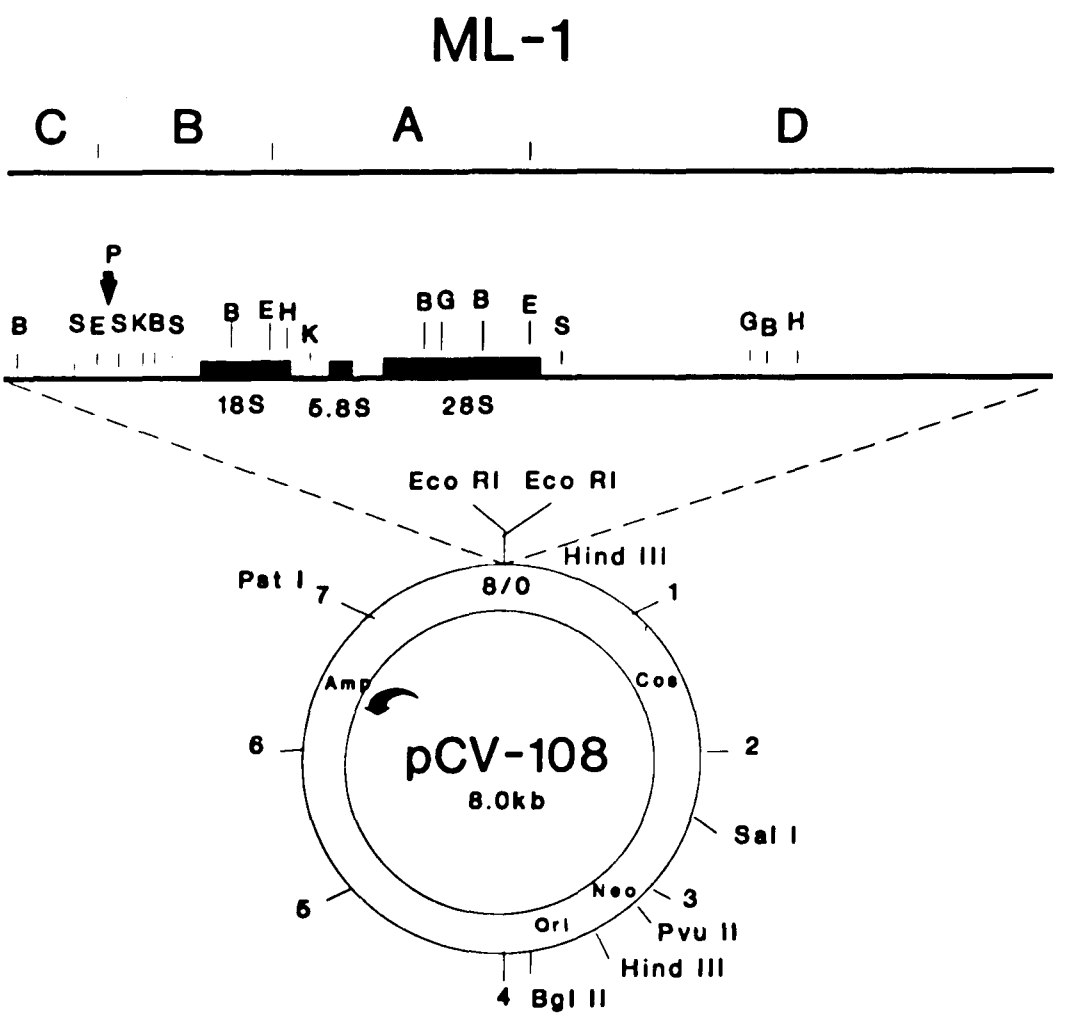

ML-1
C
B
A
D
P
B
SESKBS
B EH
K
BG B
E
S
GB H
18S
5.8S
28S
Eco RI Eco RI
Hind III
Pst I
7
8/0
1
Amp
Cos
6
pCV-108
8.0kb
2
Sal I
5
Neo
3
Ori
Pvu II
Hind III
4
Bgl II

Figure 8. Cosmid ML3. Cosmid ML3 contains a portion of the rDNA repeat unit ligated into pCV108 (vector). This cosmid contains the entire transcriptional unit which has its origin within the B region of rDNA. The transcription initiation site is indicated by the arrow (P=Promotor). Transcription should proceed from the B region through the A region to the first termination site located in the D region. The transcriptional unit is flanked by a portion of the D region and the C region attached to another portion of the D region. There are rearrangements in both the C and D regions (Areas with rearrangements are indicated by an *). This cosmid is 44.9 kb in size. The restriction sites indicated are *Bam*HI(B), *Bgl*I(G), *Eco*RI(E), *Hind*III(H), *Kpn*I(K) and *Sal*I(S).

ML-3

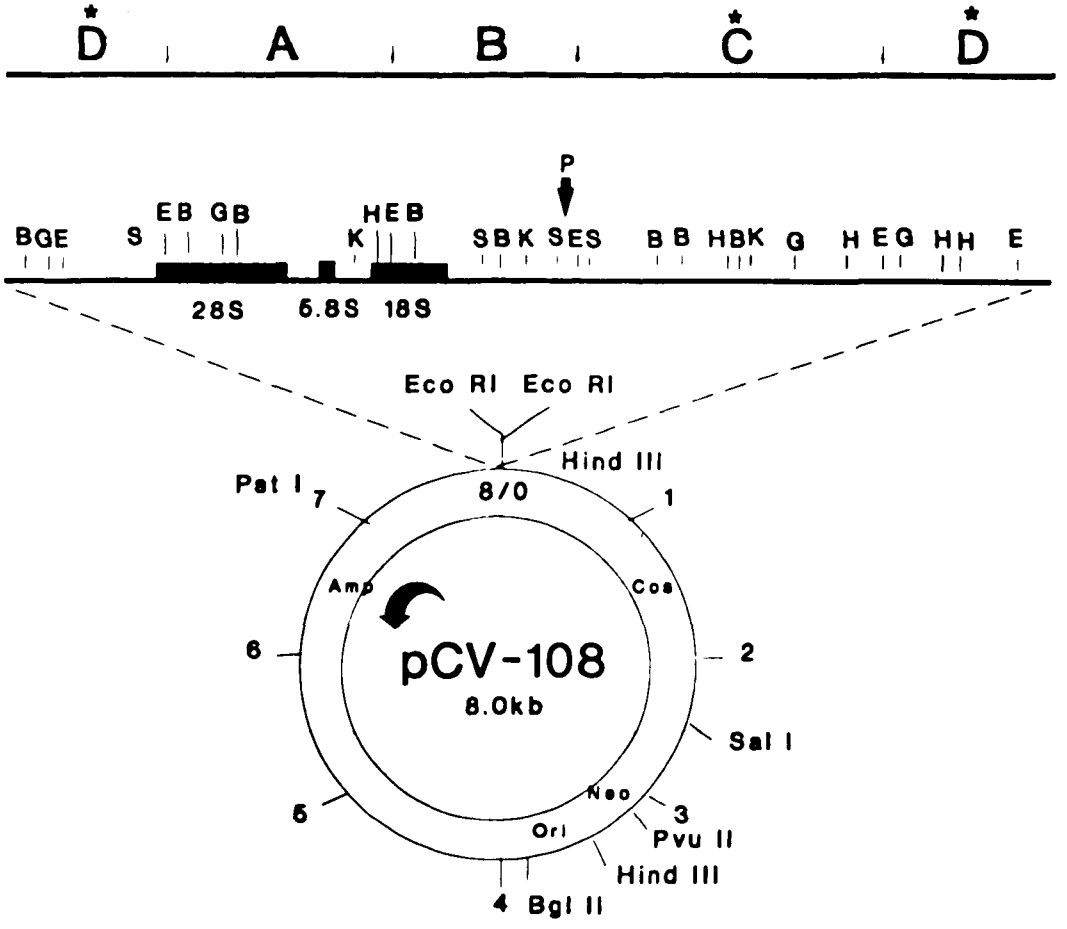

D
A
B
C
D
P
BGE
S
EB GB
HEB
K
SBK SES
B B HBK
G
H EG
HH
E
28S
5.8S
18S
Eco RI Eco RI
Hind III
Pst I
7
8/0
1
Amp
Cos
6
pCV-108
8.0kb
2
Sal I
5
Neo
3
Pvu II
Ori
Hind III
4 Bgl II

Figure 9. Cosmid ML4. Cosmid ML4 contains a portion of the rDNA repeat unit ligated into pCV108 (vector). This cosmid contains part of the transcriptional unit. The promotor region is deleted. The promotor is normally found in the early portion of the B region of rDNA. The remaining portion of the transcriptional unit (B, A and approximately 300 bp of the D region) is flanked by portions of both the vector and D regions. The D region is attached to the C region. There are rearrangements in both the C and D regions [Areas with rearrangements are indicated by as (*)]. This cosmid is 36-37 kb in size. The restriction sites indicated are *Bam*HI(B), *Bgl*I(G), *Eco*RI(E), *Hind*III(H), *Kpn*I(K) and *Sal*I(S).

ML-4

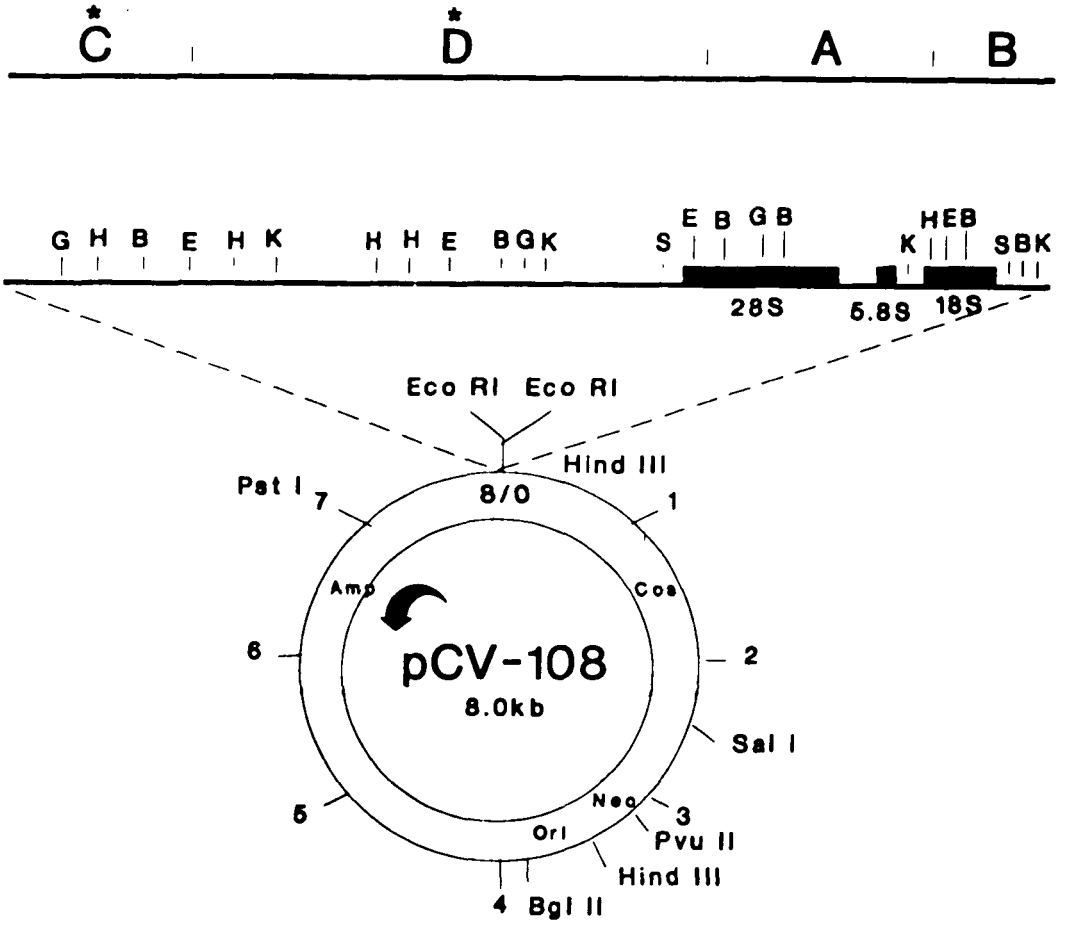
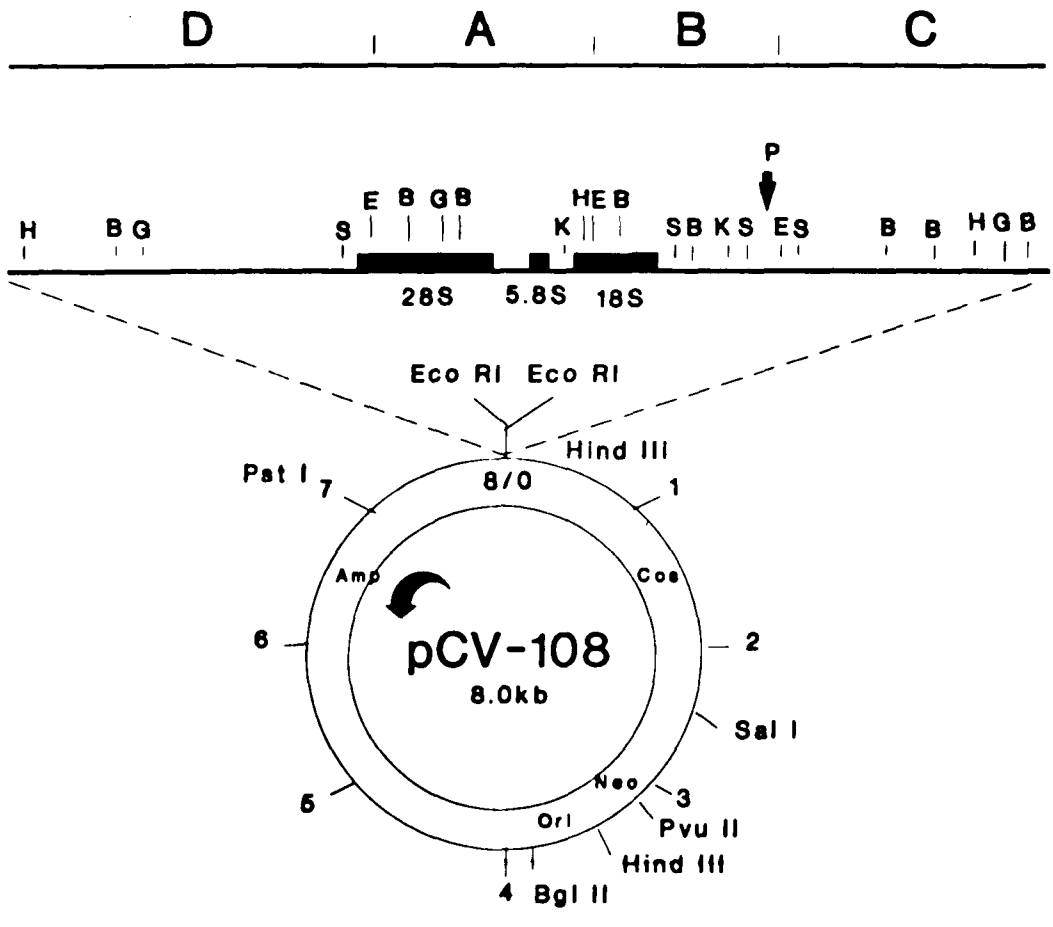

C
D
A
B
G H B E H K
H H E BGK
S E B G B
K HEB
SBK
28S
5.8S
18S
Eco RI Eco RI
Hind III
Pst I
7
8/0
1
Amp
Cos
pCV-108
8.0kb
6
2
Sal I
5
Neo
3
Pvu II
Ori
Hind III
4 Bgl II

Figure 10. Cosmid ML6. Cosmid ML6 contains a portion of the rDNA repeat unit ligated into pCV108 (vector). This cosmid contains the entire transcriptional unit which has its origin within the B region of rDNA. The transcription initiation site is indicated by the arrow (P=Promotor). Transcription proceeds from the B region through the A region to the first termination site located in the D region. The transcriptional unit is flanked by portions of both the C and D regions. This cosmid is 28.6 kb in size. The restriction sites indicated are *Bam*HI(B), *Bgl*I(G), *Eco*RI(E), *Hind*III(H), *Kpn*I(K) and *Sal*I(S).

ML-6


D
A
B
C
P
H
B G
S
E B G B
H E B
K
S B K S E S
B B
H G B
28S
5.8S
18S
Eco RI
Eco RI
Hind III
Pst I
7
8/0
1
Amp
Cos
pCV-108
8.0kb
6
2
Sal I
5
Neo
3
Ori
Pvu II
Hind III
4
Bgl II

B. Transfection of Cosmid DNA into Human Diploid Fibroblasts.

1. Optimal Conditions.

Preliminary experiments were performed to determine the optimal DNA concentration and time required for successful transfection (Table 2). The optimal conditions for HAL cells were 30μg of DNA for 3 hours.

The optimal time period was determined since lipofectin is toxic to cells and each cell line has a different sensitivity. Long exposure times put undue stress on the cells decreasing the efficiency of transfection, whereas short exposure times do not allow appropriate fusion between cell membrane and liposomes. The concentration of DNA is also important. Liposomes become large at high concentrations of DNA and could be too large for adequate uptake into the cell. Conversely, a low concentration results in cells acquiring liposomes devoid of DNA. The optimal conditions for HAL cells were determined to be 30μg of DNA for 3 hours. Under these conditions eight clones were produced.

2. Analysis of Transfections

ML1 and ML4 DNAs were successfully transfected into HAL cells. ML1 DNA contained an intact transcriptional unit, was not rearranged and had the largest insert size. ML4 DNA did not have an intact transcription unit; the promotor was missing. The transfection efficiency for the two DNAs averaged 6.6×10^{-6}. Clones chosen for analysis were the first to emerge from clonal expansion indicating the most rapid rate of growth. These clones were named 1K1 (ML1) and 4K2 (ML4).

When a cloned cell line was grown in a T-25 flask, it was considered an

Table 2

LIPOSOME-MEDIATED TRANSFECTION

	Exposure time in hours		
	3	6	9
Amt DNA(μg)		Number of clones	
10	2	1	0
20	7	nd[1]	nd
30	8	nd	nd

[1] nd: the experiments were not done

established cell line, and named by a three digit alphanumeric code (Table 3). The first digit represented the last number of the name given to the bacterial clone from which the DNA was derived. The second digit was a series name and was represented by a K for clones containing cosmid DNA and a T for plasmid clones (see appendix). The third digit was a number assigned to the clone based upon the order in which it was transferred into a T-25 flask. Mixed cell populations were isolated and cultured. These cells served as a source of additional clones and are listed in Table 3.

There were 4 attempts to transfect ML1 into HAL cells and two were successful. The transfection efficiency for ML1 was 7.5×10^{-6} (Table 4). Pure cell line 1K1, 1K2, 1K3, and 1K4 were all isolated from mixed cultures which had been reseeded onto plates. A schematic representation of the lineage of both pure and mixed cell lines from ML1 transfected HAL cells are summarized in Figure 11.

The transfection of ML4 into HAL cells was accomplished by three experiments yielding a transfection efficiency of 5.7×10^{-6} (Table 4). Pure cell line, 4K1, was isolated from a plate containing cells from the initial transfection. This cell line was lost when the cells went into crisis after entering a T-25 flask but prior to having a sufficient quantity to make stocks. Three additional pure cell lines were isolated from plates in which mixed cultures were reseeded. These cell lines were 4K2, 4K3, and 4K4. A schematic representation of the lineage of both the pure and mixed cell lines from ML4 transfected HAL cells are summarized in Figure 11.

Three attempts were made to transfect ML3 and ML6 cosmid DNA into HAL cells. The transfection efficiency for ML3 and ML6 was 3×10^{-6} and 1.3×10^{-6},

Table 3

Clones obtained from transfection with cosmids ML1, ML3, ML4 and ML6

A. Pure Clones

DNA Construct	Clones
ML1	1K1
	1K2
	1K3
	1K4
ML3	3K1[1]
ML4	4K1[2]
	4K2
	4K3
	4K4
ML6	----

B. Mixed cell populations

DNA Construct	Clones	# of Clones
ML1	ML1(1) clone 4	3
ML4	ML4(1) clone 4	2-3
ML4	ML4(2) clone 2	2-3
ML4	ML4(2) clone 2&3 (reseeded)	2

C. Mixed clones obtained by reseeding transfected cells

DNA Construct	Mixed clones	Source
ML1	ML1TGLP orig	Original clones regrown
ML1	ML1TGLP #1	Seeded from ML1TGLP orig
ML1	ML1TGLP #2	serial dilution
ML4	ML4TGLP orig	Original clones regrown
ML4	ML4TGLP #1	Seeded from ML4TGLP orig
ML4	ML4TGLP #2	serial dilution
ML4	ML4TGLP #3	serial dilution

[1]lost due to contamination.
[2]Clone 4K2 went into crisis after being placed in a flask, but prior to having enough cells to "freeze down".

Table 4

Transfection efficiencies for cosmids ML1, ML3, ML4, and ML6

Cosmid	# of clones/ $5x10^5$ cells	Transfection efficiency/plate	Average efficiency
ML1	9	$1.8x10^{-5}$	$7.5x10^{-6}$
	6	$1.2x10^{-5}$	
	0		
ML4	3-4	$7.0x10^{-7}$	$5.7x10^{-6}$
	5	$1.0x10^{-5}$	
	0		
ML3	3	$6.0x10^{-6}$	$3.3x10^{-6}$
	0		
	2	$4.0x10^{-6}$	
ML6	0		$1.3x10^{-6}$
	0		
	2	$4.0x10^{-6}$	

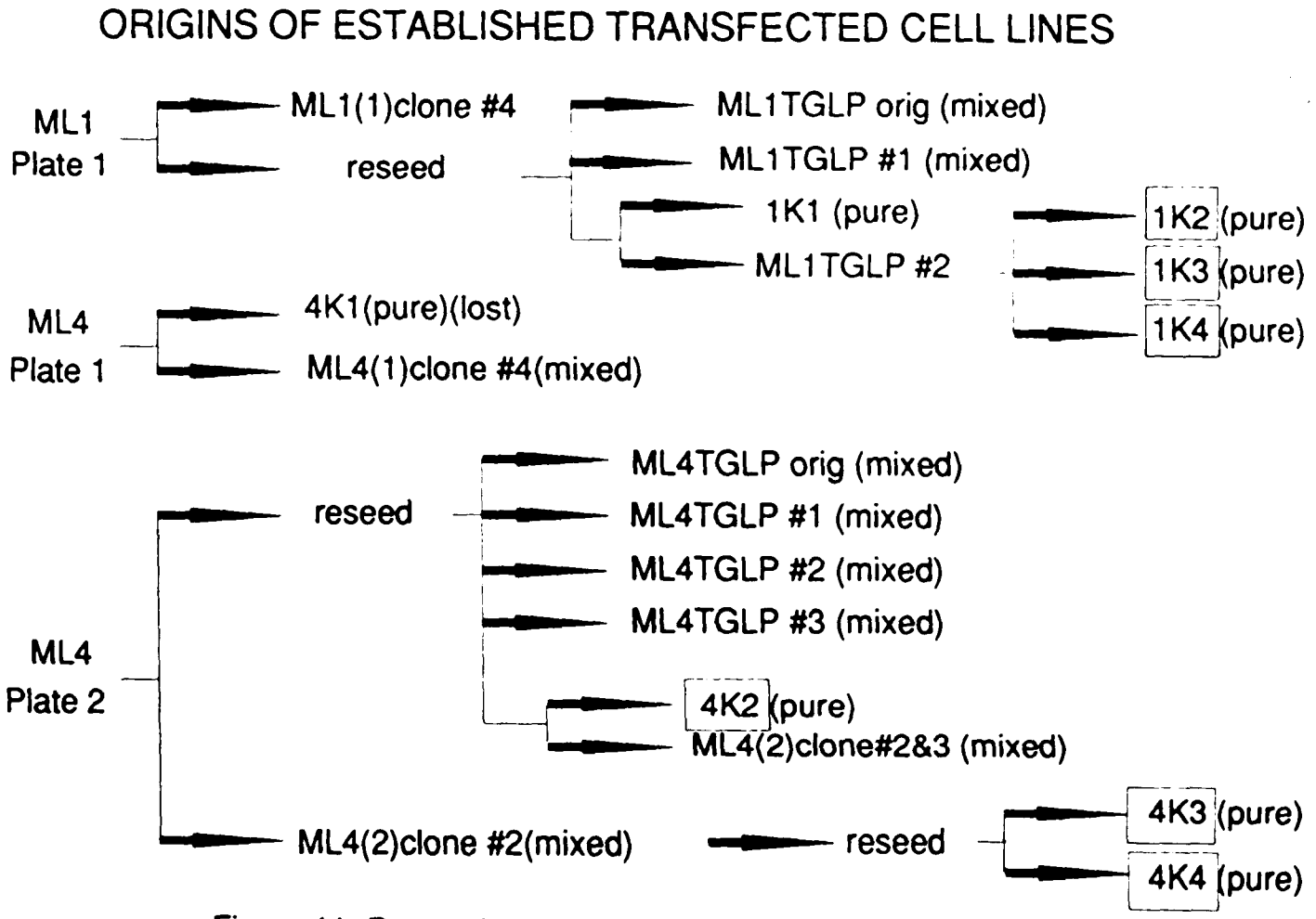


Figure 11. Parental lineage of ML1 and ML4 cells

respectively. One pure clone from ML3 transfections survived clonal expansion, but was lost due to contamination. No established cell lines were produced from ML6 transfection. Further attempts at transfecting ML3 and ML6 were deemed unnecessary because experiments involving restriction enzyme and Southern analysis indicated that ML3 contained substantial rearrangements and ML6 lacked adequate representation of rDNA (See section A of Results.).

C. Analysis of rDNA Insertion Site

1. Determination of the Recombination Site (collaboration with Michael Hadjiargyrou).

The object of these experiments was to determine the site within the cosmid at which recombination with the human genome occurred. This site was determined by using Southern hybridization of the vector sequences to the HAL DNA. The restriction fragments obtained were compared to those expected based on our analysis of the cosmid and known restriction sites in native rDNA. Ribosomal DNA was not used as a probe since the endogenous native rDNA is in a 400 fold excess compared to donor sequences and the probes would selectively hybridize to rDNA.

Cosmid pJB8 was used as a probe because it gives the maximum homology to the vector without hybridizing SV40 sequences known to be present in HAL. HAL was initially immortalized by a plasmid (pMK16) which contained SV40 sequences. The vector pCV108 was derived from pJB8 (5.4 kb) by the addition of SV40 sequences and therefore pJB8 was selected as the probe (Note: this plasmid was unavailable for earlier experiments since the stock cultures we

received from American type culture collection were ambiguous relative to the expected restriction pattern). HAL genomic DNA was a control for pMK16 fragments since there was 1.8 kb homology between pMK16 and pJB8. The bands were expected in all transfected cell lines since they were derived from HAL cells.

The restriction enzymes *Bam*HI, *Bgl*I, *Eco*RI, *Hind*III, *Kpn*I and *Sal*I were employed for Southern analysis to determine which portion of the cosmid ligated into the HAL genome. The results indicated that in both 1K1 and 4K2 the insertion occurred within the vector portion of the cosmid. If the vector were intact, an 8 kb band with homology to pJB8 would be expected when the genomic DNA was digested with *Eco*RI. Conversely, if the cosmid was spliced into the genome within the vector, then 2 bands would be expected, each containing a portion of the vector, as well as genomic DNA. In both 1K1 and 4K2, the 8 kb band was absent, but there were two additional bands which were not present in HAL cells. 1K1 DNA produced a DNA of 10.8 kb, as well as another high molecular weight band (>20 kb). There were two bands which corresponded to pMK16. Restriction of 4K2 DNA produced DNA banding at 14.2 kb, a higher MW band (>20 kb), and two bands with homology to pMK16 DNA. These results were consistent with cleavage of the vector DNA during insertion into the human genome, leaving the integrated rDNA repeat unit intact.

The conclusion drawn from the Southern analysis was that the cell lines, 1K1 and 4K2, contained a large intact repeat unit. This result was highly desirable because it would permit the maximum amount of interaction between the rDNA sequences and any factors required for NOR formation.

2. Chromosome Localization of Integrated DNA.

a. Location of vector sequences. Cytological hybridization was used to localize the insertion site. Two different methods were employed. The first method used ^{125}I-dCTP labelled pBR322 as a probe. PBR322 DNA has 3.2 kb direct homology to the cosmid vector (pCV108) and 1.8 kb homology to pMK16. The second method employed a non-radioactive method with biotin-labeled pJB8 DNA used as a probe. Plasmid pJB8 had 5.4 kb homology to the cosmid vector formed by ligating SV40 sequences into pJB8. There was also 1.8 kb homology to pMK16.

Chromosome 5q21 is the site of integration of pMK16 (Patsalis, unpublished). This site served as an internal control for hybridization *in situ*.

The chromosomal regions of 1K1 which accumulated a significant number of grains during the radiolabelled hybridization *in situ* are indicated in Table 5. These were at the middle of the p-arm of chromosome 3 and the expected site on chromosome 5q. The X^2 values for each chromosomal region are plotted in Figure 12. Photographs of the labelled chromosomes are given in Figure 13. The results from the radiolabelled cytological hybridization for 4K2 were ambiguous.

The positive regions for each chromosomal region following enzymatic cytological hybridization in 1K1 and 4K2 are plotted in Figures 14 and 15. The previous results were confirmed. The ML1 insertion site was located above the centromere in chromosome 3 in 1K1 (Figure 16). In addition, the site in 4K2 was localized to chromosome 4 (Table 5; Figure 17).

b. Location of rDNA. The rDNA transcriptional unit was localized to the same chromosomal regions as the vector sequences in both 1K1 and 4K2 cell

Table 5

Chromosomal location of cosmid insertion

A. Chromosomal regions of transfected cell lines which contained significant numbers of grains following hybridization *in situ* using ^{125}-IdCTP labelled pBR322 DNA as a probe.

Cell line	Chromosome	Region	Number of grains - expected background[2]	X^2
1K1[1]	3	2	18	72.6
	5	3	14	43.6

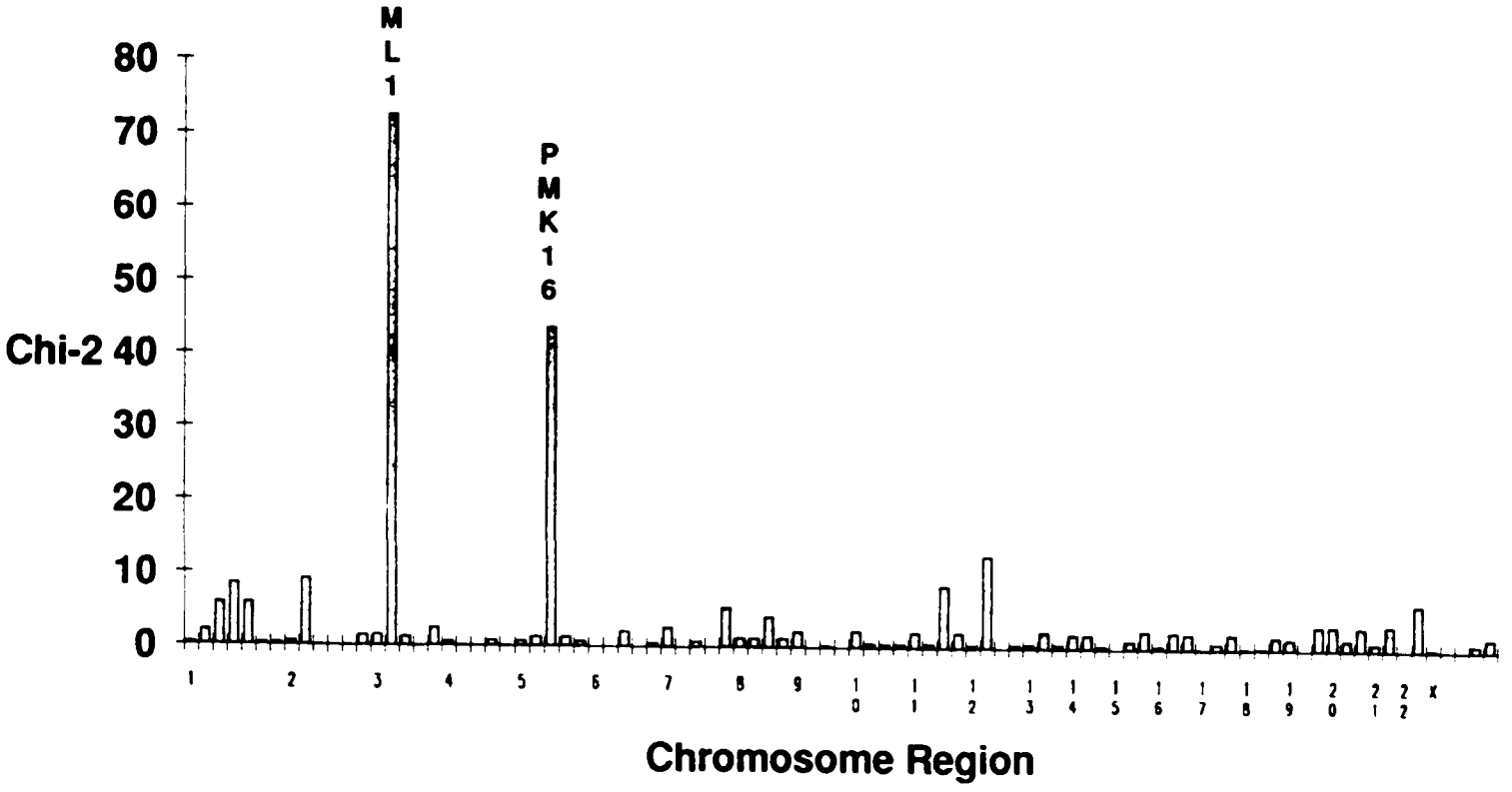
B. Chromosomal regions of transfected cell lines stained on both sister chromatids when pJB8 is used as a probe in cytologic hybridization

Cell line	Chromosome	Region	% stained
1K1[3]	3	3	41%
4K2[4]	4	4	50%

[1] Data based upon the analysis of 31 plates
[2] The expected values are 5 grains and 4 grains for each region of chromosome 3 and 5, respectively.
[3] Data based upon the analysis of 22 metaphase plates
[4] Data based upon the analysis of 28 metaphase plates

Figure 12. Analysis of 1K1 cells following cytological hybridization. The histogram shows X^2 values for each chromosomal region in 1K1 cells. These values were obtained from a radiolabelled hybridization *in situ*. The probe used was pBR322 which has homology to cosmid, ML1, and pMK16 DNA. pMK16 is located in chromosome 5 (Patsalis, unpublished). There is a peak in the p-arm of chromosome 3 which corresponds to the ML1 insertion site.

Analysis of 1K1 Cells following Cytological Hybridization



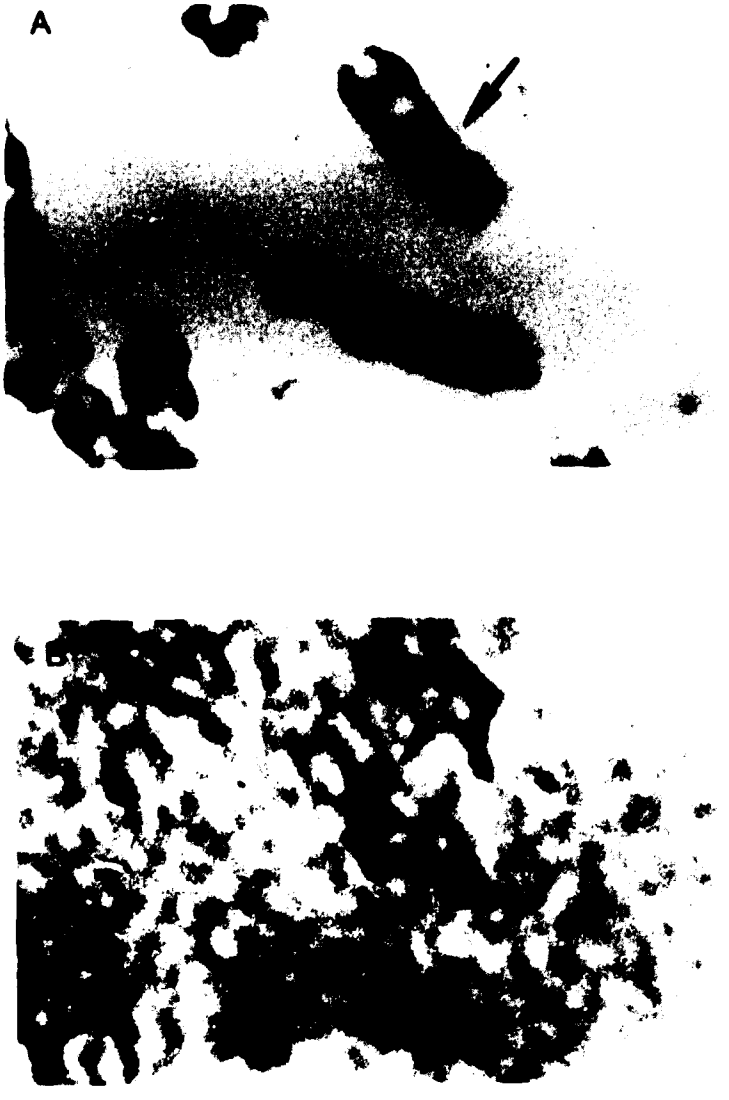


Figure 13. Hybridization to chromosome 3. Photographs of chromosome 3 in 1K1 before (A) and after (B) cytological hybridization with pBR322. (arrow indicates ML1 insertion site).

Figure 14. Hybridization to 1K1 cells. The histogram gives results from an enzymatic cytological hybridization of probe pJB8 to chromosomal spreads of 1K1 cells. The results are plotted as the % of plates in which deposited stain appeared on both sister chromatids relative to chromosome region. Chromosome 3 in the region of the p-arm near the centromere had the highest percent stain.

Analysis of Data: Enzymatic Cytologic Hybridization to 1K1 Cells

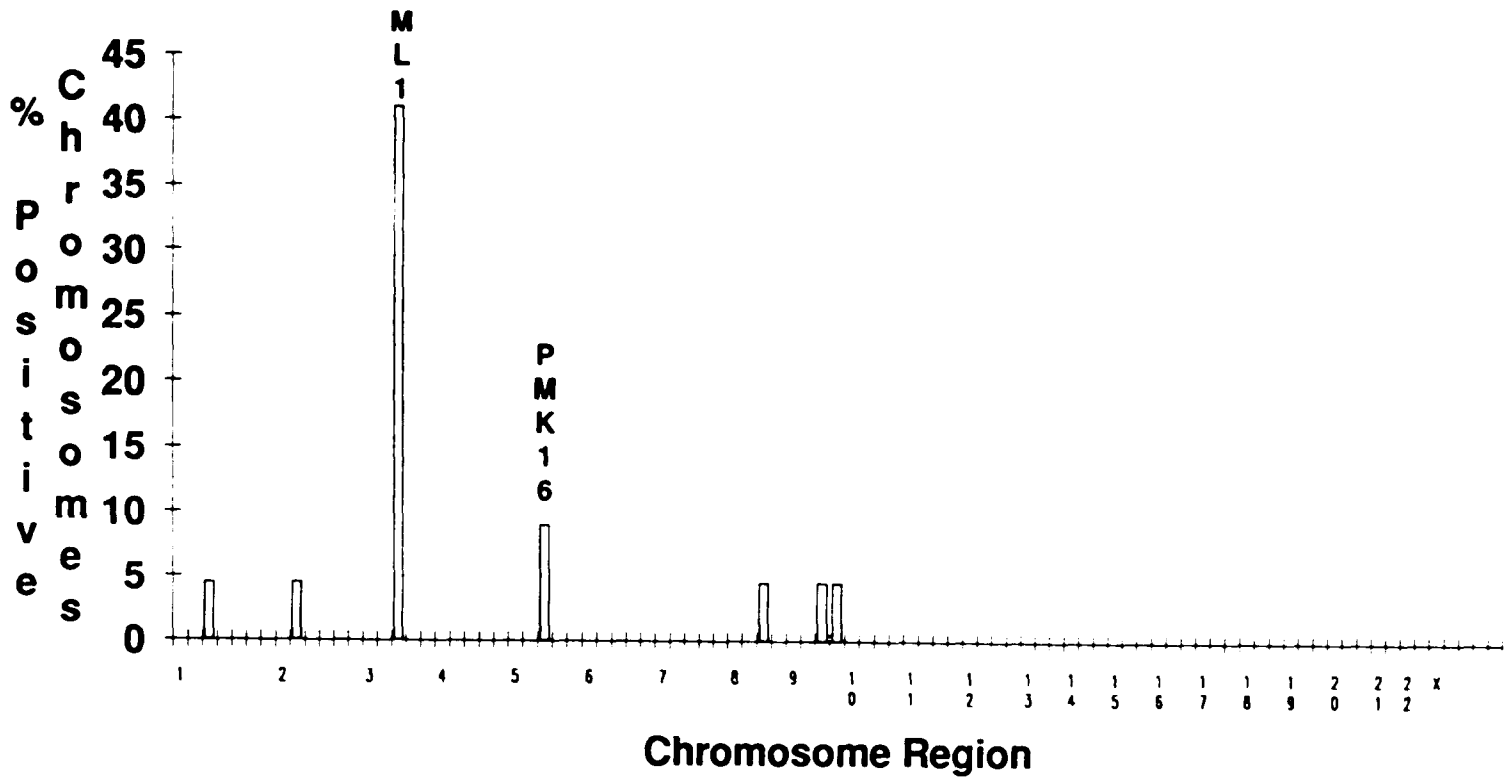


Figure 15. Hybridization to 4K2 cells. The histogram shows the results from an enzymatic cytological hybridization of probe pJB8 to chromosomal spreads of 4K2 cells. The results are plotted as the % of plates in which deposited stain appeared on both sister chromatids verses chromosomal regions. The insertion site is in the q-arm of chromosome 4.

Results of Enzymatic Cytologic Hybridization to 4K2 Cells

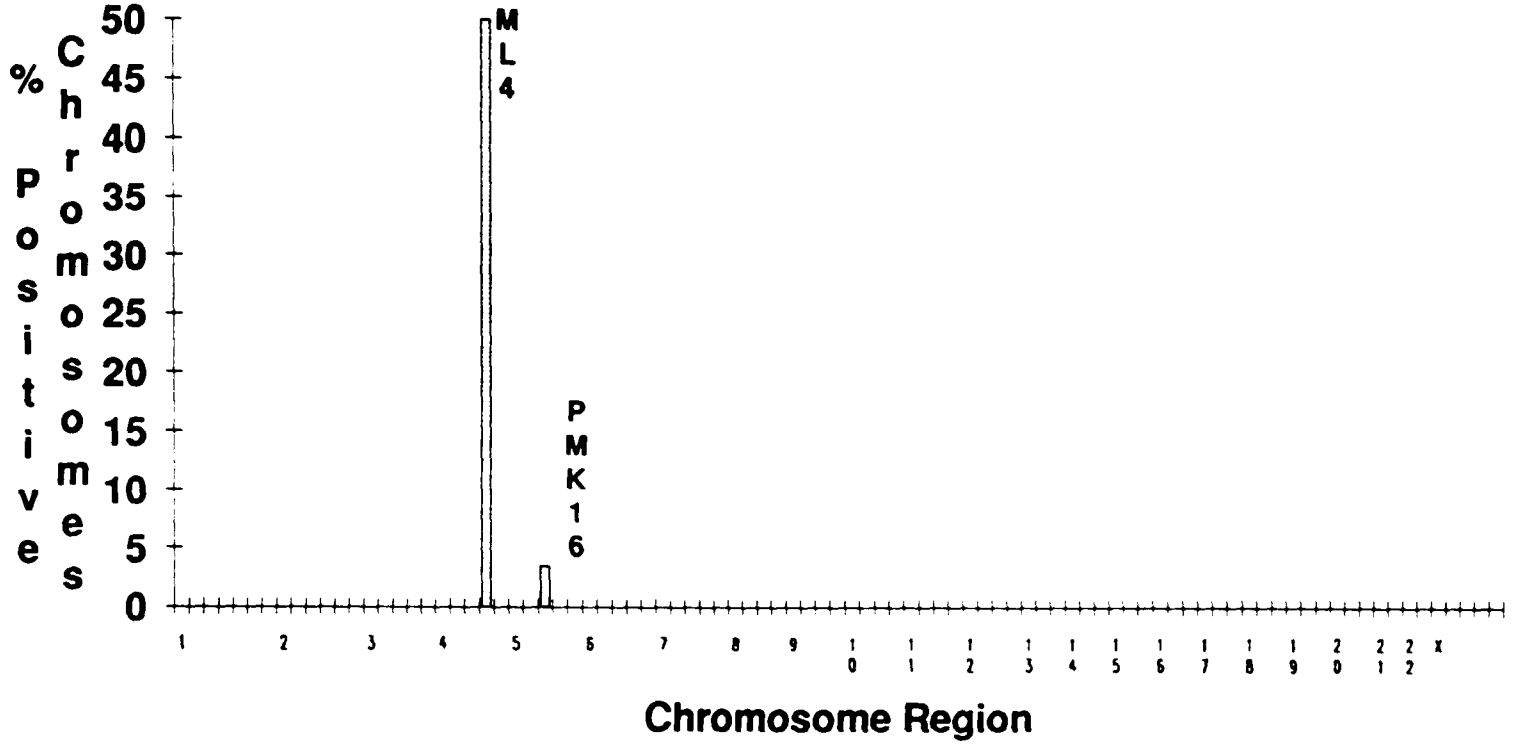


Figure 16. Enzymatic hybridization to chromosome 3. Photographs of chromosome 3 in 1K1 before (A) and after (B) enzymatic cytological hybridization of 1K1 with pJB8. (arrow indicates ML1 insertion site)

Figure 17. Hybridization to chromosome 4. Photographs of chromosome 4 in 4K2 cells before (A) and after (B) enzymatic cytological hybridization with pJB8. (arrow indicates ML4 insertion site).

lines. Equal concentrations of DNA from both regions A and B were combined (for maximum homology) and used as a probe in an enzymatic hybridization *in situ* to determine if rDNA was present in the ML1 insertion site in chromosome 3 of 1K1 cells and the ML4 insertion site of chromosome 4 in 4K2 cells (Table 6, Figures 18 and 19).

The cosmid ML1 DNA was localized specifically to chromosome 3p12 in 1K1 cells and chromosome 4q31-32 in 4K2 cells. As expected, the acrocentric chromosomes acquired large amounts of the probe and all acrocentric chromosomes gave positive results for Eco A and Eco B DNA.

D. Transcriptional Analysis.

1. Silver Staining

Silver staining was employed to determine if the inserted DNA was transcriptively active. The insertion region in chromosome 3 in 1K1 stained positive with silver, but the insertion region in chromosome 4 in 4K2 did not. The remnants of nucleoproteins on endogenous NOR regions selectively acquire large amounts of silver. Most silver stained chromosomes were identified by group names because G-Banding techniques destroy the nucleoproteins.

The normal pattern of silver staining in a typical HAL cell is shown in Figure 20. Four of these chromosomes were participating in satellite association and have formed a ring in which the satellite regions are touching.

The data clearly indicate that the rDNA from ML1 was actively transcribed by pol I since chromosome 3 in 1K1 was silver stained. In 1K1 cells, sixty-three chromosome 3's were positively identified and 32 potentially identified from 84

Table 6

Cytological hybridization of Eco A and Eco B to non-acrocentric chromosomes

Chromosome	region	Cell line	% stained
3	3	1K1[1]	47%
4	4	4K2[2]	31%
t(11:22)	2	1K1,4K2[3]	19%

[1] Data based upon the analysis of 19 metaphase plates
[2] Data based upon the analysis of 42 metaphase plates
[3] Data based upon the analysis of 42 identified t(11;22)

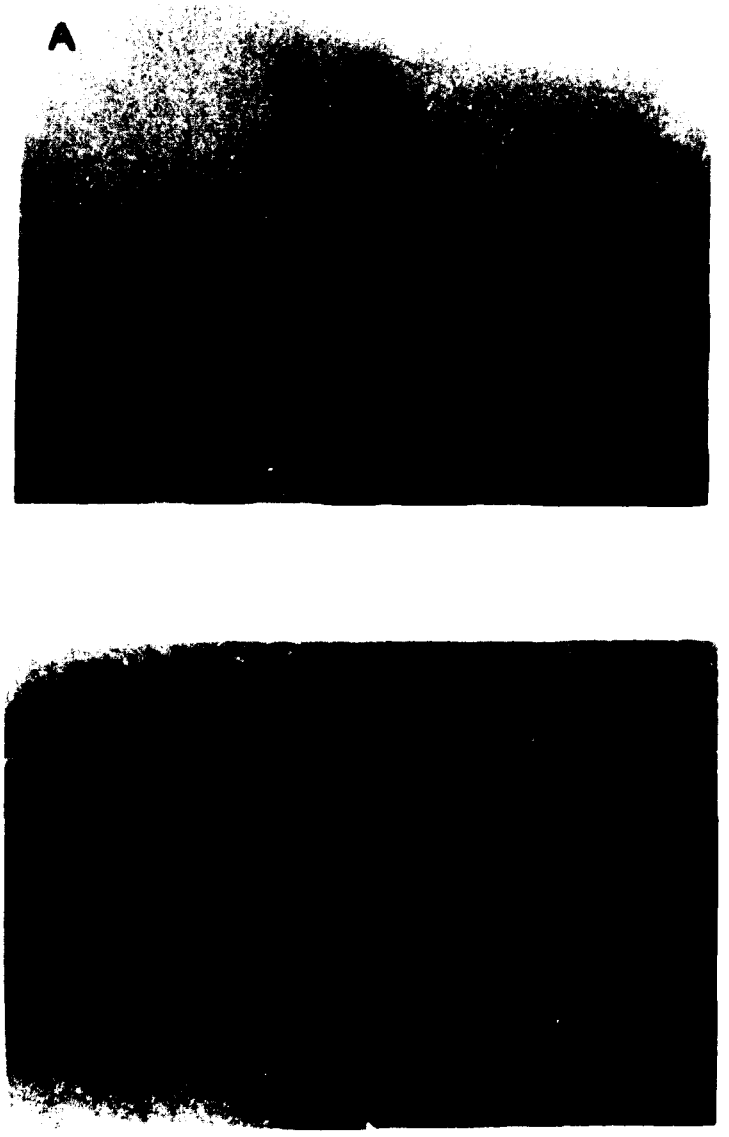


Figure 18. Cytologic hybridization to chromosome 3. Photographs taken of chromosome 3 before (A) and after (B) enzymatic cytological hybridization of 1K1 with Eco A and Eco B. (arrow indicates site of ML1 insertion).

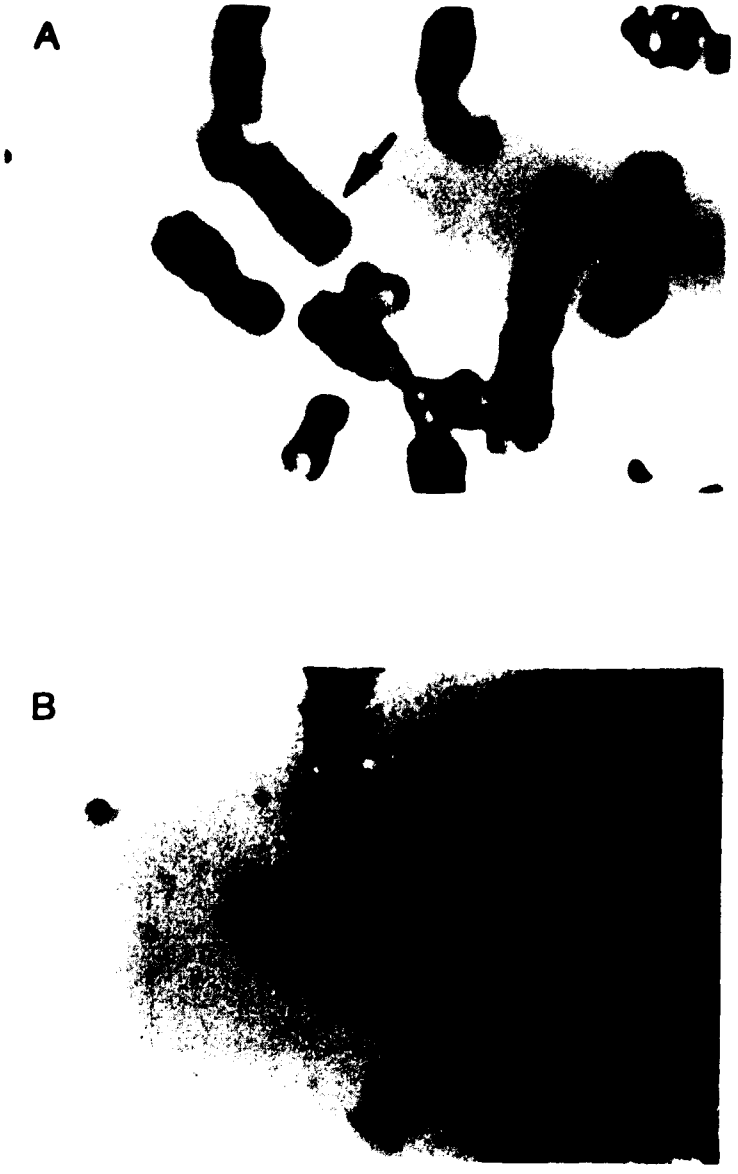


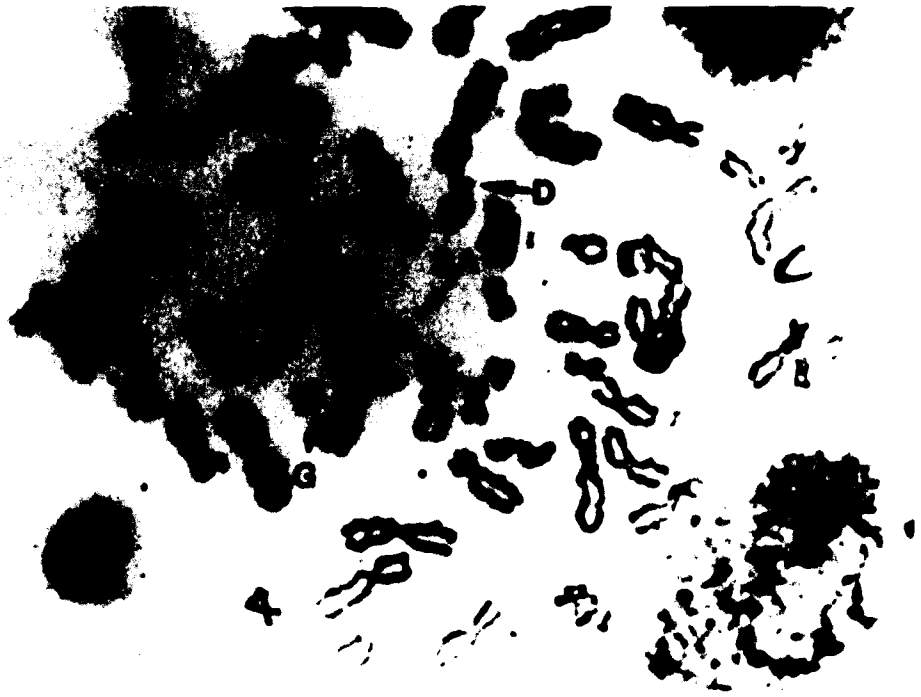
Figure 19. Cytologic hybridization to chromosome 4. Photographs taken of chromosome 4 in 4K2 cells before (A) and after (B) enzymatic cytological hybridization with Eco A and B. (arrow indicates site of insertion of ML4).

Figure 20. Analysis of silver staining

A. Silver stained metaphase plate from HAL cells. A metaphase plate from HAL in which the acrocentric chromosomes were stained with silver stain. The D-labelled and G-labelled arrows point to single silver stained D-group and G-group chromosomes. The SA labelled arrow shows 4 G-group acrocentric chromosomes participating in satellite association. These chromosomes have formed a ring.

B. Silver stained chromosome 3 from 1K1. A photograph of chromosome 3 from 1K1 which has silver deposits over the region of cosmid integration in both sister chromatids (arrow).

A



B

plates. Eight out of the 95 chromosomes had recognizable silver deposits on both sister chromatids in the region corresponding to the insertion site of ML1 (Table 7 and Figure 21). The percent of rDNA bearing chromosome 3's which stain positive with silver was 17%. The silver deposits over the rDNA insertion site in chromosome 3 appeared less dense than over the satellite regions of the acrocentric chromosomes. This was due to one copy of rDNA being present in each sister chromatid of chromosome 3 compared to the 30-50 copies present in each acrocentric chromosome. The distribution of silver stain on 1K1 chromosomal groups showed two nonacrocentric chromosomal regions which contained higher than background levels of silver deposits. These chromosomes were chromosome 3 at the region of insertion of ML1 and the centromeric region in the C-group size chromosome, t(11;22). T(11;22) stained positively in 4K2 cells, but no other chromosome showed specific staining (Table 7; Figure 22).

2. Northern Analysis of Transcripts from HAL, 1K1 and 4K2 Cells

a. Background. Northern hybridizations were employed to determine if transcripts from transfected cell lines contained detectable cosmid sequences. There was the potential for transcription into the vector sequences since a small percentage of pol I molecules transverse the downstream terminator sites. It was also possible that during transfection into HAL cells some deletions occurred in ML1. In this scenario, 1K1 cells would initiate transcription at the promotor region and transcription would proceed until it came to an rRNA termination sequence or until transcription ceased due to physical constraints. In these experiments, HAL cells served as controls for any transcripts common to HAL-derived cell lines and

TABLE 7

Comparison of silver staining between 1K1 and 4K2

	1K1	4K2
Number metaphases examined	84	33
Identified chromosomes 3	63	38
Chromosomes 3 (tentative identification)	32	2
Chromosomes 3 silver stained (region 2)	8	0
Chromosomes 3 silver stained (region 3)	2	0
B-group chromosomes identified	ND	69
B-group chromosomes-silver stain (region 4)	0	0
C-group chromosomes-silver stain (centromere)		
[chromosome t(11;22)]	7	4
D-group: silver stained	105	45
G-group: silver stained	170	61

ND: not determination

Note: Human chromosomes fall into 7 groups which are A (1,2,3); B (4,5); C (6,7,8,9,10,11,12,X); D (13,14,15); E (16,17,18); F (19,20); and G(21,22). Abnormal chromosomes were placed into normal groups based upon the group characteristics (with the exception of the abnormal 7 which was identifiable as having two centromeres). Chromosome 3 was identified on the basis of size, position of centromere and rudimentary banding pattern. Chromosome 4 was identified as a B-group chromosome, but it cannot be differentiated from chromosome 5. It could be differentiated from its abnormal homolog and a second abnormal B-group chromosome based on size.

Figure 21. Silver deposits over chromosomal regions in 1K1 cells.

Silver deposits present in both sister chromatids in 1K1 cells. There were 84 plates analyzed. The abnormal chromosomes in HAL were placed in "normal" chromosomal groups based upon size and position of the centromere with the exception of the abnormal 7 chromosome. This chromosome has two centromeres and therefore did not fall into a defined group. The acrocentric chromosomes stained positive with silver and are indicated by a star on the chart. In HAL cells there is an abnormal acrocentric chromosome which resembles a C-group chromosome. This chromosome, t(11;22), contains rDNA. The process of silver staining does not allow for the clear differentiation of the t(11;22) chromosome from other C-group chromosomes. There were two nonacrocentric chromosomal regions which contained higher than background levels of silver deposits. These chromosomes were chromosome 3 at the region of insertion of ML1 and the centromeric region in the C-group chromosomes. These C-group deposits are probably over the t(11;22) chromosome.

DISTRIBUTION OF SILVER DEPOSITS OVER CHROMOSOMAL GROUPS FROM 1K1 CELLS

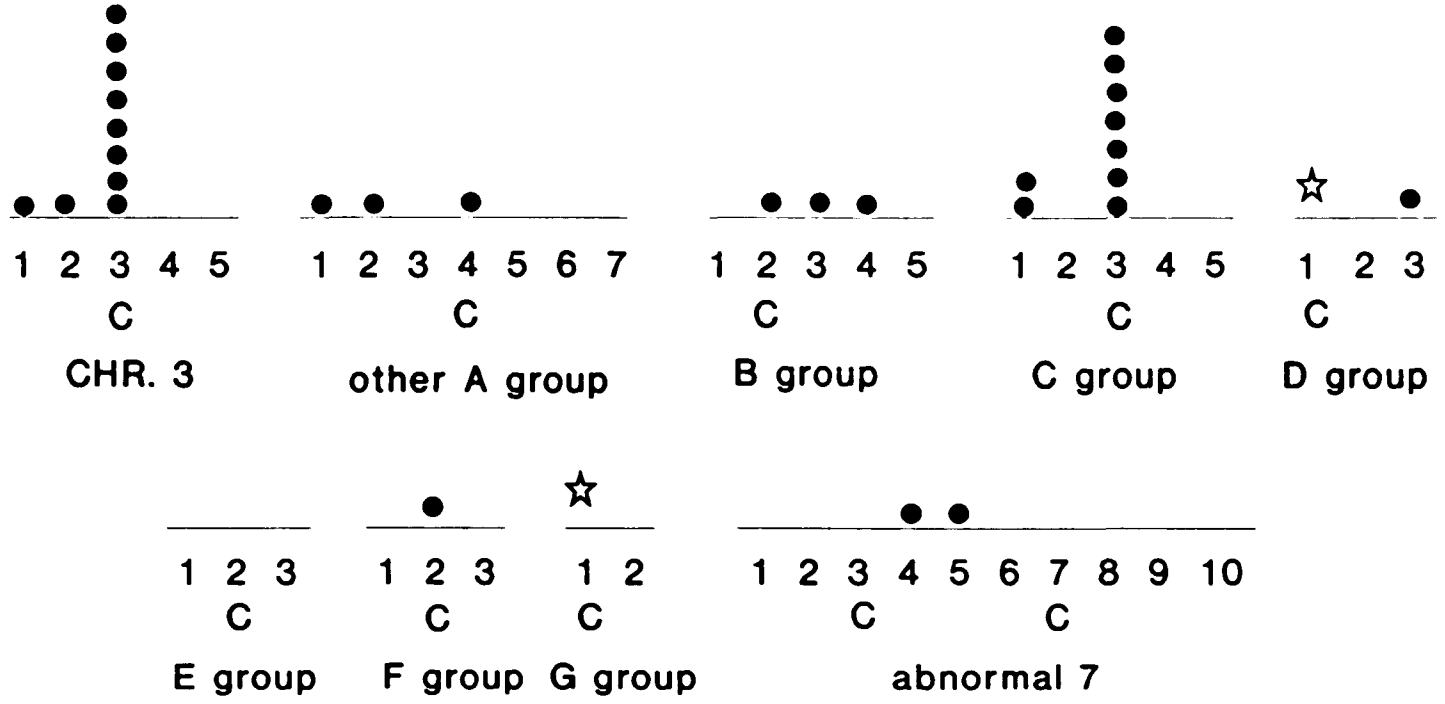
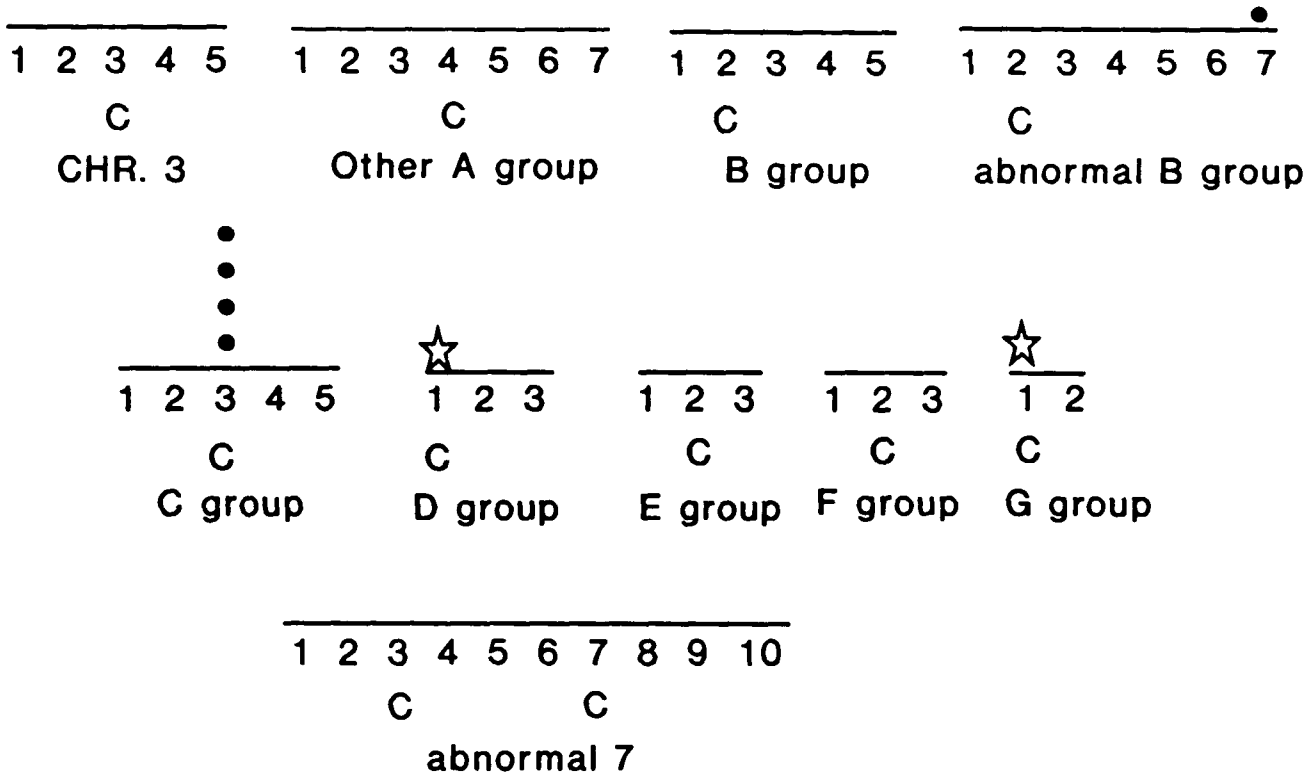


Figure 22. Silver deposits over chromosomal regions in 4K2 cells. There were 33 plates analyzed. A star represents the endogenous rDNA regions which have very large numbers of positively stained chromosomes. Only one chromosomal region was greater than background. This region is in the centromeric region of C- group chromosomes. These deposits are probably over the t(11;22) chromosome which contains rDNA. There was one other chromosome which was positive for silver stain. This region is at the terminus of the q-arm of one of the abnormal B-group chromosomes, but is at background levels. The insertion site for ML4 does not stain positive with silver stain.

DISTRIBUTION OF SILVER DEPOSITS OVER CHROMOSOME GROUPS FROM 4K2 CELLS



4K2 served as a negative control for 1K1.

The autoradiograms from Northern hybridizations were exposed over various time intervals ranging from 30 minutes to 2 weeks (in order to increase the probability of detecting aberrant transcripts). The rationale for the time intervals is that the probability of detecting a transcript present in low copy number in a short exposure is small. However, longer exposures also increase the probability that a band will become obscured by other bands over time. Several types of probes were used to determine whether a transcript from the integrated rDNA could be detected. None gave definitive results.

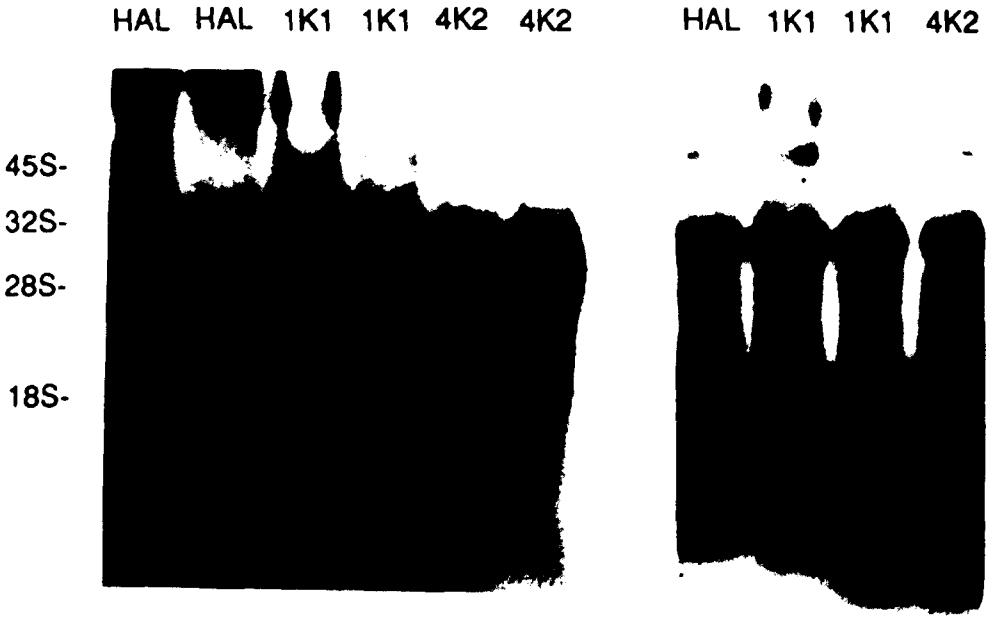
b. Hybridization using Eco A and Eco B. Aberrant rRNA transcripts were not detected from any of the 3 cell lines examined when Eco A or Eco B were used as probes in spite of extended autoradiographic exposure times. For long exposures, the 18S and 28S bands were removed from the blot prior to exposing an autoradiogram.

Eco A has homology to 45S and 32S rRNA intermediates. There is also homology between Eco A and the 28S transcript (with slight homology to 18S rRNA). The bands were present in all 3 cell lines as expected (Figure 23). The 28S band was more intense because it was a stable end product of rRNA processing and was therefore more abundant than the intermediates. The homology between Eco A and the 18S transcript is limited to a few hundred base pairs which explains the reduction in intensity of this band compared to the 28S gene. When Eco B was used as a probe, the 45S and 18S rRNA was visualized (Figure 23).

Figure 23. Northern analysis of total RNA from HAL, 1K1 and 4K2.

A. Probed with Eco A. The 45S band is a small, sharp band. The 28S rRNA gives an extremely strong signal. There is low homology to 18S rRNA which accounts for the less intense signal.

B. Probed with Eco B. The autoradiogram of the Northern blot shows Eco B hybridized to RNA isolated from HAL, 1K1 and 4K2 cell lines. The 45S band is a small sharp band. The 32S rRNA intermediate has hybridized and gives a strong signal. The 28S rRNA is absent. The 18S band gives an extremely strong signal.



A B

There was also a band present in the region corresponding to the 32S intermediate which was probably due to sequence similarities among the transcribed spacer regions of the rDNA repeat unit. The results indicated that no detectable aberrant rRNA transcripts were produced from either ML1 or ML4.

c. Hybridization using the 2.1 kb *Sal*I fragment. When the 2.1 kb *Sal*I fragment was used as a probe, there were no aberrant transcripts detected. The *Sal*I fragment was used to determine transcripts of a size that would not be masked by the 18S and/or the 28S rRNA transcripts.

The results of Northern analysis indicated that there weren't any detectable differences from HAL, 1K1 and 4K2 transcripts when probed with the *Sal*I fragment (Figure 24). There is a positive band which corresponds to the 32S rRNA intermediate and is probably due to sequence homology between The *Sal*I fragment and the transcribed spacer region within the A region of rDNA.

d. Hybridization using vector DNA. There were no detectable differences in transcripts of nontransfected or transfected cells when vector sequences, pSV2neo and pJB8, were used as probes. When pSV2neo was used as a probe, three bands were detected in all three cell lines (Figure 25). One of the bands gave an intense positive band located just above the 18S rRNA transcript. The band is about 2.3 kb and is not present in RNA isolated from another human cell line (HL-60) which does not contain SV40. This transcript is SV40 in origin and is from the early region of SV40. This has been confirmed with related experiments performed by Steven Gold in which the SV40 early region was used as a probe against HAL cells.

Figure 24. Northern analysis RNA transcripts-I. Total RNA from HAL, 1K1 and 4K2 was probed with *Sal*I fragment of Eco B. A print of an autoradiogram of Northern blot in which the 2.1 kb *Sal*I fragment of Eco B was hybridized to RNA isolated from HAL, 1K1 and 4K2 cell lines. The uppermost band is the 45S band. The 32S band shows homology to this region and may be due to cross homology between the ETS and ITS regions of rDNA. The 20S rRNA intermediate is clearly visible, but neither 18S nor 28S rRNA transcripts are observed. The location of 45S, 28S, 20S and 18S are marked.

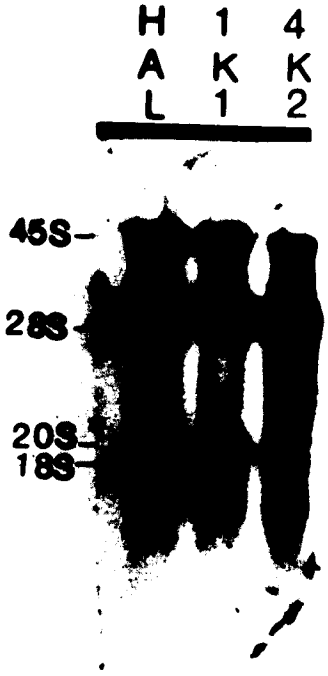

HAL
1K1
4K2
45S
28S
20S
18S

Figure 25. Northern analysis of RNA transcripts-II. Total RNA from HAL, 1K1 and 4K2 was probed with pSV2neo. There are three bands present in each lane of the autoradiogram in which pSV2neo was used as a probe. The center band is 2.3 kb and has very strong homology to the probe. The upper and lower bands are 3.7 and 1.8 kb, respectively. There are no additional bands found in 1K1 or 4K2.


HAL 1K1
18S

Four bands were obtained in all three HAL-derived cell lines when pJB8 was used as a probe. The bands were not observed in HL-60 RNA, an unrelated human cell line (Figure 26). The bands were in the same regions as those observed when pSV2neo was used as a hybridization probe and are probably due to transcription of SV40 DNA which was used in the initial immortalization of HAL.

e. Summary. The preceding section demonstrated that there was no difference between rRNA transcripts among cells that were transfected with promotor plus or promotor minus rDNA and those of nontransfected cells. These results are consistent with the expected results since the donor rDNA transcriptional unit is unaltered in 1K1 cells and should not be present in 4K2 cells.

E. Participation of Donor rDNA in Nucleolar Formation

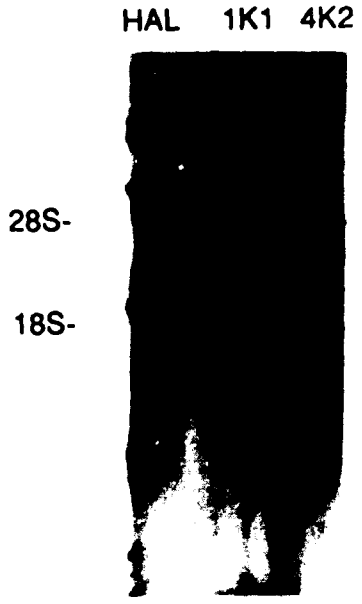
The results show that a partial rDNA repeat unit containing an intact transcriptional region acts as a nucleolar organizing region and that the promotor is required. These results also indirectly prove that the transcriptional unit of ML1 is functional.

1. Isolation of Nucleolar DNA

The cosmid insertion site within chromosome 3 of 1K1 cells participated in nucleolar formation. ML1 contained the promotor region of rDNA and was actively transcribed by pol I in 1K1 cells. 4K2 cells, which did not contain a promotor region were not transcribed.

Southern blot hybridizations of DNA from genomic, nucleolar enriched and nonnucleolar DNA from HAL, 1K1 and 4K2 cells were compared. They indicated that vector sequences from ML1 were present in nucleolar enriched DNA

Figure 26. Northern analysis of RNA transcripts-III. Total RNA from HAL, 1K1 and 4K2 was probed with pJB8. There are four bands present in each lane of the autoradiogram. The uppermost and lowermost bands correspond to the 3.7 and 1.8 kb bands observed when pSV2neo is used as a probe. The center bands are 2.4 and 2.2 kb, respectively. There are no additional bands found in 1K1 or 4K2 lanes.



pJB8

at a much higher level than in HAL or 4K2 (Table 8) (collaborative experiments with M. Hadjiargyrou). Sonication was used in the process of isolating nucleoli which results in the formation of single strand breaks (Beebee, 1986) yielding fragments of different lengths. These breaks were controlled for by comparing the nucleolar DNA fraction to the nonnucleolar DNA portion which was present during the sonication. Vector sequences from pMK16 and ML4 were found mostly in the nonnucleolar chromatin. These experiments prove that a partial rDNA repeat with an intact promotor and transcription region can perform as a nucleolar organizer.

2. Direct examination of nucleoli

Cytogenetic analysis of nuclei stained for nucleoli indicated that the ML1 cosmid behaved as a nucleolar organizing region. The nucleoli were differentially stained using a combination of methyl green and pyronin Y stains. Photographs of the nuclei were made prior to hybridization studies for later comparison of the same nuclei after hybridization. Biotinylated pJB8 was employed as a probe in an enzymatic cytological hybridization. Nuclei were examined for the presence of doublets (paired dots of stain). One pair of doublets was expected for the cosmid vector. A second pair of doublets could be present representing pMK16, but this site does not contain rDNA and is present in both cell lines examined. Nuclei containing more than two pairs of doublets were rejected since it could not be unequivocally determined if the extra doublets were background or the cell was a tetraploid. Nuclei were also rejected if they were during manipulation of the slide and no doublets were present in the undamaged regions. In these experiments, 4K2 was used as a negative control.

Table 8

Southern analysis of genomic, nonnucleolar and nucleolar DNA hybridized with pJB8.

	CELL LINE								
	HAL			1K1			4K2		
EcoRI fragment size (kb)	G[1]	ENC[2]	NUC[3]	G	ENC	NUC	G	ENC	NUC
>20				+	-	+	+	+	-
14.2							+	NO	-
10.8				+	-	+			
3.9	+	+	-	+	NO	-	+	+	-
3.4	+	+	-	+	+	-	+	+	-
Fragments	NO	NO	NO	NO	NO	+	NO	+	N0

* NO (Not obtained)

[1] G: Genomic DNA
[2] ENC: Non-nucleolar DNA
[3] NUC: Nucleolar DNA

The results indicated that the cosmid is present in the nucleolus 45% of the time in 1K1 cells and 16% of the time for 4K2 cells (Table 9 and Figure 27). This data demonstrated direct evidence that vector sequences were located in the nucleoli of 1K1 cells.

The data obtained from both molecular and cytological analyses of nucleoli clearly indicated that cosmid ML1 was in the nucleolus, and thus a functional rDNA site was present. ML4 does not participate in nucleolar activity and therefore it can be concluded that the promotor region of rDNA is required for nucleolar formation.

F. Satellite Associations among Endogenous rDNA Sites and "Newly" Created rDNA Sites.

The cosmid insertion site within 1K1 cells participated in satellite association, whereas the site within 4K2 did not. The mean frequency of random associations between endogenous rDNA bearing chromosomes and selected nonacrocentric chromosomes was calculated as 2.86 for 1K1 cells and 1.8 for 4K2 cells, respectively (Table 10 and Table 11). The chromosomes monitored were; 1, abnormal 7, 9 and abnormal 11, with the addition of chromosome 3 for 4K2 cells (see appendix A(1) for HAL karyotype).

Chromosome 3 showed a significant number of associations in the p-arm of 1K1 cells with a frequency of 13% above background (Figures 28 and 29). This number was derived by combining the proportion of associations above background of the contiguous regions near the insertion site.

The region of insertion of the promotor (-) cosmid, ML4, did not undergo satellite associations in 4K2 cells (Figure 30). This region and contiguous regions

Table 9

In situ hybridization of pJB8 DNA to nucleoli

	CELL LINE	
	1K1	4K2
# nuclei analyzed	23	62
# with doublets in nucleolus	9	5
# with doublets in nuclei	20	31
# without doublets in nuclei	3	31
% nuclei with doublets in nucleolus	45	16

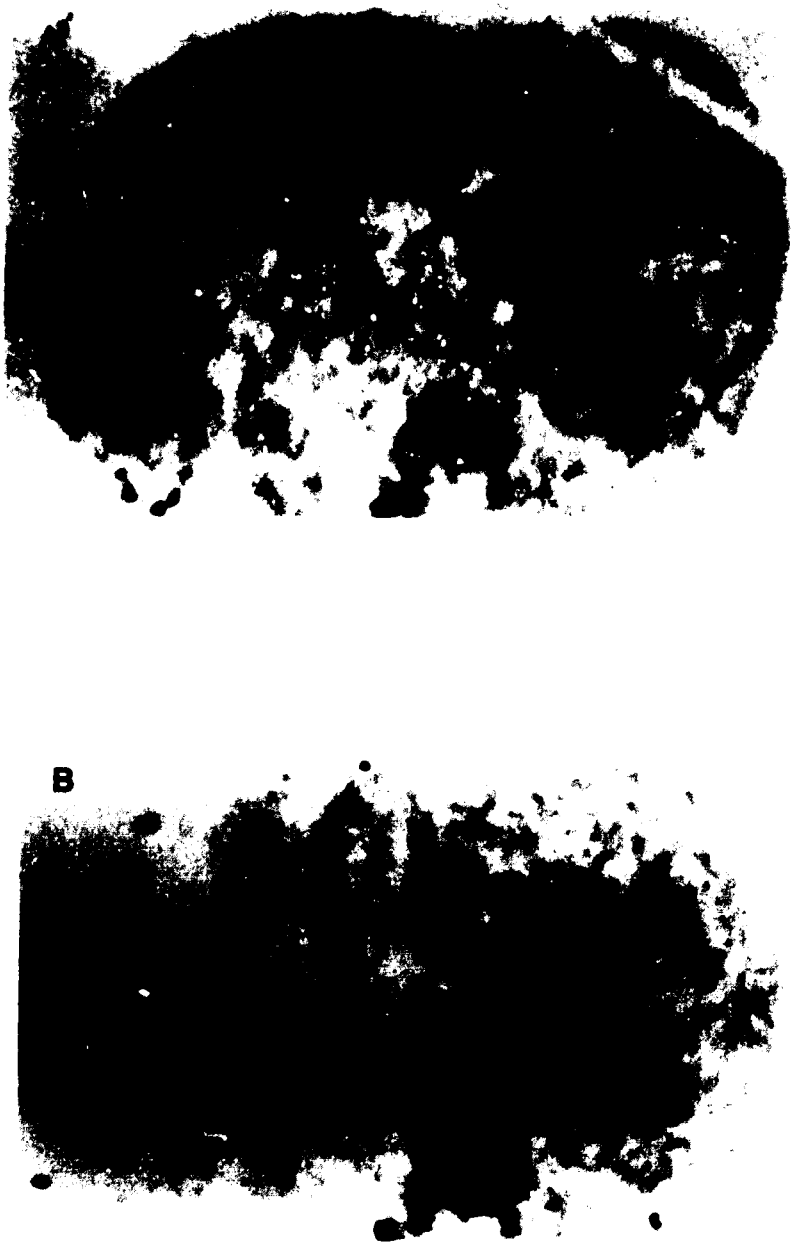


Figure 27. Cytological hybridization to nucleoli. Photographs taken before (A) and after (B) nucleoli were subjected to cytological hybridization with pJB8.(Arrow indicates nucleolus in which a stained doublet was located)

Table 10

Determination of Random Association Frequency for 1K1

Chromosome (region)		# Chromosomes identified	# Random associations	% in random association
Chromosome 1		154		
region	(1)		5.0	3%
	(2)		1.0	1%
	(3)		5.0	3%
	(4)		11.0	7%
	(5)		7.0	5%
	(6)		2.0	1%
	(7)		4.0	3%
Chromosome Abn 7		85		
region	(1)		2.0	2%
	(2)		0.0	0%
	(3)		0.0	0%
	(4)		2.0	2%
	(5)		1.0	1%
	(6)		2.0	2%
	(7)		2.0	2%
	(8)		1.0	1%
	(9)		1.0	1%
	(10)		0.0	0%
Chromosome Abn 11		81		
region	(1)		4.0	5%
	(2)		2.0	2%
	(3)		1.0	1%
	(4)		1.0	1%
	(5)		0.0	0%
	(6)		1.0	1%
	(7)		2.0	2%
Chromosome 9		167		
region	(1)		7.0	4%
	(2)		7.0	4%
	(3)		4.0	2%
	(4)		5.0	3%
Random Association Frequency				
total			80	
mean			2.86	
Stdev			2.7	

*The centromeric region of Human chromosome 1 undergoes nonrandom associations with human acrocentric chromosomes (Shaw, 1961)

Table 11

Determination of Random Association Frequency for 4K2[1]

Chromosome (region)	# Chromosomes identified	# Random associations	% chromosomes in random association
Chromosome 1	166		
region (1)		6.0	4%
(2)		2.0	1%
(3)		2.0	1%
(4)		6.0	4%
(5)		1.0	1%
(6)		2.0	1%
(7)		4.0	1%
Chromosome 3	145		
region (1)		6.0	4%
(2)		1.0	1%
(3)		3.0	2%
(4)		1.0	1%
(5)		4.0	3%
Chromosome Abn 7	88		
region (1)		3.0	3%
(2)		2.0	2%
(4)		0.0	2%
(6)		2.0	2%
(9)		1.0	1%
(10)		2.0	2%
Chromosome Abn 11	80		
region (1)		1.0	1%
(2)		2.0	3%
Chromosome 9	11[1]		
region (1)		3.0	3%
(2)		2.0	2%
(3)		1.0	1%
(4)		2.0	2%
Random Association Frequency			
total		59	0.47
mean			1.79
Stdev			1.8

[1] Regions where there was no association were omitted.

Figure 28. Satellite association to region of insertion. Satellite association between an acrocentric chromosome and the region of ML1 insertion in a metaphase plate from 1K1. Association between the satellite region of acrocentric chromosome 22 and the p-arm of chromosome 3 near the region of ML1 insertion. In a metaphase plate the chromosomes tend to appear randomly spread with many chromosomes residing in close proximity. This background level of random association must be considered when determining if a chromosome is involved in nonrandom association. The background level is obtained by determining the frequency at which a given chromosomal region is in association with the satellite region of an acrocentric chromosome. The region is considered to be in association if the proximity of the two chromosomes is equal to or less than the length of chromosome 21. The nonacrocentric chromosome is divided into sections the size of a number 21 chromosome. The section of the nonacrocentric chromosome scored as being in association is the section closest to the satellite region. The arrow indicates the point of association between chromosome 22 and chromosome 3.

Figure 29. Frequency of satellite association among 1K1 chromosomes containing rDNA. The region of insertion of ML1 into chromosome 3 in 1K1 cells is very close to the dividing line between regions 3(2) and 3(3). Both of these regions showed a high percentage of associations above background which gave the combined rate of 13% above background. Background associations (random associations) occurred at a rate of 2.86. Background was established by determining the frequency at which selected nonacrocentric chromosomes appeared to be in association.

SATELLITE ASSOCIATION AMONG rDNA BEARING CHROMOSOMES IN 1K1 CELLS

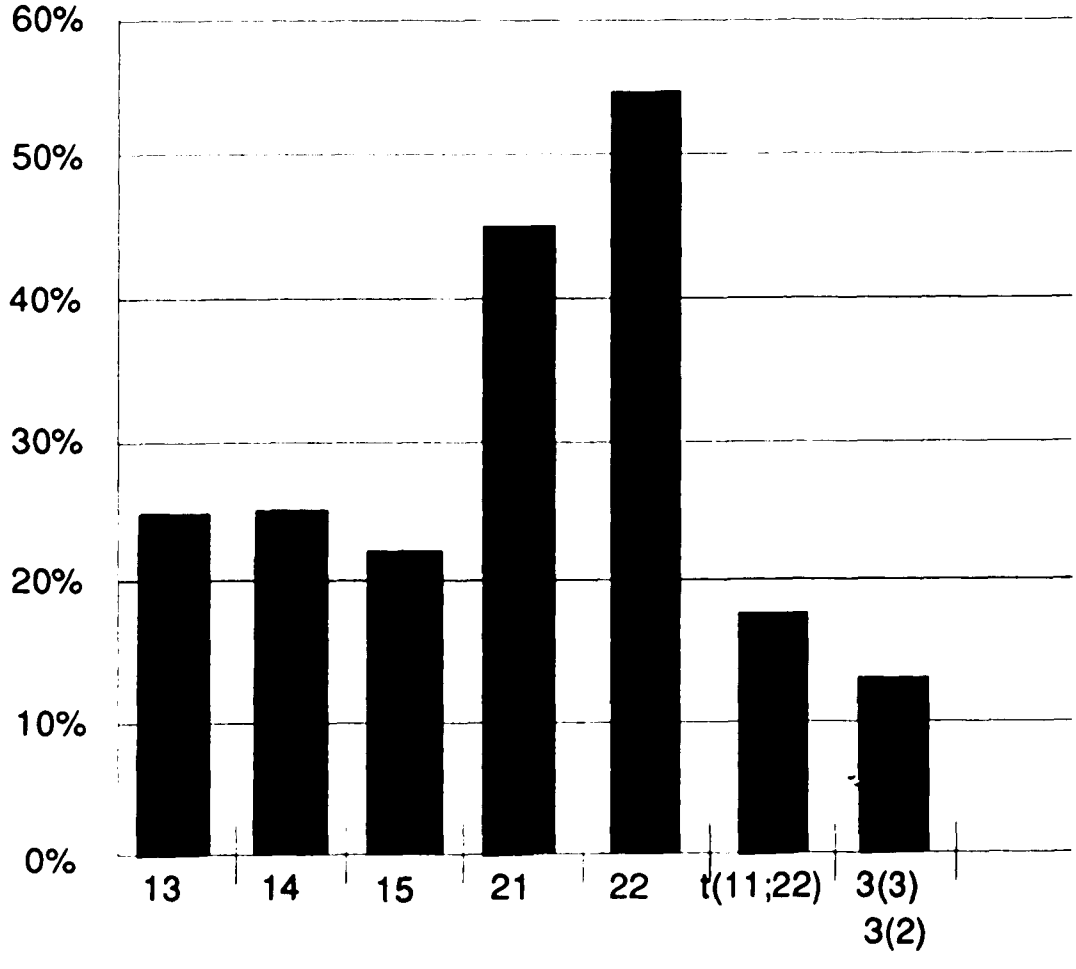
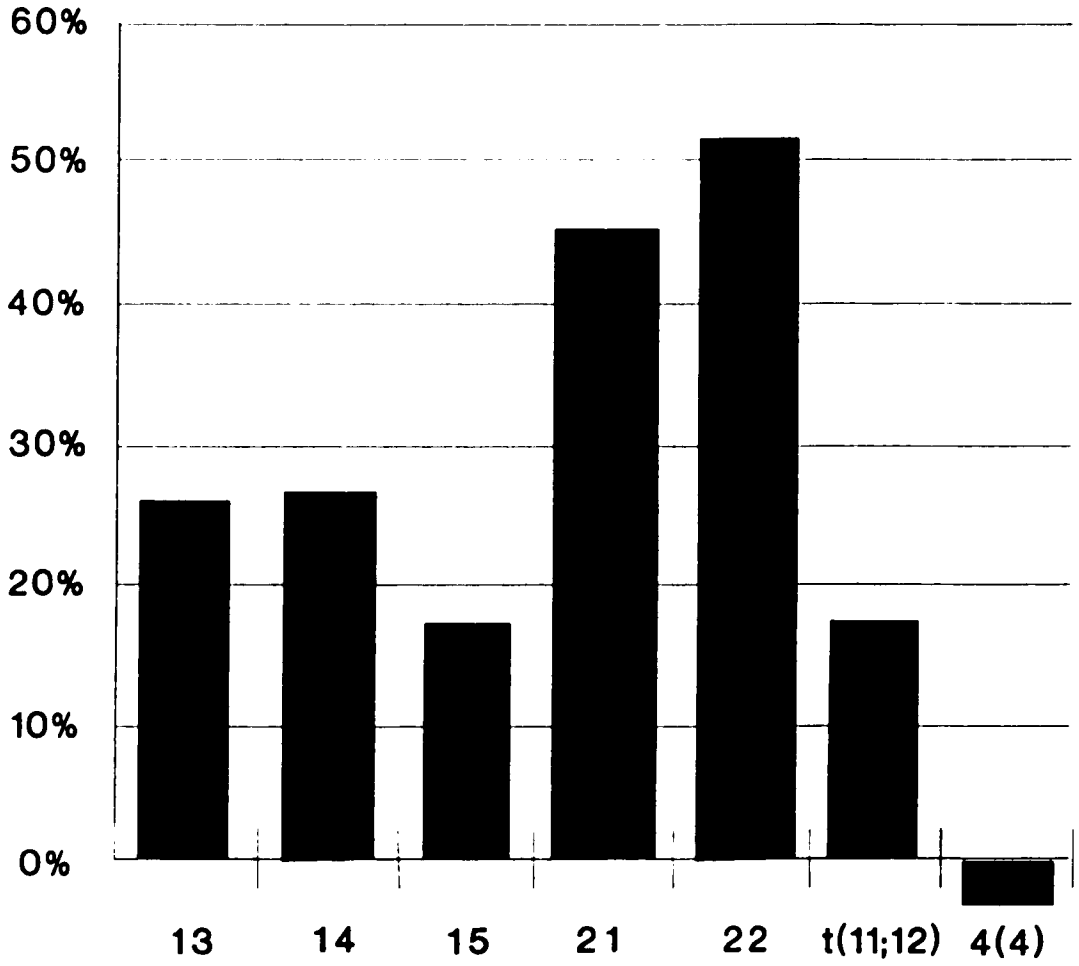


Figure 30. Frequency of satellite association among chromosomes containing rDNA in 4K2 cells. The chromosomes compared are the 13, 14, 15, 21, 22 and t(11;22). ML4 inserted into region 4(4) of chromosome 4 in 4K2 cells. Neither this region nor the contiguous regions on either side had associations above background levels. Background associations (random associations) occurred at a rate of 1.79. Background was established by determining the frequency at which selected nonacrocentric chromosomes appeared to be in association.

SATELLITE ASSOCIATION AMONG rDNA BEARING CHROMOSOMES IN 4K2 CELLS



were in association at background levels. The frequency of satellite association among the endogenous rDNA-bearing chromosomes of 4K2 cells was comparable to that of 1K1 cells.

The results show that transcription is a requirement for NOR participation. The chromosome containing the inserted DNA with a promotor region participated in satellite association. Further, the results show that the entire repeat unit is not a requirement for SA. (Tables 12,13). Satellite association involving the ML1 insertion site was in the range of that observed for the endogenous rDNA-bearing site on a translocation chromosome, where presumably rDNA genes had been lost as a result of the translocation. Satellite association of chromosome 3 in 4K2 cells was at background levels, which indicated this area does not inherently undergo SA in HAL-derived cell lines (Table 11).

G. Summary of Results and Conclusions

The results of this study conclusively demonstrated that a single rDNA repeat unit is capable of forming an active nucleolar organizing region and participates in nucleolar formation and resultant satellite association. The functional insertion site contained an entire transcriptional unit with the promotor region, the transcribed spacers, the three rRNA genes and both upstream and downstream termination sequences. A second insertion site which lacked the promotor region, but retained most of the rDNA unit, was also analyzed. In both cell lines, the insertion occurred within the vector, thereby yielding the largest contiguous stretch of rDNA possible. Both vector DNA and rDNA were mapped to chromosome 3 in 1K1 cells and chromosome 4 in 4K2 cells. Silver stain analysis demonstrated that

Table 12

Frequency of participation of rDNA containing chromosomes in satellite association

Cell line - 1K1

Chromosome (region)	# of Chromosomes identified	# of associations	Associations -bkgrd[1]	Proportion identified chromosomes in association
Acrocentrics				
13p	32	11	8	25%
14p	140	38	35	25%
15p	153	37	34	22%
21p	130	61	58	45%
22p	120	69	66	55%
t(11;22)	57	13	10	18%
NON-ACROCENTRICS				
3(2)	157	17	14	9%
3(3)		9	6	4%
Total		28	0	13%

[1]The expected random association of chromosomes in the plates analyzed is 2.86

Table 13

Frequency of participation of rDNA containing chromosomes in satellite association

Cell line - 4K2

Chromosome (region)	# of chromosomes identified	# of associations	Associations -bkgrd[1]	Proportion identified chromosomes in association
Acrocentrics				
13p	19	7	5	26%
14p	175	49	47	27%
15p	168	30	28	17%
21p	118	55	53	45%
22p	90	50	48	53%
t(11;22)	63	13	11	17%
Non-acrocentrics				
4(4)	71	0	-2	-3%

[1]The expected random association of chromosomes in the plates analyzed is 1.79

the cell line containing inserted DNA which included a promotor region was being actively transcribed by RNA polymerase I. Northern analysis revealed no detectable differences among the transcripts produced by HAL, 1K1 and 4K2 cell lines. Molecular and cytological analyses of nucleoli demonstrated the presence of vector sequences in the nucleoli of 1K1 cells and gave direct evidence that the insertion site is a nucleolar organizing region. Further, the newly created NOR site participated in satellite association with endogenous NORs substantiating that rDNA is the basic element necessary for satellite association.

DISCUSSION

A. Overview

The results of this study prove that tandem repetition of rDNA is not a requirement for nucleolar formation. A single rDNA repeat unit which contains a promotor region is capable of forming a fully active NOR which interacts with other NORs to form nucleoli. The "new" NOR resulting from transfection is transcriptively active and participates in SA with other acrocentric chromosomes.

Transfection was accomplished by using cosmid DNA derived from the Lau and Kan cosmid Library. Four of the ten cosmids were analyzed to determine those most suitable for transfection. Two cosmid DNAs with homology to the Eco A and Eco B were selected on the basis of size and the presence of an intact transcription unit. ML1 had the largest non-rearranged insert containing the entire transcriptional unit, but ML4 was missing the promotor region. These were transfected into HAL cells using liposome-mediated gene transfer (review see Mannino and Gould-Fogerite, 1988). Analysis using Southern hybridizations showed that both cosmids inserted within the vector.

The next aspect of the analysis involved locating the region of cosmid insertion into the human genome. The insertion site for ML1 DNA was located in the pericentromeric region on the p-arm of chromosome 3 in 1K1 cells and on the q-arm of chromosome 4 in 4K2 cells.

Transcriptional analysis of the transfected rDNA was made since nucleoli form around actively transcribing rRNA genes (reviewed in Scheer and Benavente, 1990). Several methods were used to determine if rDNA was transcriptively active.

The first method involved silver staining which specifically identifies proteins involved in NOR formation (Goodpasture and Bloom, 1975). It was demonstrated that the transfected rDNA was a functional NOR on the basis of silver staining of the nucleolar proteins. The insertion site containing rDNA with a promotor gave positive stain. This was direct evidence for rRNA transcription by RNA polymerase I and indicated that a single transcription unit was responsible for NOR formation.

Another method for determining transcriptional activity was direct analysis of the rRNA transcripts via Northern hybridization analysis. This analysis, however, failed to detect differences among transcripts produced from the parental HAL cells and those from either transfected cell line. It is assumed that the transfected rDNA containing the promotor and downstream terminators produced a functional fully sized transcript.

A third method for determining if the inserted rDNA was transcriptively active was based on detection of DNA with homology to the cosmid DNA in nucleoli. Only actively transcribing rRNA genes transcribed by pol I are expected to participate in nucleolar formation (reviewed in Scheer and Benavente, 1990). The nucleoli form around the actively transcribing genes and therefore vector sequences which were contiguous to the inserted genes should be present in the nucleoli if the transfected site was transcriptively active. The results of both cytological and molecular analysis of nucleoli indicated that vector sequences from the promotor plus DNA are located within the nucleoli of 1K1 cells. These were not present in the nucleoli of 4K2. The cytological analysis involved differential staining between nucleoli and the rest of the nuclei. These nuclei were then subjected to cytological

hybridization to detect vector sequences. The molecular analysis involved isolation of nucleoli. Through this procedure, the DNA was partitioned into two fractions, one containing nucleolar enriched DNA and second containing nonnucleolar chromatin. These factions were compared for the presence of vector sequences using Southern analysis. The results established that ML1 DNA was present in nucleoli, and therefore functioning as a fully active NOR.

The next step was to determine if the "newly" created NOR participated in SA with the other acrocentric chromosomes. The results of this analysis were indisputably positive. The level of SA was somewhat lower than for the other rDNA bearing chromosomes as expected. The region on chromosome 3 containing rDNA with a promotor region participated in SA with a level that was 13% above background.

B. Selection of DNA for Transfection

Screening of a cosmid library was done in order to obtain large portions of the human rDNA repeat unit for transfection into human fibroblast cells. Ten cloned DNAs with homology to Eco A and Eco B were isolated and analyzed in depth. Three of these had a complete transcriptional unit with flanking NTS DNA. A fourth clone was missing the promotor region but had most of the transcriptional unit including all three rRNA genes, downstream termination sequences and most of the NTS. ML1 was chosen for transfection because of its intact promotor region and large size (32 kb). Other DNAs were large, but contained additional unexpected restriction sites indicating substantial rearrangements of the rDNA. These were rejected. Clone ML4 contained some rearrangements in the NTS

region, but was used for transfection because it lacked the promotor, transcription initiation region and a small segment of the ETS. By using a segment of rDNA that can not initiate transcription, we could determine for certain that nucleolar formation is due primarily to rDNA transcription rather than some other aspect of rDNA or its surrounding regions.

There is one other study in which rDNA was isolated from the same library (Renalier *et al.*, 1989). Thirteen rDNA-containing cosmids with a transcription initiation region were isolated. Based upon preliminary findings, five were evaluated for structural heterogeneity. The results indicated that all five of the clones contained rearrangements within the NTS. The additional sites are presumed to be due to mutations or rearrangements of DNA present prior to or induced during the construction of the library. The NTS region of cloned rDNA has previously been shown to undergo spontaneous rearrangements during propagation in plasmid form (Erikson and Schmickel, 1985).

A YAC library has also been screened with the purpose of isolating tandemly repeated rDNA units (Labella and Schlessinger, 1989). The rationale for this study was to isolate tandemly repeated rDNA and analyze the function of the NTS in rRNA production and polymorphism. A total of 27 clones was isolated of which thirteen contained most or all of the repeat unit. The maximum number of contiguous repeat units obtained was 1.5. The authors speculate that either there are complete singular rDNA repeat units interspersed throughout the genome or the YAC clones were originally produced from tandemly repeated rDNA which underwent deletions during propagation of the YACs.

C. Transfection Experiments

Human rDNA was integrated into human fibroblasts using liposome-mediated gene transfer. This technique utilizes the synthetic cationic lipid DOTMA mixed with a phospholipid which is purchased under the name Lipofectin (Felgner *et al.*, 1987, Felgner and Holm, 1989). Lipofectin forms a lipid bilayer which encapsulates the DNA. The first step in encapsulation involves spontaneous binding of positive charged lipid to negative charged DNA which yields 100% entrapment of DNA (Felgner *et al.*, 1987). The DOTMA also enables the complex to bind and trasverse the plasma membrane. Optimal conditions must first be determined for a given cell line since different lines have different sensitivities to the liposome/DNA complex. It has been shown that levels of gene expression of transfected DNA increase linearly with increasing DNA concentration (10μg was the maximum tested) (Maurer, 1989). The optimal conditions for HAL cells were determined to be 30μg lipofectin/30μg cosmid DNA for 3 hours. The transfection efficiency of plasmids into macrophages using liposome-mediated gene transfer is reported to be between 1 and 2 x 10^{-5} (Belkowski *et al.*, 1989). This efficiency is higher than was obtained in the present experiments (average 6.6 x 10^{-6}).

D. Transcription.

A major aspect of this project was to examine the relationship between rDNA and nucleolar formation. The transfection of rDNA from one species into a second species is very useful for studying rDNA transcription because the differences between the spacer regions enables the detection of transcripts only from the transfected rDNA. This presents a problem if the goal is to examine

nucleolar formation under optimal and species-specific conditions. This was circumvented in the present experiments by the use of cytological techniques.

The linkage between rDNA transcription and nucleolar formation was first suggested based upon studies involving mouse-human cell line. In one study, both human and mouse rDNA were shown to be present in the cell line, but only mouse rRNA was detected (Miesfeld *et al.*, 1984). The authors subjected the DNA to a variety of tests to determine whether the lack of human rRNA transcription was due to a block at transcriptional initiation, elongation or nucleolar formation. The authors determined that the problem was due to the lack of or inhibition of a species-specific factor. This was accomplished by two types of experiments. The first experiment involved S_1 nuclease protection assays. The mouse rDNA was protected from S1 nuclease and therefore was identified on Southern blots where the transcription initiation region was used as a probe. The human rDNA was digested by S1 nuclease, indicating that it was not protected by the transcription initiation factors. The second involved *in vitro* transcription analysis to determine whether the rDNA *per se* or transcriptional factors were preventing human rRNA expression. The mouse rDNA actively transcribed in the presence of cellular extract, whereas the human rDNA did not. When extracts containing human transcription factors were added, the human rDNA from the hybrid became transcriptively active. This led to the conclusion that there are species-specific transcriptional factors required for transcription of rDNA (Miesfeld *et al.*, 1984). The species-specificity of rDNA transcription was later shown to be due to one of the transcriptional binding factors (Bell *et al.*,1988; Learned *et al.*,1986; Learned *et*

al.,1985).

There is a correlation between silver staining and the activity of rRNA genes. This was shown in a study using mouse-human heterokaryons. In these hybrids, the human chromosomes dominate and the mouse chromosomes are repressed. Therefore, the human rDNA was actively transcribing, but the mouse rDNA was repressed (Miller *et al.*, 1976). The human acrocentric chromosomes stained positive with silver, but the mouse rDNA-bearing chromosomes did not. The authors concluded that silver stain detects regions of rRNA gene activity. The proteins surrounding an active NOR were shown to silver stain to the highest degree during prophase and diminish during later stages (Schwarzacher *et al.*, 1978).

In the present study, the ML1 insertion site containing a promotor region stained positive with silver indicating the presence of an active NOR. The low copy number of rDNA in both chromosome 3 of 1K1 contributes to a low overall activity of rRNA in that region and is probably responsible for the low level of detectability by silver stain. This is consistent with reports of a threshold at which low levels of rRNA synthesis cannot be detected by silver stain (Schmiady *et al.*, 1979). This threshold is intrinsically linked to the process of silver staining. Silver is reduced and precipitated over a specific region during the initial stage of staining and then the silver continues to precipitate increasing the size of the mass. This mass is dependent upon the amount of the stainable material present and the size of the area where the precipitate can form (Schwarzacher *et al.*, 1978).

There have been four previous studies where rDNA from one species was

integrated into the genome of a related species. Transfection of mouse rDNA into CHO (Chinese hamster ovary) cells showed a correlation with the amount of the repeat unit present and the level of mouse rDNA transcribed, with a maximum reaching 3-10% of endogenous rDNA (Raziuddin *et al.*, 1989). The level of rRNA transcription was determined by hybridizing spacer sequences unique to mouse rRNA as a probe. This alleviated the potential problem associated with high levels of endogenous mature rRNA, since mouse and CHO mature rRNA are very similar. In the second study, mouse rDNA containing the promotor, transcription initiation region and part of the ETS were transfected into CHO cells. The rDNA was amplified by a stepwise increase in the selection media and analyzed for transcriptional activity (Dhar *et al.*, 1985). The authors reported an interrupted pattern of silver stain along the region of amplified rDNA indicating that CHO machinery is capable of transcribing mouse rDNA, but at a fraction of the expected rate.

In another study, a plasmid containing 1.2 kb of human rDNA including the promotor region was transfected into Chinese hamster cells (Dhar *et al.*, 1985). Their results demonstrated transcription of donor rDNA by RNA polymerase II, not by RNA polymerase I. The authors based their conclusions on drug inhibition studies. Actinomycin D at low levels blocks pol I transcription; α-amanitin blocks both pol II and pol III transcription. The results of nuclear run-on assays demonstrated that the transfected human rDNA was transcribed in the presence of actinomycin D, but not in the presence of alpha-amanitin. These results were the opposite of those obtained in experiments where HeLa cells were used as a

control. The transfected human rDNA in the hybrid cells did not silver stain, but the endogenous CHO rDNA did. The authors concluded that the transfected rDNA was transcribed by RNA polymerase II alone and that rDNA regions able to be transcribed by pol I will be silver stained. RNA polymerase I requires a protein complex bound to a specific region of the promotor. The regions are the core element and upstream control element. First, upstream binding factor 1 (UBF1) recognizes and binds to these sequences, then a second protein (SL1) which is species-specific binds to UBF1. This allows RNA polymerase I to attach and begin species-specific transcription (Bell et al., 1988; Learned et al.,1986; Learned et al., 1985).

In the fourth study, it was shown that mouse rDNA was accurately transcribed and processed in rat cells. The level of transcription of mouse rRNA depended on the clone studied, but varied from 1/2-1/6.5 to 1/20-1/65 when compared with transcription in mouse cells (Vance *et al.*, 1985). These studies indicate the feasibility of using closely related species for rDNA transcription analysis, but the quantity of rDNA transcription from the heterologous rDNA is reduced. These results indicate that factors not present in the host cell are required for proper transcription of donor rDNA.

The presence of a transcriptionally active region at the insertion site was also sought using Northern blot analysis to determine if rRNA transcripts of a different size could be recognized in the transfected 1K1 and 4K2 cells by hybridization with the Eco A and Eco B region DNA. This was not the case. Additional experiments were done which utilized parts of the vector as a probe.

The rationale for these experiments was to determine if vector sequences were being transcribed since not all pol I transcripts terminate at the first termination site (Pfleiderer *et al.*, 1990). No unusually sized transcripts were observed. There are several possible reasons for the failure to find transcripts of unpredicted size, including the obvious possibility that the transcripts formed from the inserted sites were the same size as those normally found in the cell. Another possibility is that the level of transcript from the insertion site was so low as to be undetectable or was masked in a Northern blot where huge amounts of the normal transcripts were observed.

E. Nucleolar Formation

The present study is the first to incorporate human rDNA into human cells. The rationale for using human to human transfections was to take advantage of the species specificity of rRNA transcription and to examine nucleoli function. One previous study involved the incorporation of a single rDNA repeat unit from Drosophila into the genome of Drosophila (Karpen *et al.* 1988). The rDNA contained the entire transcriptional unit plus flanking 5' and 3' spacer regions. The authors differentiated between the endogenous transcripts and the transcripts from the inserted rDNA by using strand specific probes on polytene chromosomes. A comparison between the level of cytologic hybridization to probes which recognized only rDNA and probes that recognized both rDNA and rRNA was made. The insertion sites were located in regions other than the NOR and within euchromatic regions, and were transcriptively active. The rDNA insertion sites formed "mininucleoli" that stained with an antibody specific for a nucleolar antigen.

Ribosomal rDNA deficient mutants of Drosophila were partially rescued by transformation with a single rDNA repeat unit, indicating that the repeat unit was functioning in the production of ribosomes. Their results conclusively demonstrated that a single rDNA repeat unit is capable of behaving as a nucleolar organizing region. This region actively transcribes and is surrounded by at least one antigen found in nucleoli (Karpen *et al.* 1988).

Our experiments also confirm that a single rDNA repeat unit is able to function in nucleolar organization. Nucleolar participation was substantiated by detecting vector sequences directly in the nucleoli. The cytological method involved hybridization *in situ* of vector sequences to nuclei which had been previously stained for nucleoli. The results demonstrated that 45% of the nucleoli from 1K1 stained positive. The molecular analysis demonstrated that DNA from isolated nucleoli had more vector sequences present than nonnucleolar DNA.

All studies, including the current one, demonstrated that transcription of cloned mammalian rDNA occurs *in vivo* and that tandem repetition is not an absolute requirement for rRNA gene expression. The complete function of full length rRNA transcripts in nucleolar organization or in satellite associations, however, was not determined in the previous studies. The present study was the only study to date to examine the relationship between rDNA and normal cellular processes.

F. Satellite Association

One aspect of rDNA-bearing acrocentric chromosomes of many species is their participation in satellite associations in metaphase. Ferguson-Smith and

Handmaker (1961) were the first to report that all 5 pairs of acrocentric chromosomes in humans were satellited. They also showed that the acrocentric chromosomes participated in nonrandom associations at the satellite region, which they termed satellite associations. The associations could lead to abnormal segregation or exchange by failure to dissociate resulting in chromosomal anomalies (reviewed in Jacobs *et al.*, 1976). DNA homology among rDNA- bearing chromosomes was shown to be the critical factor in satellite associations (Henderson *et al.*, 1973, Warburton *et al.*, 1976, Henderson and Atwood, 1976).

The present study is the first in which transfected rDNA was examined for participation in satellite associations. The results show that a chromosome containing a single rDNA transcriptional unit including the promotor, will participate in satellite associations. Chromosome 3 in 1K1 cells participated in satellite association with other acrocentric chromosomes at a level of 13% above background compared to other non-acrocentric chromosomes. Conversely, the region of insertion of ML4 in chromosome 4 did not participate in satellite associations. Thus, the presence of rDNA, its interactions within the nucleolus, and transcriptive activity are causative factors in satellite associations among rDNA-bearing chromosomes.

The correlation between the degree of silver staining and the participation of acrocentric chromosomes in SA (Miller *et al.*, 1977) holds true for the experiments we performed. The lower frequency of SA for the ML1 insertion site is accompanied by a lower degree of silver staining when compared to that of the endogenous acrocentric chromosomes. A direct correlation also exists between SA

and NOR activity in mitotic chromosomes as determined by silver staining (Guichaoua *et al.*,1986). The NOR on chromosome 3 participates in SA at 13% above background. This result can be compared to the levels for endogenous rDNA-bearing chromosomes which ranged from 18%-55% for 1K1 to 17%-53% for 4K2. The presence of a single copy of the rDNA transcriptional unit on each sister chromatid of the ML1 insertion site resulted in a lower participation in SA than for the other acrocentric chromosomes.

The frequency of satellite association is higher for the G-group chromosomes than for the D-group chromosomes in both 1K1 and 4K2 cell lines which is comparable with other studies which used normal white blood cells (Jacobs *et al.*, 1976). The frequency of SA involving the ML1 insertion site [as well as t(11;22), which has a reduced rDNA copy number] is consistent with other findings which have reported a correlation between the amount of rDNA present and the SA frequency of a given chromosome (Warburton *et al.*, 1976). This leads to individual differences between chromosomes (reported by Warburton *et al.*, 1976 and Jacobs *et al.*, 1976).

G. Summary, Prospectives

The present experiments were based on the hypothesis that rDNA homology, rDNA content and transcriptional activity play a role in the formation of the functional nucleolus and in associative interactions among human rDNA-bearing chromosomes (Henderson *et al.*, 1973, Warburton *et al.*, 1976). The focus of the problem addressed dealt with the factor or factors of the rDNA complex which contribute to nucleolar organization and resultant satellite associations.

Our analyses demonstrate that the critical DNA to participation in nucleolar formation and resulting satellite association is rDNA. More specifically, the presence of the promotor, along with the rest of the transcribed spacer and resultant transcription determines the activity of a given rDNA site.

The mechanisms involved in the relationship between nucleolar organization and satellite associations remain partly unknown. Further identification of various aspects of rDNA function and nucleoli formation can be investigated with the availability of large intact regions of the human rDNA repeat unit. Future experiments will be performed in which point mutations are created within the promotor and/or other regions of the transcriptional unit. The mutated segments can be analyzed by both molecular and cytological techniques to determine whether any particular sequence of the transcribed spacer is solely responsible for rDNA function in nucleolar formation and satellite associations.

APPENDIX

A. Features of the HAL Karyotype.

1. Abnormal Chromosomes of HAL Cells.

HAL is a pseudodiploid cell line whose karyotype has been extensively analyzed (Figure 31) (Patsalis, unpublished). There are several abnormal chromosomes in HAL cells, including aberrations of the acrocentric chromosomes. HAL cells also contain double minutes. Double minutes (DMs) are characterized as small, paired, autonomously replicating structures. The relevant abnormal chromosomes include a missing Chromosome 13, 13q-, $4q^+$, abnormal 7, abnormal 11 and t(11;22). The 13q- is not always able to be differentiated from chromosomal fragments which contributes to the low number of 13's analyzed for satellite association. The t(11;22) contains rDNA, participates in SA and appears to stain positive with silver and has been included in the cytological analysis involving 1K1 and 4K2. The $4q^+$ is the homolog for the normal 4 chromosome. Therefore all data obtained for SA within the 4K2 cell line was based upon the actual ML4 insertion site. The abnormal 7 chromosome has two centromeres, is the largest chromosome in HAL. The abnormal 11 is as large as chromosome 1.

2. Analysis of Double Minutes.

HAL, 1K1 and 4K2 all possess double minutes containing rDNA. When transfected cells remain under selection for increasing concentrations of drug resistance they develop double minutes which contain amplified portions of the transfected DNA (Crouse *et al.*,1983; Carroll *et al.*, 1988.). For this reason, it is important to determine if HAL and HAL-derived cell lines contain rDNA in their

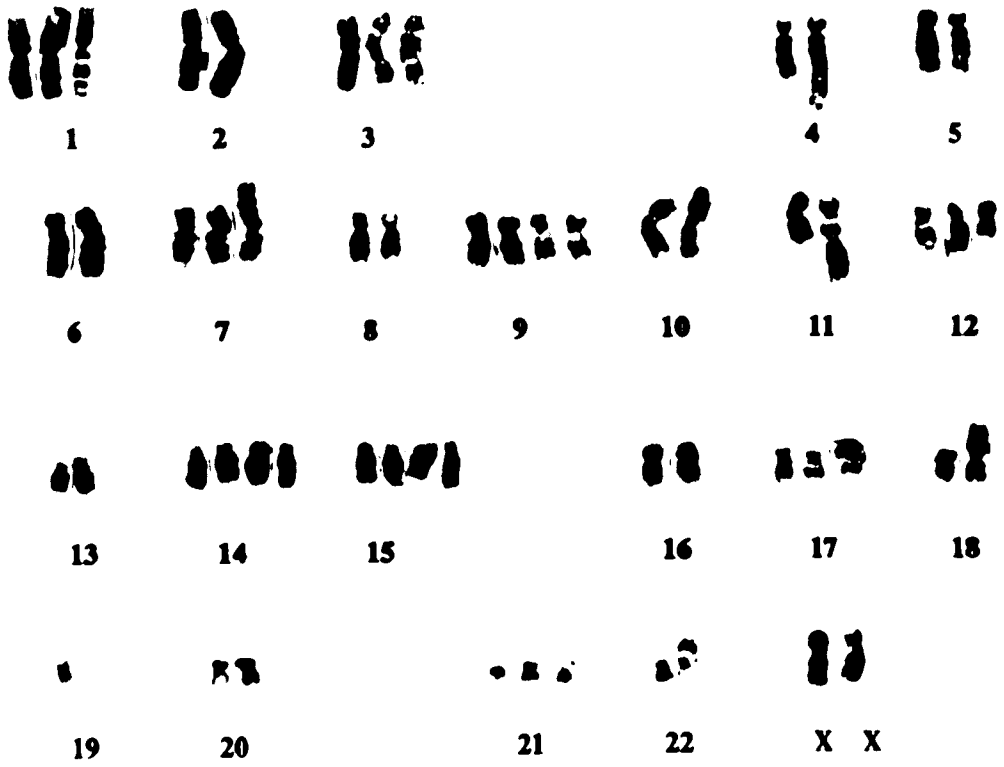


Figure 31. Karyotype of HAL cells.

DMs. When chromosomes are subjected to silver stain, the chromosomal regions that are negative for silver stain become golden in color. In some plates, this background stain may not be dark enough to visualize the small double minutes. Therefore, in this analysis the percent silver positive DMs is based upon the number of plates analyzed, whereas the number of double minutes in satellite association are compared to the number of plates containing recognizable double minutes.

The percentage of double minutes staining positive was comparable in all three cell lines (15%, 9.5% and 12% for HAL, 1K1 and 4K2, respectively). Only one DM stained in any given metaphase plate in any cell line. 1K1 was the only cell line in which a sufficient number of plates were analyzed to unequivocally determine that the DMs undergo SA. The percentage of identifiable DMs undergoing SA was 13% for 1K1.

The origin of a double minute present in all three cell lines might be from one of the abnormal acrocentric chromosomes in which the centromere and part of the NOR have remained. The evidence for this hypothesis is that the DMs stain positive in all three cell lines and were observed in association in both 1K1 and 4K2 (though the numbers in 4K2 were to low to be sure the association was nonrandom).

B. Analysis of rDNA Containing a Partial Transcriptional Unit

1. Transfection of Eco B into HAL cells.

a. Rationale. Cosmids which contained a complete transcriptional unit with intact termination sequences for rDNA were used in the study presented in this

dissertation. The results indicated that this unit was fully functional and behaved as a nucleolar organizing region. The next questions to be asked pertain to which portions of the transcriptional unit are required to elicit NOR activity. Is the entire transcriptional unit necessary for nucleolar formation and satellite association? Are there specific sequences within the repeat unit required for NOR formation? Is there a graded effect correlated with the length of the repeat unit? Do duplications and/or deletions within various portions of the repeat unit increase or inhibit NOR formation? Initial experiments were performed.

The Eco B fragment was subcloned into pSV2neo (performed by Michael Hadjiargyrou). The rationale for using Eco B is to provide cell lines in which the promotor is present and the 18S gene is incomplete. There are two reasons for this. The first is that plasmid constructs containing mouse rDNA transfected into CHO cells produced transcripts containing vector sequences only when the rDNA was ligated to the vector within the 18S gene (Raziuddin *et al.*, 1989). The explanation given for this occurrence is that other transcripts containing vector sequences are processed and degraded at a rapid rate. A second reason for choosing an incomplete 18S gene is based on data from Drosophila. A large percentage (approximately 60%) of the rRNA genes contain non-rDNA sequences within the 28S rRNA gene. These genes are seldom transcribed (Reviewed in Glover, 1981).

b. Transfection. pSV2neo was used as the vector because it contained the geneticin resistance marker and had an *Eco*RI cloning site. Two of the resulting

clones were chosen which contain the insert in opposite orientation. These plasmids were referred to as T1 and T4 (Figures 32 and 33). Both of these plasmids were transfected into HAL cells (Table 14).

Cultures from T1 were referred to as the 1T series whereas those obtained from T4 were the 4T series. A limited number of pure clones were cultured with the rest being mass cultured (Table 15). The rationale for the mass culture was to increase the probability of finding polymorphisms among the rRNA transcripts.

2. Analysis of Cell Lines containing T1 and T4

The pure cell lines 1T2 and 4T2 were subjected to Southern analysis to determine the plasmid region in which insertion occurred in the HAL genome. The results indicated that 1T2 had 2 copies of the insert. One copy was spliced within the vector region yielding an intact B region whereas the second copy was spliced within the B region. 4T2 had one copy which was spliced within the rDNA region

3. Northern Analysis of RNA from T series Transfectants

RNA isolated from both pure clones (1T2, 4T2) and mass cultured clones (1TA, 4TA) were analyzed by northern hybridizations for the presence of aberrant RNA transcripts. In these experiments, HAL RNA was used as a control.

a. rRNA analysis. Northern analysis did not reveal any length heterogeneity among rRNA transcripts. Eco A, Eco B and the *Sal*I fragment from Eco B were used as probes to determine if there were rRNA polymorphisms within the transcriptional region. Eco A was not introduced unto the transfected cells and served as a negative control. The *Sal*I fragment from Eco B was used as a probe to determine if any aberrant transcripts would become visible if exposure time

Figure 32. Schematic representation of plasmid T1. Plasmid T1 was constructed by the ligation of Eco B into the unique *Eco*RI site of pSV2neo. Transcription would commence at the promotor (p=promotor) and continue into the incomplete 18S gene and then into vector sequences (indicted by solid arrow). Restriction sites *Bam*HI(B), *Eco*RI (E) and *Sal*I(S) present in Eco B are shown.

T-1

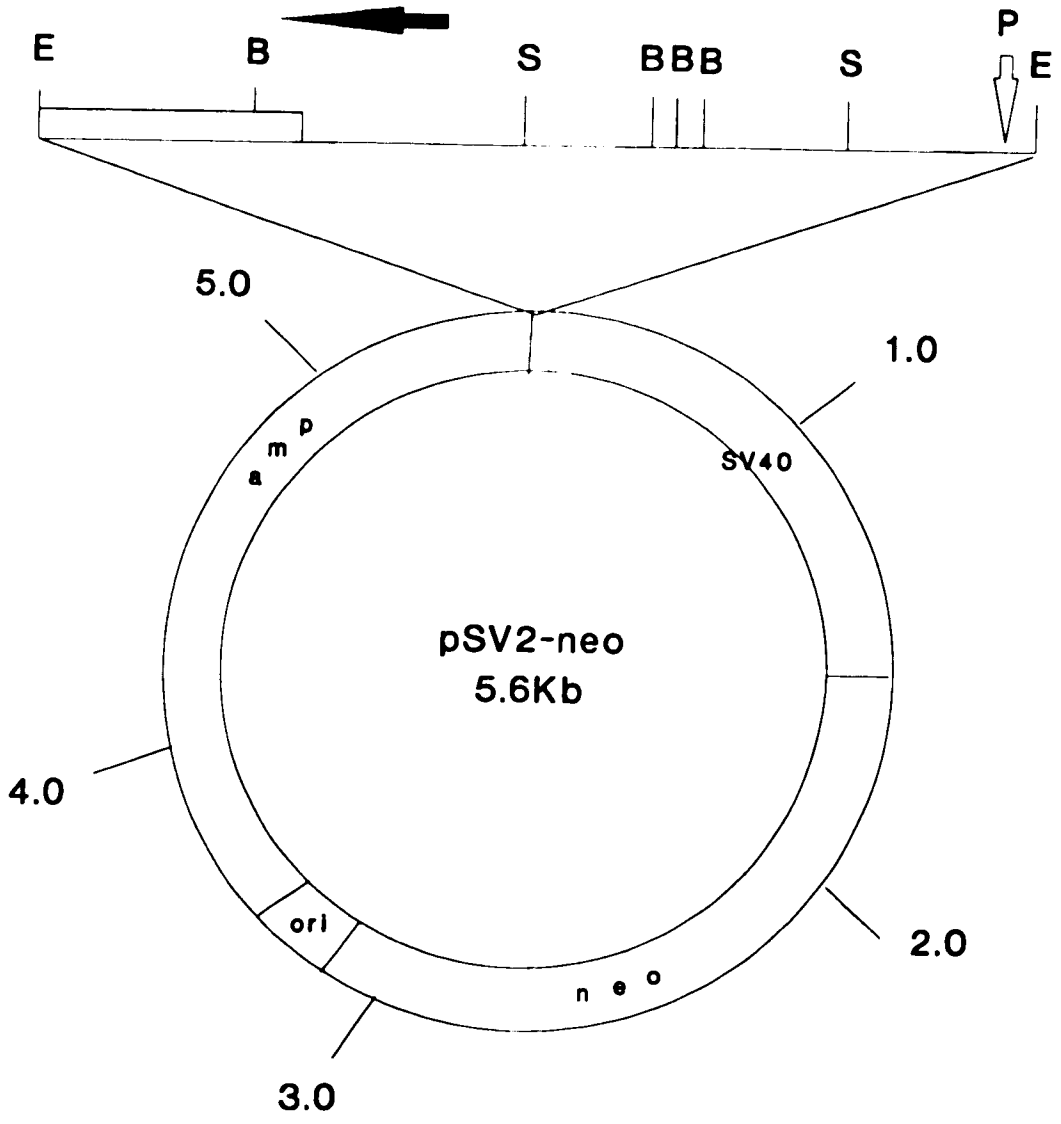


Figure 33. Schematic representation of plasmid T4. Plasmid T4 was constructed by the ligation of Eco B into the unique *Eco*RI site of pSV2neo. Transcription would commence at the promotor (p=promotor) and continue into the incomplete 18S gene and then into vector sequences (indicted by solid arrow). Restriction sites *Bam*HI(B), *Eco*RI (E) and *Sal*I(S) present in Eco B are shown.

T-4

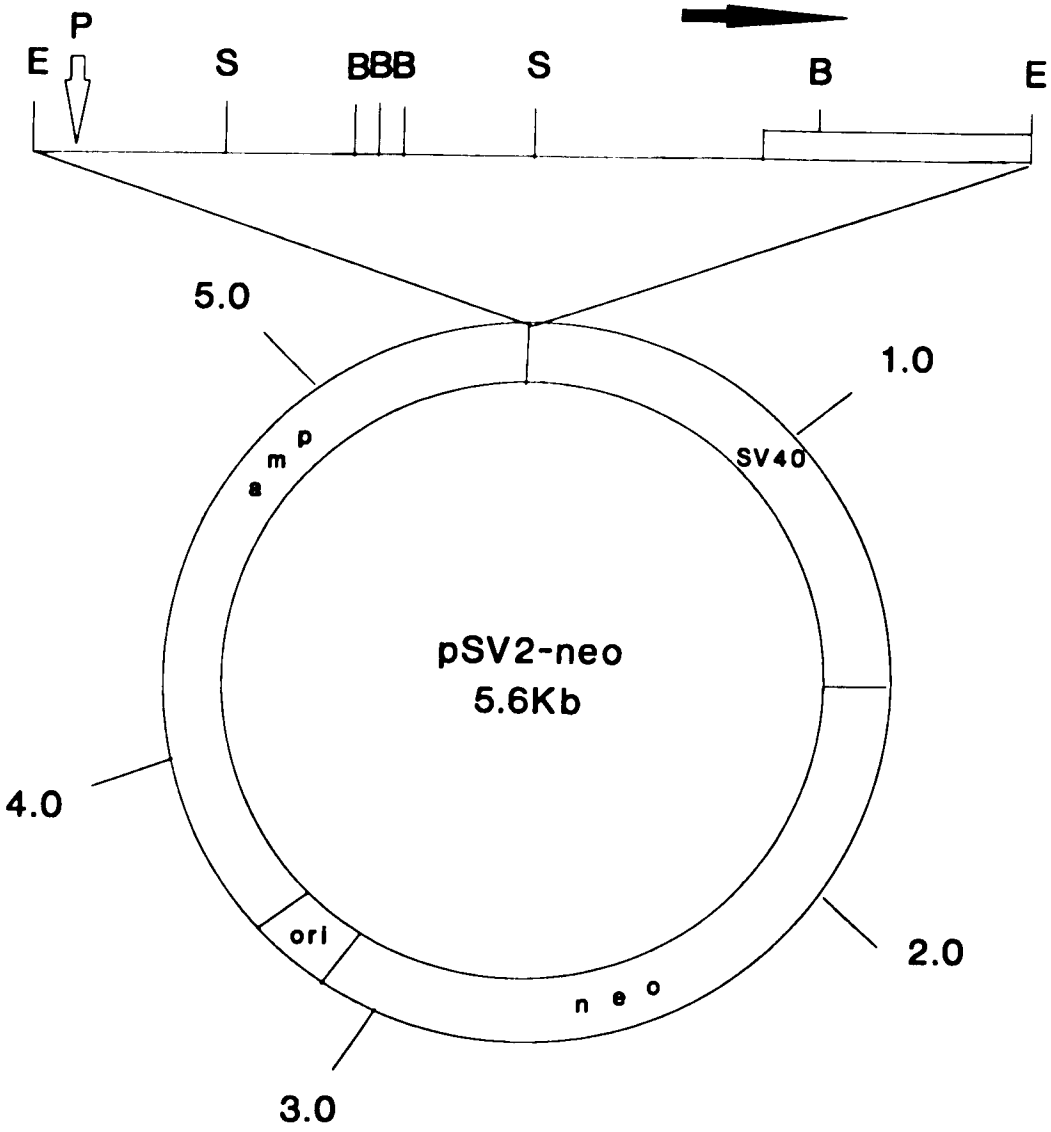


Table 14

Transfection efficiencies for plasmids T1 and T2 into HAL cells

Plasmid	DNA concentration	#clones/plate	Transfection efficiency/plate
T1	15µg	9	1.8×10^{-5}
	10µg	3	6.0×10^{-6}
	5µg	0	0
T4	15µg	4	8.0×10^{-6}
	10µg	3	6.0×10^{-6}
	5µg	0	0

* ALL transfections were done with 30µg of lipofectin

Table 15

Established cell lines obtained from T-series transfections

Pure clones	DNA Transfected
1T1	T1
1T2	T1
4T1	T4
4T2	T4
4T3	T4

Mass Cultured Lines	DNA Transfected
1TA	T1
4TA	T4
4TB	T4

could be increased and if there were any abnormal transcripts with the same molecular weight as the 28S or 18S rRNA. Aberrant transcripts were not detected. These results were identical to those obtained for cosmid clones (K-series).

b. Vector analysis. pSV2neo and pJB8 were used as probes for northern analyses to determine if transcription proceeded into vector sequences. pSV2neo was used since the T-series used pSV2neo as vector. pJB8 was not homologous to SV40 and therefore would have detected any transcripts which occurred at the same molecular weight as the SV40 early region transcripts. The bands detected with pSV2neo were identical in all 5 cell lines.

When pJB8 was used as a probe there appeared to be a higher molecular weight band in RNA from cell line 4T2. The other cell lines did not possess this band nor was this band observed on autoradiograms from pSV2neo. This band could not have been masked by positive pSV2neo bands since their molecular weight is lower. In addition, cell line 4T2 ligated into the human genome within the insert and therefore transcription from the T4 rDNA promotor into the vector could not have occurred. The resulting transcripts would be of variable length without homology to vector sequences. Further experiments would have to be performed to explain the ambiguity of these results.

C. Analysis of Isolated Nucleoli for Vector DNA.

Nucleoli were isolated from pure clones T1 and T4. The results indicated there was an equal concentration of vector sequences in both nucleolar and nonnucleolar DNA. This level was lower than for 1K1 nucleolar DNA, which indicated that this DNA was not transcribing to as high of a degree. This result was

consistent with the correlation observed between the level of mouse rDNA transcription and the size of the rDNA repeat unit transfected into CHO cells (Razuiddin *et al.*,1989). Further experiments must be performed to confirm that these vector sequences are within the nucleoli.

In summary, the experiments with the T series indicated that vector sequences were not detected in RNA from clone 1T2 nor mass cultures 1TA and 4TA. Clone 1T2 had a contiguous stretch of transfected DNA which contained an intact promotor and an 18S gene ligated to the vector. The high molecular band produced in 4T2 when hybridized with pJB8 was not present with pSV2neo and may be an artifact. There was some preliminary evidence which suggested that the truncated rDNA transcriptional units were transcribing, but at a lower level than for transfectants containing the entire transcriptional unit.

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