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Molecular analyses of a transfected partial human rDNA repeat unit in human fibroblasts: NOR formation

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MOLECULAR ANALYSES OF A TRANSFECTED PARTIAL HUMAN rDNA REPEAT UNIT IN HUMAN FIBROBLASTS: NOR FORMATION

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

MOLECULAR ANALYSES OF A TRANSFECTED PARTIAL HUMAN rDNA REPEAT UNIT IN HUMAN FIBROBLASTS: NOR FORMATION

by

Michael Hadjiargyrou

Adviser: Professor Ann S. Henderson

A functional comparison was used to investigate the relationship between ribosomal DNA (rDNA) and the function and formation of the nucleolus, as well as corollary events, such as satellite associations. Cloned human rDNAs were isolated from a cosmid library and characterized. Analyses showed that one clone contained an intact transcribed spacer region and most of the nontranscribed spacer region. The second contained a transcribed spacer and much of the nontranscribed region, but lacked the promotor. These were transfected into human fibroblasts. Molecular analyses determined that one to two copies of the transfected DNA is present in the genomic DNA of the transfected cells. Preliminary results with silver staining showed that the rDNA containing a promotor region is transcribed. Further molecular and cytological analyses demonstrated that this rDNA displays Nucleolar Organizer Region (NOR) activity and participates in satellite associations. In contrast transfected rDNA lacking a promotor is not transcribed, and does not participate in NOR activity. We conclude from this study that tandem repetition is not an absolute requirement for rDNA

function and that transfected rDNA containing a promotor region can establish an active NOR region.

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INTRODUCTION

I. Background

The genes encoding human ribosomal RNA (rRNA), 18S, 5.8S and 28S rRNA, are present in clusters on the telomeres of the short arms (p-arms) of the five pairs of the D and G group human acrocentric chromosomes, 13, 14, 15, 21 and 22 (Henderson *et al.*, 1972, Evans *et al.*, 1974). The regions containing rDNA are called Nucleolar Organizer Regions or NORs, a term first proposed by McClintock in 1934. As early as 1931, Heitz established a relationship between the nucleolus and specific chromosomal regions, indicating the involvement of these regions in the construction of the nucleolus during the telophase stage of mitosis. During metaphase, NORs appear as clear constrictions (secondary constrictions) on chromosomes. This condition may be due to the low degree of condensation of DNA or to the protein content in the secondary constrictions (Busch and Smetana, 1970).

The functional NOR is conceived as a region of the chromosome containing rDNA genes and associated chromatin fibrillar material. The nucleolus is formed when the NOR is active in ribosome biogenesis, or shortly before activation, presumably as a means of anticipating activity (Schwarsacher and Wachtler, 1987). More specifically, nucleolar reformation at telophase begins with the aggregation of nucleolar material into pre-nucleolar bodies, which unite around the NORs into the developing nucleolar body (De La Torre and Gimenez-Martin, 1982).

At the molecular level, NORs are comprised of many rDNA transcriptional

units aligned as repeats. There are between 30-50 rDNA transcriptional units in tandem array per NOR or a total of 200-300 gene units per human genome (Schmickel and Knoller, 1977). Electron microscopy, molecular techniques and R-looping analysis of rDNA proved to be direct methods of visualizing the organization and structure of rDNA. The rDNA genes were characterized initially as four *Eco*RI fragments designated A, B, C and D (Wellauer and Dawid, 1979). Subsequently, each fragment was cloned and measured as 6.0, 7.3, 12.0 and 19.0 kb (kilobase) to give a total of approximately 44 kb for each gene unit (Figure 1). The results of the molecular analyses were in agreement with previous measurements by electron microscopy (Wilson *et al.*, 1978, Erickson *et al.*, 1981).

Each complete ribosomal gene unit is divided into a 13 kb transcribed region and a 31 kb nontranscribed spacer (NTS) region (Figure 2). Ribosomal DNA is transcribed by RNA polymerase (pol) I in a telomere to centromere direction (Worton *et al.*, 1988). Transcription by RNA pol I gives rise to the large 45S rRNA precursor which is processed and yields 18S, 5.8S and 28S mature rRNAs (Figure 3). Transcribed spacers are represented in the primary transcript, but not in the mature ribosomal RNA. On the other hand, large portions of DNA (nontranscribed spacers) associated with each ribosomal RNA gene, are not transcribed at all (Federoff, 1977).

The transcribed region is divided into the external transcribed spacer (ETS), the rRNA genes and two internal transcribed spacers (ITS). Within the ETS is the origin of transcription and a promotor 3' of the 18S rRNA gene. The promotor and

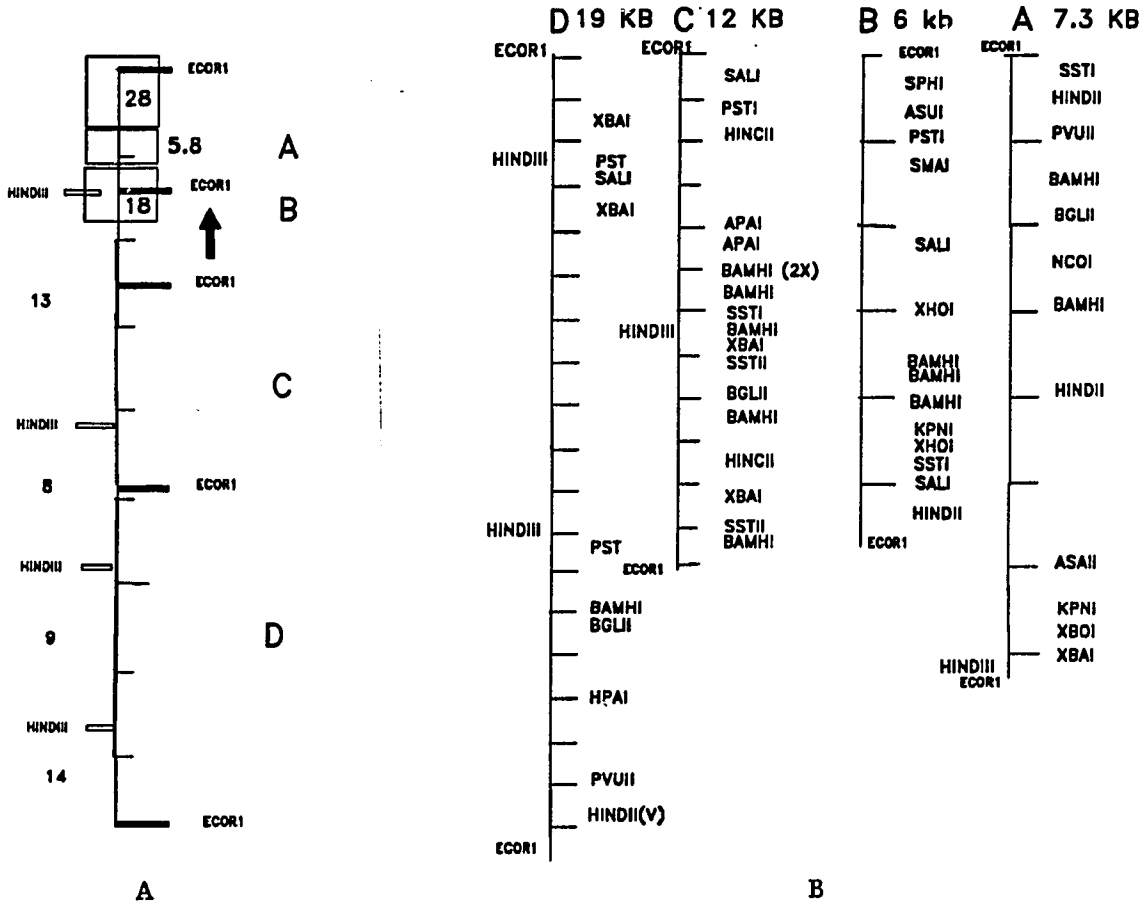


Figure 1. Restriction endonuclease map of the rDNA repeat unit and of the *Eco*RI A, B, C and D regions.

A. Restriction endonuclease map of one 44 kb rDNA repeat unit defined by the *Eco*RI regions.

B. Restriction endonuclease map of each of the *Eco*RI rDNA regions.

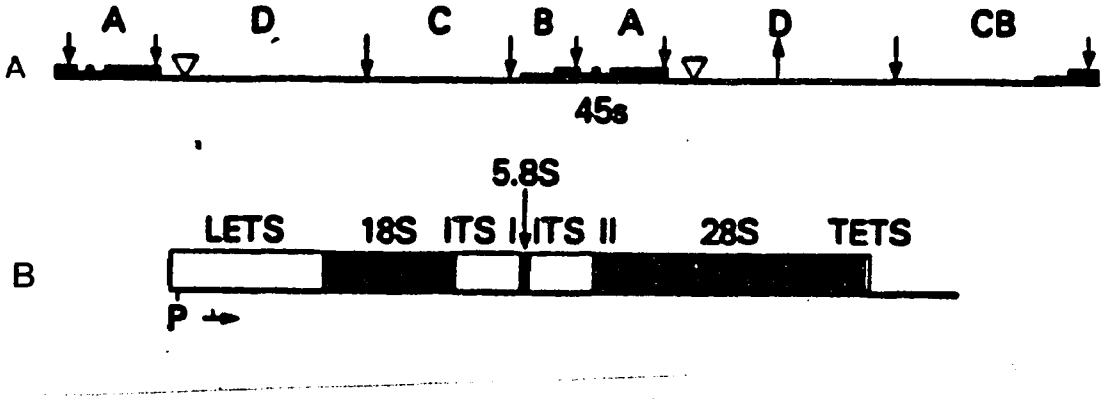


Figure 2. Schematic representation of the rDNA repeat unit and the 45S primary transcript (Schmickel, 1987).

A. The complete ribosomal DNA repeat showing the four *Eco*RI regions.

B. The 45S primary transcript shown in detail, as defined by the *Eco*RI B and A regions, the promotor (P), the left and terminal external transcribed spacers (LETS and TETS), the internal transcribed spacers (ITS I and ITS II) and the rRNA genes (18S, 5.8S and 28S).

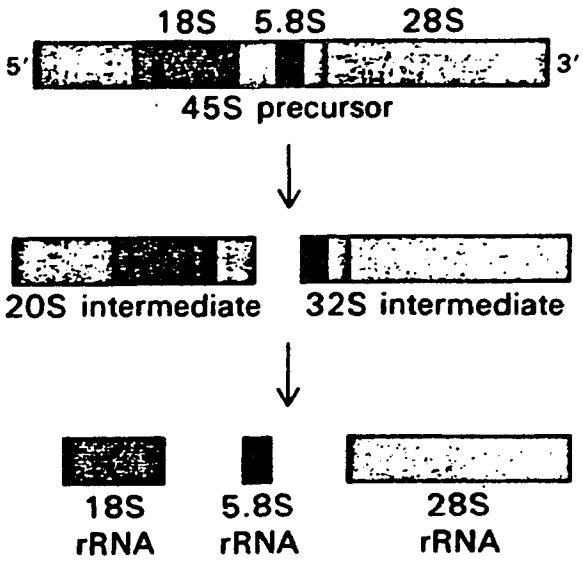


Figure 3. Schematic representation of human ribosomal RNA processing (Stryer, 1988).

Formation of mature human ribosomal 18S, 5.8S and 28S rRNAs from the 45S primary transcript through the 20S and 32S intermediates.

surrounding DNA has been sequenced and found to consist of 75% guanine and cytosine, as well as a number of short direct and inverted repeats and palindromes (Financsek *et al.*, 1982). The 5' ETS was also sequenced and found to be very rich in guanine and cytosine (G + C) and very poor in adenine (A) (Renalier, *et al.*, 1989).

The human rDNA promotor contains two distinct control elements designated as the core and the upstream control element (UCE) that serve as target sites for binding cellular sequence-specific DNA binding proteins (Haltiner *et al.*, 1986). The core element extends from nucleotides -45 to +18, while the UCE is located between nucleotides -156 to -107. These share no sequence homology.

The core element appears to be an essential signal for RNA pol I transcription because mutations in this region cause a 5-100 fold reduction in the level of transcription. The UCE plays an important role in modulating the efficiency of transcription, but it is not an absolute requirement for transcription *in vitro* from the human ribosomal promotor. The role of UCE depends on the core element in a directional and distance dependent manner. The two control elements operate in concert to promote efficient transcription by RNA pol I (Haltiner *et al.*, 1986).

In addition to the control elements of the human ribosomal promotor, two transcriptional factors have been purified and shown to recognize and bind to both UCE and core elements. The sequence-specific DNA binding proteins are designated SL1 and UBF1. UBF1 activates RNA pol I transcription through direct interaction with both UCE and core elements while SL1 plays a role in the promotor recognition

process and is required to reconstitute transcription *in vitro*. These studies suggest that protein-protein interactions between UBF1 and SL1 mediate the function of the UCE as well as the core element. Thus, functional cooperation between these two transcriptional factors mediate human rRNA synthesis (Learned *et al.*, 1985, Learned *et al.*, 1986, Bell *et al.*, 1988).

Transcription of rDNA comprises about 50% of cellular RNA synthesis (Grummt, 1989). The extremely high transcriptional activity results from the need of the cell to produce more than a million ribosomes per cell cycle which requires an efficient, quick and coordinated transcriptional apparatus (Grummt, 1989). Ribosomal DNA transcription, with associated processing and initial packaging of transcripts with ribosomal and non-ribosomal proteins, occurs within the nucleolus in an ordered manner and under defined topological conditions (Hadjiolov, 1985, Sommerville, 1986).

Termination sites for human rRNA transcription have also been found. A termination site, designated as the "Sal box", is present about 350 nucleotides downstream from the 28S RNA gene extending into the 3' terminal spacer (Bartsch *et al.*, 1987). More recently, Safrany *et al.*, (1989), found a putative termination site at the same position, necessary for approximately 90% of pre-rRNAs terminated. Three additional 3' termini found within the heterogeneous spacer region (about 930-1110 nucleotides downstream from the 3' end of the 28S rRNA gene) are also involved in termination, which suggests that a small percentage of human rDNA

transcription terminates at multiple sites (Safrany *et al.*, 1989).

In addition to these downstream termination sites, another, nearly identical termination site, is found upstream of the transcription start site (Pfleiderer, *et al.*, 1990). The 5' upstream terminator, which has a 10-11 base identity with the 3' downstream Sal box terminators, is an active terminator that stops pol I molecules which have started in the spacer separating individual transcription units. Thus, it prevents transcriptional interference with preinitiation complexes at the rDNA promotor.

The NTS contains several *Alu* repeats, as well as DNA sequences that are unique to the human rDNA repeat unit. Other DNA sequences are present that have homology to scattered DNAs elsewhere in the genome (Higuchi *et al.*, 1981, Wilson *et al.*, 1984, Erickson and Schmickel, 1985, Dickson *et al.*, 1989, Sylvester *et al.*, 1989, Safrany and Hidvegi, 1989). It is probable that all functions of the NTS have not yet been fully determined. One function assigned to the NTS is that it includes signals for the initiation of rRNA synthesis. One speculative function for the NTS is that it contains sequences that could be involved in nucleolar organization. Other suggested functions of the NTS include: (a) modulation of tissue-specific or metabolic responsive expression of rDNA; (b) a concentration of hot spots of recombination; (c) initiation sites for DNA replication; (d) a role in DNA or chromosome packing and (e) the promotion of alternative conformations of DNA, including Z-DNA.

As a consequence of the molecular analyses of rDNA, a large amount of data

is available related to variation and heterogeneity of ribosomal genes and associated sequences. Heterogeneity in rDNA structure was first shown in *Xenopus laevis*, where the length of the large NTS varies among repeats (Wellauer *et al.*, 1974, Morrow *et al.*, 1974). Later research focussed on mammalian rDNA, especially in mice and humans. For example, Arnheim and Southern (1977), using the restriction endonucleases *Eco*RI and *Hind*III, showed that individual mice or humans had a heterogeneous pattern of restriction fragments that resulted from differences in the NTS DNA. In the same study, results using mouse-human hybrid cell lines suggested that by sharing certain structural features, genetic exchanges could occur between ribosomal repeats on non-homologous chromosomes. Cytological analyses which show that non-homologous chromosomes containing NORs can associate during cell division (Fergunson-Smith and Handmaker, 1961). The "satellite associations" probably result from the interaction of rDNA sequences of non-homologous acrocentric chromosomes that cooperate in the formation of a single nucleolus in interphase, providing a possible mechanism for genetic exchange.

Evidence for genetic exchanges between ribosomal sequences was demonstrated by Arnheim *et al.* (1980). Through the use of restriction enzyme analysis of ribosomal genes in humans and apes, it was shown that different regions of the ribosomal gene families have different evolutionary patterns. The transcribed regions are less prone to nucleotide substitutions, although other small changes occur. This is consistent with the finding that the 18S and 28S rRNA genes are highly conserved during evolution

(Sinclair and Brown, 1971). Further, this study showed that differences among species can exist in the structure of the NTS; at least some regions of the rDNA genes undergo concerted evolution (reviewed in Dover, 1982). Within a single species, individual members of a multigene family are far more similar to each other than would be expected if each gene had evolved independently of other family members.

A number of other studies have defined polymorphisms, heterogeneity and fragment size variations in the human rDNA family (Arnheim and Southern, 1977, Krystal and Arnheim, 1978, Wilson *et al.*, 1978, Wellauer and Dawid, 1979, Schmickel *et al.*, 1980, La Volpe *et al.*, 1985). Polymorphisms are uniformly distributed over the NOR-containing chromosomes, consistent with the model of genetic exchange between rDNA sequences on non-homologous chromosomes (Krystal *et al.*, 1981). Evidence for the discrete and limited nature of NTS length polymorphism in humans is provided through restriction pattern analysis of genomic DNA in a study by Ranzani *et al.*, (1984). A basis for the discrete size variation in human spacer rDNA is known by the analysis of the cloned rDNA NTS region (Erickson and Schmickel, 1985). By selective cloning of parts of the NTS region, it was shown to be variable in both size and sequence composition (Sylvester *et al.*, 1986). Finally, a recent study compares the transcribed spacer sequences with those of variable regions of rRNA and with those of the NTS. The results support the hypothesis that the variable regions are descended from transcribed spacers on the basis of base content, sequence characteristics and sequence variability (Gonzalez *et*

al., 1990).

Molecular analysis of cloned human 18S and 28S rDNA genes has been carried out and critical information on restriction enzyme sites, nucleotide sequences, possible 3-D structure and their molecular evolution is available (Nazar *et al.*, 1975, Wilson *et al.*, 1978, Erickson *et al.*, 1981, Torczynski *et al.*, 1985, McCallum and Maden, 1985, Gonzalez and Schmickel, 1986, Gorski *et al.*, 1987, Maden *et al.*, 1987). The human 18S rRNA gene is 1868 base pairs (bp) long, with a G + C content of 56.0% (Torczynski *et al.*, 1985). This is very close to the G + C content of mouse (55.97%) (Raynal *et al.*, 1984) and that of rat (55.6%) (Torczynski *et al.*, 1983), but higher than that of frog (53.8%) (Slim and Maden, 1981), or yeast (45.0%) (Rubtsov *et al.*, 1980). The overall sequence comparison of the human 18S rRNA gene with that of mouse and rat reveals a 98.8-99.0% homology with specific regions present between conserved regions called "variable regions" (Chan *et al.*, 1984, Torczynski *et al.*, 1985, McCallum and Maden, 1985, Gonzalez and Schmickel, 1986). This indicates a common evolutionary ancestor as well as very low sequence divergence rate between mammalian genes.

Gonzalez and Schmickel (1986) investigated the secondary structure of 18S rRNA genes and found them to be remarkably well preserved throughout evolution changing very little over 3 billion years, pointing to the significance of the structure for rRNA function. The authors propose a secondary structure for the human 18S rRNA gene that is composed of double stranded regions and many hairpin loops.

The small 160 bp 5.8S rRNA gene has also been isolated and sequenced (Nazar *et al.*, 1975). When compared to frog and chick, the sequences are 97% and 99% homologous, respectively (Khan and Maden, 1977). These results indicate that the 5.8S rRNA gene has remained very stable during vertebrate evolution. A secondary structure for this gene was also proposed (Nazer *et al.*, 1975). Within the secondary structure, there are five base paired regions, with three forming a folding at the termini and two forming secondary hairpin loops. One stable region is very G + C rich and the other less stable region is A + U rich.

The 28S rRNA gene contains 5025 bp and is composed of conserved and variable regions (Gonzalez *et al.*, 1985). The regions differ in composition, degree of conservation and evolution. When human 28S rDNA is compared with the corresponding available rDNA sequences from the mouse, rat, frog, yeast and bacteria (Gorski *et al.*, 1987), the degree of homology between the conserved regions is extremely high. The human sequences are 99.0% homologous to those of the mouse and rat and 96% to that of frog. The variable regions show less homology, they are less numerous, contain variations in their sequences and are conserved over shorter evolutionary distances (Gorski *et al.*, 1987, Maden *et al.*, 1987). The secondary structure of the human 28S rRNA gene, together with the 5.8S rRNA gene, has been proposed to be a bimolecular structure (Gorski *et al.*, 1987). It appears to be a rather complex structure with various domains, hairpin loops and a large number of helixes.

II. Rationale for research

Several aspects of the function and morphology of rDNA-bearing chromosomes have led to a hypothesis which relates their functional characteristics to a role in nondisjunction and translocations (reviewed in Jacobs *et al.*, 1976). Chromosomal anomalies due to meiotic nondisjunction (for example, trisomy 21 or Down Syndrome) are the most frequent cause of human spontaneous abortions (reviewed in Mirre *et al.*, 1980). NOR-containing chromosomes are involved in about 40% of human lethal trisomies. Robertsonian-like translocations are the most frequent form of chromosome rearrangement in humans. They result from different forms of exchange occurring within (presumably) the centromeric or p-arm regions of two acrocentric chromosomes, giving rise to a metacentric or submetacentric element (John and Freeman, 1975). In 90% of these translocations, non-homologous acrocentrics are involved, with t(13;14) and t(14;21) as the most frequently observed translocations (Mattei *et al.*, 1979). The acrocentric chromosomes recombine so that they are assumed to have one functional centromere, although two morphologically distinct centromeres may be present.

One possible causative factor in both nondisjunction and Robertsonian translocation lies in the DNA homology among rDNA-bearing chromosomes. This could initiate satellite association during normal nucleolar organization, but lead to abnormal segregation or exchange by failure to disassociate. Critical to this hypothesis is the finding that human rDNA-bearing chromosomes are often observed to be

interconnected to each other by strands that contain nucleoprotein and rDNA (Henderson *et al.*, 1973). The interpretation of these interconnections is that strand proximity offers an opportunity for rDNA complexity (and recombination) between NOR-containing chromosomes. The presence of the interconnections also explains the persistence of satellite associations into metaphase (Warburton *et al.*, 1976). This suggests that rDNA homology and rDNA content in chromosomes plays a role in the formation of the functional nucleolus. Analysis of a double-satellited chromosome reveals an increase in rDNA content and association frequency, providing evidence for the proposal that the frequency with which a given chromosome becomes involved in satellite associations or its tendency to remain in association on the metaphase plate may be related to the rDNA content (Henderson *et al.*, 1976).

Recently, two studies investigated the involvement of rDNA in nucleolar formation and provided more support for a hypothesis which implicates rDNA as a primary participant in nucleolar organization. The first study concluded that NOR activity is an intrinsic property of the rDNA or its RNA products (Karpen *et al.*, 1988). The second study similarly concluded that the primary determinants of nucleolus development and maintenance appear to be the growing transcripts, be it the pre-rRNA transcript molecules themselves or specific proteins bound to their 5' termini (Scheer and Benavente, 1990).

A further investigation of rDNA involvement in nucleolar organization and satellite association provided the basis for this study. The specific aim was to identify

DNA homologies which are responsible for associative interactions among human rDNA-bearing chromosomes. The primary question targeted was: what is the molecular basis for associations between rDNA-bearing chromosomes? The specific problems addressed were to determine: (a) if the critical DNA (to association) is rDNA *per se*; if this is the case, what DNA sequences and how many copies are critical for association between chromosomal regions carrying rDNA and (b) if a special sequence within the rDNA complex is required or if the entire repeat unit or if multiples of the repeat unit are necessary. A combination of molecular techniques and cytological hybridization with cytogenetic analysis were used in this study.

III. Research findings

This investigation was divided into three parts. First, DNA was isolated from a human cosmid library which contained a partial rDNA repeat unit (Lau and Kan, 1983). Second, a new and functional (and ultimately manipulative) NOR site was established by transfection into chromosomes of human fibroblasts. Third, the molecular and cytological analysis of the new NOR site was carried out.

The first part of this study sought the possible isolation of an entire rDNA repeat unit from the cosmid library. Instead, three clones were isolated, with an entire intact transcribed spacer and the majority of the nontranscribed spacer. A fourth clone contained the rDNA transcribed spacer intact, but the promotor was absent. Large parts of the NTS were also present. From the first three, the one with the largest rDNA unit was used in the transfection of human fibroblasts. The single clone with

the absent promotor was also used for transfection because it served as a control in the functional analyses of the transfected rDNAs. It is proposed that if the promotor is absent, no transcription of the exogenous rDNA will occur and thus, no participation in satellite associations or nucleolar formation is expected.

The second part sought to introduce both DNAs into human fibroblasts using lipofection (Felgner, *et al.*, 1987). Both DNAs were successfully introduced into human cells (by Kathi McDowell). Several clones were isolated from each set of transfections, but only those with a rapid rate of growth were further analyzed.

The third part sought to determine the presence of the exogenous rDNA in human chromosomes, genomic DNA and in the nucleolar DNA of the transfected cells. In addition, the transcriptional activity and the frequency of participation in satellite associations of the transfected cells was also determined (by Kathi McDowell). The integration site of the cosmid DNA containing the intact rDNA transcribed spacer with promotor and parts of the NTS was mapped to the middle of the p-arm of the 3 chromosome. One to two copies of cosmid DNA were detected in the genomic DNA of the transfected cells. The integrated DNA was transcribed and participated in both satellite associations and nucleolar formation. One copy of the DNA lacking the promotor was present in the genomic DNA. The site of integration was mapped to the q-arm of an abnormal 7 chromosome. The integrated DNA was not transcribed and did not participate in satellite association and nucleolar formation, as expected if transcriptional activity is a factor. In short, these analyses indicate that the

critical DNA to association is rDNA, more specifically, the presence of the promotor along with the rest of the transcribed spacer and their transcription determines the involvement of the rDNA in satellite associations and nucleolar formation.

MATERIALS AND METHODS

Most of the laboratory methods outlined are modifications of those described in various laboratory manuals (Schleif and Wensink, 1981, Maniatis *et al.*, 1982, Davis *et al.*, 1986, Ausubel *et al.*, 1987).

I. Tissue Culture

All tissue culture work was done inside a tissue culture hood under sterile conditions. A human B-cell derived lymphocytic cell line, IB4, a promyelocytic human cell line, HL-60 and an SV40 *ori*$^-$ transformed human fibroblast cell line, HAL, have all been used in this study. IB4 and HL-60 cells were cultured in T-25 [25 cm^2, (cm=centimeters) Corning], T-75 (75 cm^2, Falcon) and T-175 (175 cm^2, Falcon) flasks containing 1X RPMI 1640 medium with L-glutamine (Gibco), 10% fetal or newborn calf serum (Gibco) and 1.0% of 100X antibiotic-antimycotic solution (Sigma). Both of these cell lines were grown in the presence of a gas mixture (5.0% CO_2, 20% O_2, 75% N_2, T.W. Smith) in a 37°C incubator. HAL cells were grown in the presence of 1X D-MEM/F-12 medium (Dulbecco's modified Eagle medium containing nutrient mixture F-12 [Ham] 1:1 with 15mM (millimolar) HEPES buffer with L-glutamine, Gibco), 10% fetal calf serum and 1.0% antibiotic-antimycotic solution. This cell line was grown in the presence of the same gas mixture as above but in a 35°C (optimum temperature) incubator.

IB4 and HL-60 cells are suspension cultures and are easy to maintain. The cells were fed twice a week by transferring 1/3 of the overgrown culture into a new flask

and by adding 2-3x more fresh medium. The fibroblastic HAL cells were fed three times a week; once a week, all of the medium was poured out and the same amount of fresh medium is added; in the second feeding the medium was poured out and the cells were trypsonized for 2-3 min (minutes) in the presence of 1X trypsin-EDTA (0.25% trypsin, 1mM EDTA (ethylenediamine-tetraacetic acid), Gibco). These cells were transferred to a sterile 15 ml (milliliters) polypropylene tube and centrifuged at 1,000g for 5 min. The trypsin was discarded and the pellet was washed and resuspended in fresh medium. The resuspended cells were transferred into new flasks and diluted with fresh medium.

Transfection of the isolated library clones (circular vector and insert DNA) into HAL cells was accomplished by Kathi McDowell using a lipid-mediated DNA transfer method, lipofection. This method makes use of a synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). This lipid forms liposomes which interact with DNA spontaneously to form lipid-DNA complexes (with entrapment of the DNA), fuse with culture cells and facilitate the delivery of functional DNA into the cell (Felgner, *et al.*, 1987). Electroporation and protoplast fusion were also used as means of DNA transfer into IB4 and HAL cells but no viable cells were obtained. Following selection, few clones were isolated. The HAL transfected cloned cells were also cultured under the same conditions as those for HAL cells and in the presence of the selective agent G418 [300 μg/ml (μg=micrograms)] at all times.

II. Bacterial Strains

The following *Escherichia coli* strains were used in this investigation: a) ED8767 (*sup*E44, *sup*F58, *hsd*S3, *rec*A56, *gal*K2, *gal*T22, *met*B1), a recombination deficient suppressing strain used for propagation of bacteriophage lambda vectors, b) HB101 (*sup*E44, *hsd*S20, *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20, *xyl*-5, *mtl*-1), a suppressing strain commonly used for large scale production of plasmids and c) LE392 (*sup*E44, *sup*F58, *hsd*R514, *gal*K2, *gal*T22, *met*B1, *trp*R55, *lac*Y1), a suppressing strain commonly used to propagate bacteriophage lambda vectors and their recombinants.

III. Plasmid DNA Isolation

A. Small scale plasmid DNA isolation

The small scale plasmid DNA isolation method is also known as a "minipreparation". Growth of *Escherichia coli* bacteria cells was achieved using the method outlined in Maniatis *et al.*, (1982). Bacteria containing recombinant plasmids were grown in 2.0 ml LB medium (1.0% Bacto-tryptone [Difco], 0.5% Bacto-yeast extract [Difco], 100mM NaCl, adjusted to pH 7.5 with NaOH) containing ampicillin (Boehringer Mannheim Biochemicals, BMB) at 50-75 μg/ml for 18 hrs at 250 rpm (revolutions per minute) in a incubator with aeration at 37°C (these were the standard conditions used for bacterial growth at all cases unless specified otherwise). 0.5 ml of the culture was transferred to a 1.5 ml microcentrifuge tube (Fisher), adjusted to 7.0% DMSO (dimethylsulfoxide) and stored at -70°C as a stock culture. The remaining 1.5

ml culture was subjected to alkaline lysis to extract the plasmid DNA (Birnboim and Doly, 1979). The cells were centrifuged at 13,000g for 2 min in a microcentrifuge tube to pellet the cells. The supernatant was discarded and the pelleted cells were suspended and lysed in 100 µl (microliters) of an ice-cold solution of 50mM glucose, 10mM EDTA, 25mM Tris-Cl (pH 8.0), 4 mg/ml (mg=milligrams) lysozyme (Reheis) and 150 µg/ml pancreatic RNase A (BMB). Incubation was for 5 min at RT. 200 µl of an ice-cold solution of 0.2N NaOH, 1.0% SDS was added to denature proteins. The tube was inverted rapidly 2-3x to mix the contents and incubated for 5 min on ice. 150 µl of an ice-cold solution of 3M KOAc (adjusted to pH 4.8 with glacial acetic acid for a final concentration of 3M with respect to potassium and 5M with respect to acetate) was added to the lysates and the tube was briefly vortexed and incubated on ice for 5 min. Centrifugation at 13,000g for 5 min at 4°C pelleted the proteins and chromosomal DNA. The supernatant was transferred to a fresh tube and extracted once with an equal volume of 1:1 phenol:Sevags (Sevags is 24:1 chloroform:isoamyl alcohol). Two volumes of 95% ethanol were added to the aqueous phase which contained the plasmid DNA, to precipitate the DNA. The DNA was incubated for 2 min at RT and centrifuged at 13,000g for 5 min at RT (room temperature). The supernatant was discarded and the DNA pellet was washed once with 70% ethanol, air dried for 5-10 min and suspended in 5-10 µl TE [(10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)] buffer (pH 8.0). The method usually yielded 2-5 µg of plasmid DNA.

B. Large scale plasmid DNA isolation

Bacteria containing recombinant plasmids were grown with the same standard conditions described above. Between 0.1-0.4 ml (approximately 2-4 X 10^8) cells were used to inoculate 25 ml LB medium. The culture was incubated for 3-4 hrs at 250 rpm with aeration at 37°C, or until the cells reached late log phase (equal to a Klett colorimeter value of 120-170 units). All of the (25 ml) culture was used to inoculate 500 ml of ampicillin (75 μg/ml) containing LB medium. The culture was incubated for 2.5 hrs at 250 rpm with aeration at 37°C. Chloramphenicol was added (170 μg/ml, BMB) and the cells were incubated for 12-16 hrs at 250 rpm with aeration at 37°C.

Harvesting of cells was achieved by centrifugation in 250 ml capped bottles (Nalgene) at 4,000 rpm for 10 min at 4°C. The supernatants were discarded and the pellets were washed and suspended in 100 ml of ice-cold STE buffer [100mM NaCl, 10mM Tris-Cl (pH 7.8), 1mM EDTA]. The washed cells were pelleted by centrifugation at 4,000 rpm for 5 min at 4°C and the supernatants were discarded. The pellets were subjected to alkaline lysis. The pelleted cells were placed in 10 ml of an ice-cold solution containing 5 mg/ml (mg=milligrams) lysozyme, 150 μg/ml RNase A, 50mM glucose, 25mM Tris-Cl (pH 8.0) and 10mM EDTA, by pipetting up and down. The lysates were transferred into a 50 ml polyallomer tube (Nalgene) and incubated for 5 min at RT. Then 20 ml of 0.2N NaOH, 1.0% SDS (sodium dodecyl sulfate) solution was added and the tube was gently inverted several times to ensure denaturation of proteins. Following incubation for 10 min on ice, 15 ml of ice-cold 3M

KOAc was added to a final concentration 1M, the tube was inverted and incubated for 10 min on ice. The solution was centrifuged at 20,000 rpm for 20 min at 4°C to pellet the bacterial debris. The supernatant was transferred into a new tube and 0.6 volume of isopropanol was added and mixed to precipitate the DNA and left for 15 min at RT, followed by centrifugation at 12,000g for 30 min at RT to pellet the DNA. The DNA pellet was washed once with 70% ethanol, air dried for 2-3 min and dissolved in 8.0 ml TE buffer. The plasmid DNA was further purified by cesium-chloride density gradient centrifugation as described below.

C. Cesium-chloride density gradient centrifugation

Purification of closed circular plasmid DNA was accomplished by the use of ultracentrifugation in cesium-chloride gradients. One gram of CsCl was used per ml of DNA solution to give a final density of 1.55 g/ml). EtBr at 80 μg/ml was added (for visualizing bands of DNA) and the mixture was transferred to a high speed screw-capped ultracentrifuge tube (Beckman). The tube was centrifuged at 45,000 rpm for 36-48 hrs at 20°C in a Beckman Ti50 rotor. The plasmid DNA was visualized under UV light and the band corresponding to the closed circular plasmid DNA (lower band) was collected directly from the tube by using a 3cc syringe fitted with a long #18g needle. This was transferred to a 15 ml polypropylene screw-capped centrifuged tube (Corning). The plasmid sample was extracted 5-7x by adding an equal volume of 1-butanol saturated with water, followed by mixing and centrifugation at 1,500g for 3 min at RT to remove the EtBr. The extracted plasmid sample was dialyzed for 3-4

hrs against several changes of TE buffer and transferred to a 15 ml corex tube, where it was adjusted to 150mM NaCl and precipitated with 2 volumes of ice-cold 95% ethanol. Centrifugation at 9,000 rpm for 20 min at 4°C pelleted the DNA. The supernatant was discarded and the pellet was washed once with 70% ethanol. After air drying for 10-15 min, the pellet was dissolved in 0.3-0.6 ml TE buffer. The concentration was determined by spectrophotometric readings and gel electrophoresis as described in the section describing Nucleic Acid Isolation.

IV. Cosmid Library Screening

The method used to screen the cosmid library was adopted from Ausubel *et al.*, (1987). The library was constructed by Lau and Kan to allow the isolation of large fragments of human DNA, between 25-45 kb (Lau and Kan, 1983).

A. Preparation of bacteria cells

The library was grown under standard conditions. The titer of the library was determined using serial dilutions on 100 mm (millimeters) LB plates (LB medium plus 1.5% Bacto-agar [Difco], adjusted to pH 7.5 with NaOH) containing ampicillin at 75 μg/ml. The plates were incubated upside down for 18 hrs with aeration at 37°C. The colonies were counted and the number of cells was determined using the following formula:

# of cells = # of colonies x 10 x the factor of dilution.

Once the dilution factor was determined and the number of colonies and cells counted (resulting in approximately 1,000 colonies on a single LB plate), 30

LB/ampicillin plates were prepared. About 30,000 colonies were grown and screened (see below). A sintered glass Buchner funnel (Whatman) was used to filter the cells onto 100 mm nitrocellulose filters (Millipore). The nitrocellulose filters were previously marked and wetted by being placed on the surface of an LB/ampicillin plate. Once filtering was complete, the nitrocellulose filters were returned to the LB/ampicillin plates. All plates with the filters were incubated upside down for 18 hrs with aeration at 37°C.

B. Preparation of replica filters

Replica plating proceeded by designating and wetting a second set of nitrocellulose filters. The initial library filters were removed from the plates and placed on 5 sheets of 20 x 20 cm (centimeters) 3MM paper (Whatman) with the bacteria colonies facing up. The newly wetted filters were placed directly above the bacteria colonies with a 2-3 mm overlap. Three sheets of 3MM paper were added on top of the filters and a textbook was used as weight to press down on the filters, to allow transfer of the bacteria colonies to the new filters and to create replicas of the initial filters. Before removing the replica filters, 3 holes on both filters were made using an #18g needle to mark the orientation of the colonies. The filters were pulled apart and placed "bacteria side up" on the plates. Two sets of replica filters were made using this procedure. The replica filter plates were incubated upside down for 18 hrs with aeration at 37°C, while the initial filter plates were incubated under the same conditions at RT. After incubation, the initial filter plates were sealed with parafilm

and stored at 4°C, while the replica filters were screened.

Before screening, the replica filters were removed from their plates and denatured "bacteria side up" on a sheet of 46x57 cm 3MM paper soaked with 0.5M NaOH for 5 min, followed by neutralization for 5 min on a soaked 3MM paper with 1M Tris-Cl, pH 7.5 and for another 5 min on a soaked 3MM paper with 0.5M Tris-Cl/1.25M NaCl. The filters were air dried completely and then baked in a vacuum oven for 90 min at 80°C.

C. Hybridization of replica colony filters

The baked filters were divided in 6 groups (each containing 10 filters) and placed in heat-sealed plastic bags. The filters were prehybridized for 3-4 hrs at 42°C in a solution containing 50% formamide, 5X SSPE from a 20X stock solution [3M NaCl, 200mM NaH_2PO_4, 20mM EDTA (adjusted to pH 7.4 with NaOH)], 0.2% SDS, 2X Denhardt's (50X Denhardt's is 1.0% Ficoll, 1.0% polyvinylpyrolidone and 1.0% Bovine serum albumin (BSA)] and 100 μg/ml sheared denatured salmon sperm DNA (Southern, 1975). After prehybridization, the solution was removed and a freshly made solution containing the same components and the radioactively labelled probe was added. The two probes used (one for each set of replica filters) were the human *Eco*RI A and B regions of rDNA, with homology to the 28S and 18S rRNA genes, respectively (kindly provided by Dr. Schlessinger). The bags were resealed and hybridization was carried out in a shaking water bath for 12-18 hrs at 42°C.

Following hybridization, the filters were removed from the bags and washed

2x for 15 min each in 500 ml 2X SSC, 0.5% SDS at RT, 2x for 15 min in 500 ml 1X SSC, 0.5% SDS at 37°C and 1x for 30-45 min in 500 ml 0.1X SSC, 0.5% SDS at 65°C in a shaking water bath with gentle agitation. The filters were then autoradiographed as described in the Nucleic Acid Blotting section.

Ten positive colonies present in both sets of filters (same colonies that hybridized with both ^{32}P-labelled *Eco*RI A and B) were picked with a loop and regrown in 2 ml ampicillin containing LB broth for 18 hrs with aeration at 37°C. 0.5 ml of each culture was frozen down and each of the remaining culture was subjected to minipreparation, as described previously. After size and restriction enzyme analysis on agarose gels and Southern hybridization, four colonies were selected based upon the size of their inserts and their hybridization patterns to the *Eco*RI A and B probes. These were used in a large scale isolation of cosmid DNA.

D. Isolation of cosmid DNA

Large scale isolation of cosmid DNA utilized an identical procedure to that for plasmid DNA with one exception. Lysis of cells was achieved, not by alkaline conditions (as that for plasmids,) but by exposure to SDS. This method is outlined in Maniatis *et al.*, (1982). After growing and harvesting the bacteria, the cell pellets were suspended in 10 ml of an ice-cold solution of 10% sucrose in 50mM Tris-Cl (pH 8.0). Each dissolved pellet was transferred in a 50 ml polyallomer tube with 2.0 ml of 10 mg/ml lysozyme in 250mM Tris-Cl (final concentration 83mM). Immediately, 8.0 ml 250mM EDTA (to a final concentration of 150mM) was added and all solutions were

mixed by inverting the tubes several times. Incubation took place for 10 min on ice, followed by the addition of 4.8 ml 4M NaCl (to final concentration of 895mM), mixing and further incubation on ice for another 1-2 hrs to ensure complete cell lysis and digestion of proteins. The solutions were transferred to screw-capped centrifuge tubes and centrifuged for 30 min at 30,000 rpm at 4°C to pellet the bacterial debris and chromosomal DNA. The supernatants were collected, transferred and extracted once with an equal volume of phenol:Sevags and once with Sevags only. To each of the aqueous phases, 2 volumes of 95% ethanol were added, mixed and incubated for 15 min at -70°C. The samples were centrifuged at 5,000 rpm for 20 min at 4°C to pellet the nucleic acids. Supernatants were discarded and the pellets washed once with 70% ethanol and air dried for 10-15 min. Each pellet was dissolved in 8.0 ml TE buffer (pH 8.0) and purified by cesium-chloride gradient ultracentrifugation as described previously. The concentration and purity of the each cosmid DNA was determined through spectrophotometric readings and agarose gel electrophoresis.

V. Molecular Cloning

Molecular cloning techniques were used to generate rDNA fragments produced by *Eco*RI digestion of isolated crude rDNA (by multiple CsCl gradients and by selecting G + C rich fractions). The method used is outlined in the instructions of a Stratagene Cloning Systems Kit, which is in turn based on methods in Maniatis *et al.*, (1982), Frishauf *et al.*, (1983) and Glover, (1985).

A. Preparation of host cells

Two strains of *Escherichia coli* cells were used as the host cells, LE392 and P2392. Strain LE392 allows growth of both recombinant and non-recombinant clones while strain P2392 allows growth of only recombinant clones. This occurs because lambda DASH DNA (the cloning vector) utilizes the Spi system of selection. When the *red* and *gam* genes of the stuffer fragment of lambda DASH are replaced by insert DNA, the vector is able to grow on P2 lysogen (P2392) and will no longer be *s*ensitive to *P2 i*nhibition (*Spi* selection). As a result, only recombinants will grow on P2392.

Both strains were grown on LB plates under standard conditions. To ensure that all cells arose from a single colony, liquid cultures were prepared by picking a single colony from each plate and inoculating 15 ml of LB medium supplemented with 0.2% maltose and 10mM $MgSO_4$. Both P2392 and LE392 cultures were grown under standard conditions at 30°C. 5.0 ml of each culture were frozen down (to be used as stock), while the remaining cells were centrifuged at 1,000g for 10 min at RT. The supernatants were discarded and each pellet was resuspended in 0.5 volume of 10mM $MgSO_4$. The cells were then stored at 4°C.

B. Preparation of vector and insert DNA

One μg of bacteriophage lambda DASH DNA, was digested with *Xho*I for 18 hrs at 37°C to remove the stuffer region. A second enzyme, *Eco*RI, was added (with appropriate incubation salt buffer) and the sample was digested for 18 hrs at 37°C to

generate sticky ends. Parallel to the second digestion, 1 μg crude rDNA was digested with *Eco*RI for 18 hrs at 37°C. After digestion, both vector and insert DNA were mixed, adjusted with 0.5 volume of 5M NH_4Ac and precipitated with 2 volumes isopropanol. The sample was centrifuged at 13,000g for 10 min at 4°C, the supernatant discarded and the pellet was washed once with 70% ethanol, air dried for 5 min and dissolved in 3 μl TE buffer.

C. Ligation of vector and insert DNA

0.5 μl 10X ligation buffer [500mM Tris-Cl (pH 8.0), 70mM $MgCl_2$, 10mM dithiothreitol (DTT)], 0.5 μl 10mM ATP (pH 7.5), 0.5 μl T4 DNA ligase (equivalent to 3 units, BMB) and 0.5 μl water were added to the 3 μl vector-insert mixture, for a final volume of 5.0 μl. The contents were mixed and incubated for 1 hr at RT and then for 18 hr at 4°C. Both before and after ligation, 0.5 μl samples were removed from the mixture and run on a 1.0% minigel to monitor the reaction. The product of ligation was visualized under UV after staining with EtBr. The ligation reaction was judged successful since there was only one band which indicated complete ligation. Prior to ligation there were 3 bands present, one corresponding to the left arm of the vector, one to the right arm and one to the stuffer region.

D. Packaging

For packaging, a Stratagene Gigapack Gold Extracts Kit was used. For optimal efficiency 2 μl from the 5 μl ligated DNA sample were used per packaging extract. To the freeze/thaw extract 2 μl of the ligated DNA were added and immediately after

15 µl sonic extract were added. The contents were mixed and centrifuged for 3-5 sec at 13,000g to ensure that all the contents ended at the bottom of the tube. Both mixtures were incubated for 2 hrs at RT followed by the addition of 500 µl phage dilution buffer [SM, 0.5% NaCl, 0.2% $MgSO_4$, 20% 1M Tris-Cl (pH 7.5), 2.0% gelatin] and 20 µl chloroform. The solutions were mixed and centrifuged at 13,000g for 30 sec to sediment the debris. The supernatants were titered and the extracts were transferred to 100 µl of the proper host cells. After mixing, the solutions were incubated for 15 min at 37°C to allow the phage to attach to the cells. Finally, 3.0 ml of melted top agar (0.5% NaCl, 0.2% $MgSO_4$, 0.5% Bacto-yeast extract, 1.0% NZ amine, 0.7% agarose) were added to each tube, mixed and poured on dry, prewarmed 100 mm NZY [same as above, but the agarose was replaced by 1.5% agar (Difco)] plates. The NZY plates were incubated upside down for 18 hrs with aeration at 37°C.

E. Plaque transfer

Bacteriophage plaques were transferred to nitrocellulose filters using a method described in Ausubel *et al.*, (1987). Plaques corresponding to P2392 plates (recombinant clones) were transferred. Before transfer, the plates were incubated for 1-2 hrs at 4°C. The filters were labelled and placed ink side up on top of the plaques for 10 min. An 18 gauge needle was used to make orientation marks and the filters were slowly removed to avoid removal of top agar. Two replicas were prepared for each plate. The filters were air-dried plaque side up for 10 min at RT and denatured for 1-2 min on 3MM paper soaked with 0.2N NaOH and 1.5M NaCl. Neutralization

followed for 1-2 min on a 3MM paper soaked with 0.4M Tris-Cl, 2X SSC and for 1-2 min on a 3MM paper soaked with 2X SSC. All filters were air dried and baked in a vacuum oven for 90 min at 80°C.

F. Hybridization of plaque filters

The baked filters were screened using a radioactively labelled 3.5 kb rDNA fragment by the method that was previously discussed but with some modifications. Prehybridization of the filters was conducted for 3-4 hrs at 42°C in a solution containing 50% formamide, 40% dextran sulfate, 6X SSC, 0.5% SDS, 5X Denhardt's and 100 μg/ml sheared denatured salmon sperm DNA. Following prehybridization, the same solution was used for hybridization with the addition of 10mM EDTA and the labelled probe. Hybridization proceeded for 18 hrs at 42°C. Washing conditions were identical to those used for the library filters.

G. Small scale bacteriophage DNA isolation

Isolation of bacteriophage DNA was accomplished using a modified protocol from Ausubel et al., (1987). All positive clones (present in both replica filters) were picked using a pasteur pipet and transferred to a 15 ml polypropylene tube (Falcon) containing 300 μl SM buffer and 200 μl exponential P2392 cells. The tubes were incubated for 10 min at 37°C and 10 ml LB medium containing 10mM $MgCl_2$ and 1.0% glucose were added. The contents were vortexed and further incubated with the caps in a half-opened position for 18 hrs at 200 rpm with aeration at 37°C. 1.0 ml of each culture was frozen down and placed at -70°C. To each of the remaining cultures,

5.0% chloroform was added, mixed and further incubated for 10 min at the same conditions as before. Following incubation, all cultures were centrifuged for 10 min at 1,000g at RT to pellet the bacteria debris. The supernatants were transferred into polyallomer centrifuge tubes (Beckman) and centrifuged for 30 min at 30,000 rpm at 4°C. The pellets were air dried, dissolved in 200 µl SM buffer and transferred into 1.5 ml microcentrifuge tubes. To each phage suspension 200 µl of 1 µg/ml proteinase K (BMB) were added, mixed and incubated for 2 hrs at 37°C. After digestion, the phage samples were extracted once with an equal volume of phenol and once with Sevags. The aqueous phases were adjusted with 160 µl 5.0M NH_4Ac and precipitated with 1.0 ml 95% ethanol. Following mixing, the samples were centrifuged at 13,000g for 20 min at 4°C. The pellets containing the recombinant phage DNA were air dried for 10 min and dissolved in 100 µl TE buffer (pH 8.0). Both concentration and purity of all clones were determined by spectrophotometric readings and agarose gel electrophoresis. The cloned DNA samples were stored at 4°C.

VI. Molecular Subcloning

This technique was used to insert the *Eco*RI B rDNA region of the human ribosomal repeat unit into plasmid pSV2-neo (for use in transfection studies) and was based on methods outlined in Maniatis *et al.*, (1982).

A. Preparation of competent cells

The HB101 strain of *Escherichia coli* was used as the recipient cell line. The bacterial cells were grown in a 2 ml liquid LB broth overnight under standard

conditions. 500 µl of this culture was used to inoculate 50 ml of LB broth which was then grown for 2-4 hrs at 37°C. Three ml were used for each of the two transformation experiments. The cells were chilled on ice for 10 min, centrifuged at 4,000g for 5 min at 4°C and the supernatants were discarded. The pelleted cells were suspended in 1.5 ml of ice-cold sterile solution of 50mM $CaCl_2$, 10mM Tris-HCl (pH 8.0) and placed in an ice bath for 15 min followed by centrifugation at 4,000g for 5 min at 4°C. Again the supernatants were discarded and the pellets were resuspended in 200 µl of ice-cold solution of 50mM $CaCl_2$, 10mM Tris-HCl (pH 8.0) and placed in 4°C for 12-24 hrs.

B. Preparation of vector DNA

One ug of plasmid pSV2-neo was linearized with *Eco*RI and electrophoresed on a 1.0% agarose gel. The band corresponding to the linearized plasmid was excised and extracted from the gel by Geneclean. The fragment was then dephosphorylated to prevent reannealing using 1 unit calf intestinal phosphatase (CIP) in a reaction that included 20mM Tris-HCl (pH 8.0), 1mM $MgCl_2$, 1mM $ZnCl_2$. This mixture was incubated at 37°C for 30 min followed by a phenol:chloroform extraction and precipitation with ethanol. The final pellet was resuspended in 5 µl TE buffer.

C. Ligation of vector and insert DNA

The linearized plasmid was mixed with 500 ng of the *Eco*RI B rDNA region and precipitated with 95% ethanol. The sample was centrifuged at 13,000g for 15 min at 4°C, the supernatant discarded and the pellet was washed once with 70% ethanol, air dried for 5 min and dissolved in 8 µl of TE buffer. 1 µl was removed from the

sample and was used on an agarose gel in comparison to a sample taken after ligation (monitoring ligation reaction). To the 7 µl sample, 1 µl 10X ligation buffer and 10 units T4 DNA ligase was added. The contents were vortex briefly, span down and incubated for 8 hrs at 12°C.

D. Transformation of bacteria cells

To each of the 200 µl cell culture, 2.5 µl of the ligated DNA sample was added, mixed and placed on ice for 30 min. The samples were transferred into a 42°C water bath for 2 min followed by the addition of 800 µl ampicillin containing (75 µg/ml) LB broth to each tube. The samples were then incubated for 1 hr at 37°C. Following incubation, the samples were diluted serially, spread on ampicillin containing (75 µg/ml) LB plates and placed in RT until the liquid was absorbed. Finally, the plates were inverted and incubated at 37°C for 16-24 hrs.

E. Small plasmid DNA isolation

Four colonies were picked randomly from the plates and were grown in 2 ml of LB broth under standard conditions. The plasmid DNA was extracted using the same procedure as described previously. Restriction analysis, gel electrophoresis and Southern blotting were used to identify the success of the subcloning and the identity and orientation of the inserted DNA fragment.

VII. Nucleic Acids Isolation

A. Total genomic DNA extraction

Total genomic DNA extraction was achieved using a modification of the

method of Gall, (1968). Human cells (6-9 X 10^6) were centrifuged at 1,500g for 5 min at RT. The supernatant was discarded and the pellet washed and resuspended in 1X PBS (phosphate buffer saline, 137mM NaCl, 27mM KCl, 43mM Na_2HPO_4, 14mM KH_2PO_4, pH 7.3). The cells were suspended and lysed in 3-5 ml 1X Gall's buffer (10mM Tris-Cl, 10mM NaCl, 10mM EDTA, 5.0% SDS) and extracted 2x with an equal volume of buffer saturated phenol:Sevags. The aqueous phase was adjusted to 150mM NaCl and 2 volumes of ice-cold 95% ethanol were added to precipitate the nucleic acids. The nucleic acids were pelleted by centrifuging at 9,000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet was air dried for 10-15 min and then dissolved in 1.0 ml 1X SSC buffer (150mM NaCl, 150mM sodium citrate, pH 7.0). The nucleic acids were digested with 50 µg/ml RNase A for 45-60 min at 37°C, followed by pronase (BMB) digestion at 50 µg/ml for 30 min at 37°C. The digested sample was sequentially extracted with an equal volume of phenol, 1:1 phenol:Sevags and with Sevags. The DNA was precipitated with 95% ethanol, centrifuged and the pellet dissolved in 300-500 µl TE buffer. The concentration of the DNA was determined by spectrophotometric readings at 260 nm (nanometers). This is based upon the standard, OD_{260} (1.0) = 50 µg/ml double stranded DNA. The purity of the sample was determined by the ratio of readings at 260 nm and 280 nm (OD_{260}/OD_{280}). The concentration of the genomic DNA was also determined by electrophoretic quantitative analysis on a 1.0% agarose minigel with known concentrations of DNA of the plasmid pBR322. DNA sample was stored at 4°C in TE buffer.

B. Total RNA extraction

RNA extraction was accomplished by using a modification of the method of in Maniatis *et al.*, (1982). Human cells (3-6 X 10^6) were centrifuged, washed and pelleted under the same condition as those for DNA extraction. The pellet was lysed in 2-3 ml lysis buffer (100mM Tris-Cl, 100mM NaCl, 20mM EDTA, 1.0% sarkosyl, pH 9.0) and passed 5-6x through a 5cc syringe fitted with a #21g needle to ensure complete lysis. The lysates were extracted 3x with an equal volume of 1:1 phenol:Sevags and 1x with Sevags. An equal amount of ice-cold 100mM LiCl in 95% ethanol was added to the aqueous phase to precipitate the nucleic acids. The precipitated nucleic acids were placed for 30 min at -70°C and centrifuged at 13,000g for 20 min at 4°C. The supernatant was discarded and the pellet was washed once with 95% ethanol, air dried for 5-10 min and dissolved by gentle vortexing in 600 µl 50mM Tris-Cl, pH 8.0. To the dissolved pellet, 6 µl 1M $MgCl_2$ (10mM final concentration) and 20 µl DNase (BMB) at 100 µg/ml (40 units) were added. The sample was digested for 45-60 min on ice, followed by the addition of 12 µl 0.5M EDTA (10mM final concentration) and 60 µl (1/10 volume) 3M NaOAc, pH 5.2. After mixing, the sample was extracted once with an equal volume of phenol:Sevags and 1x with Sevags. The aqueous phase was ethanol precipitated, centrifuged (as discussed above) and the pellet dissolved in 50-100 µl TE buffer. Spectrophotometric readings at 280 nm were used to measure the concentration and purity of the RNA. Concentration is determined using the standard, OD_{260} of 1 = 40 ug/ml RNA. The ratio

of OD_{260}/OD_{280} was determined. A sample with a ratio of 1.9-2.0 was used. Concentration and purity were also determined through agarose gel electrophoresis. The RNA sample was stored in TE buffer at -70°C.

C. Nucleolar DNA extraction

Nucleolar DNA extraction was achieved using a modification of the methods of Beebee, (1986) and Higachi *et al.*, (1978). Human fibroblasts (1×10^8 or from 8-10 T-175 confluent flasks) were centrifuged at 1,500g for 5 min at RT, the supernatant was discarded, the pellet suspended in 1X PBS and centrifuged at 1,500g for another 5 min to pellet the cells. After the supernatant was discarded, the pellet was suspended in 3 ml of 1X RSB solution [10mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl (pH 7.6)]. The detergent Nonidet P-40 was added at 0.2% final concentration and the cells were lysed by few strokes in a glass homogenizer with a teflon pestle. Nuclei were pelleted by low speed centrifugation (2,000g) for 10 min at 4°C. The supernatant was removed and the pelleted nuclei were suspended in 3 ml of 0.34M sucrose solution containing 10mM $MgCl_2$, layered over 0.88M sucrose and centrifuged at 2,000g for 10 min at 4°C. The supernatant was discarded and the pelleted nuclei were resuspended in 3 ml of 0.34M sucrose solution containing 1mM $MgCl_2$.

The nuclei suspension was placed on ice and sonicated in a Sonics & Material Vibra Cell high intensity ultrasonic processor (Model VC 300, horn of 9.5 mm (millimeters) diameter, output control setting at 1) for 12 secs (seconds) followed by a cooling period of 30 sec on ice. The procedure was repeated 3-4 times. The

sonicated sample was layered on an equal volume 0.88M sucrose solution and centrifuged at 2,000g for 20 min at 4°C in a swinging bucket rotor.

The extra-nucleolar chromatin remained in the 0.34M sucrose layer, while nucleoli were pelleted. Both the extra-nucleolar layer and the nucleoli were collected and processed as described previously for genomic DNA (phenol-chloroform extractions, RNase, pronase, *etc.*). Throughout the procedure, the isolation of nuclei and nucleoli was monitored by light microscopy. Finally, the concentration of the DNA was determined by spectrophotometric measurements as described above.

VIII. Restriction Endonuclease Digestion

All restriction enzymes and their incubation salt buffers were purchased from BMB and BRL (Bethesda Research Laboratories). The most commonly used restriction endonucleases were *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Pvu*II, *Sal*I, *Xba*I and *Xho*I. The most commonly used salt buffers, depending on the specific enzyme, were Low [10mM Tris-Cl (pH 7.5), 10mM $MgCl_2$, 1mM Dithioerythritol (DTE)], Medium (10mM Tris-Cl, 10mM $MgCl_2$, 50mM NaCl, 1mM DTE) and High (50mM Tris-Cl, 10mM $MgCl_2$, 100mM NaCl, 1mM DTE). Each digestion reaction was prepared as indicated by the manufacturer [usually 1 unit of enzyme will digest 1 μg of DNA in 1 hr (hour)]. All reactions were incubated in a 37°C water bath for various times, depending on the amount and type of DNA. To ensure complete digestion, most reactions were incubated at longer periods of time than suggested. Total genomic DNA was always digested overnight (approximately 16-20 hrs).

Reactions involving digestion with more than one enzyme proceeded with the digestion of a low salt buffer enzyme, followed by adjusting the salt concentration to either a medium or a high value and by the addition of the second enzyme. Reactions involving two enzymes with the same salt buffer were carried out by adding the proper buffer and both enzymes.

IX. Agarose Gel Electrophoresis

Agarose gels were prepared by dissolving powdered agarose (BRL) into 1X electrophoresis buffer (TAE) buffer (40mM Tris, 20mM NaOAc, 1mM EDTA, adjusted to pH 7.2 with glacial acetic acid) and by heating until the agarose dissolves. Various concentrations of agarose gels, *i.e.*, 0.7%, 0.8%, 1.0% and 1.2% were prepared in this way depending on the size of DNA used in the analysis. After cooling for 2-3 min, the agarose solution was poured in a horizontal gel plate. The well-forming comb was positioned in the tray and the agarose was allowed to polymerize for 30 min at RT. 10-15 min prior to use, the comb was removed and the gel was immersed in 1X TAE buffer inside an electrophoresis tank.

Prior to electrophoresis, gel-loading buffer (0.25% Bromophenol blue dissolved in 1:1 1X TAE buffer and glycerol) was added to the digested and undigested DNA samples. DNA was heated for 10 min in a 68°C water bath to ensure dissociation of aggregated DNA fragments and then loaded onto the gel carefully using a Pipetman (Eppendorf). The voltage and run times for gels depended on the size of the DNA to be analyzed. Generally, analysis of small DNA fragments used 12-15 V/cm (volts per

centimeter) for 1-1.5 hrs, whereas gels with large DNA fragments were run at 2-3 V/cm for 12-16 hrs, or until the bromophenol blue had migrated 3/4 down the gel. Gels with genomic, extra-nucleolar and nucleolar DNA were run at 1.5V/cm for 12-16 hrs.

After electrophoresis, the gels were stained in a solution of 0.5 μg/ml Ethidium Bromide (EtBr) for 15-30 min at RT. The gels were rinsed 2-3x with distilled water to remove the extra EtBr. The DNA was visualized on a long wave UV (ultraviolet) transilluminator (Spectroline) and the gels were photographed using a Konica instant camera equipped with a Kodak 22A red Wratten filter with Polaroid type 667 Land film (Maniatis *et al.*, 1982).

RNA agarose gels were also prepared and photographed in exactly the same way as those for DNA. RNA formaldehyde agarose gels were prepared in a similar way in a fume hood, but with some modifications. RNase free powdered agarose (BRL) was dissolved in distilled water and heated until it was totally dissolved. Two concentrations were used, 1.0% and 1.2%, depending on the type of agarose gel. After cooling for 2-3 min, 1X MOPS buffer (40mM morpholinopropanesulfonic acid [MOPS], 10mM NaAc, 1mM EDTA, adjusted to pH 7.0 with NaOH) and 2.2M formaldehyde were added. The same steps were followed as above for setting the gel.

To the RNA samples, various amounts (depending upon the concentration of the sample) of 50% formamide (final concentration of 645mM), 2.2M formaldehyde (50mM) and 10X MOPS buffer (130mM) were added. The samples were heated for

15 min in a 65°C water bath followed by the addition of loading buffer (same as for DNA). The samples were loaded to the gel using a Pipetman. The gels were run at 3.5 V/cm for 3 hrs. To visualize the RNA, one lane was cut from the gel, stained with 0.5 μg/ml EtBr for 20 min and photographed for reference.

A computer program (using bacteriophage lambda *Hind*III fragments) as a standard was used to calculate each DNA fragment size.

X. Agarose Gel DNA Fragment Extraction

The isolation of fragments of DNA from agarose gels was accomplished using a Geneclean kit (BIO 101). The fragment to be extracted was cut from the gel, diced and transferred to a 1.5 ml microcentrifuge tube. Approximately 1.0-1.3 ml saturated NaI solution were added and the contents were mixed and incubated for 5-10 min at 50°C or until all the agarose pieces were dissolved. Then 5 μl glassmilk (DNA binding matrix) was added and the tube was vortexed and incubated on ice for 5 min with occasional mixing. After incubation, the solution was centrifuged for a few sec at 13,000g to pellet the glassmilk with the bound DNA. The supernatant was discarded and the pellet resuspended in 600 μl NEW WASH solution (NaCl, Tris, EDTA, ethanol and water; their concentrations are not provided by the company). The pellet was centrifuged for 5 sec at 13,000g, the supernatant discarded and the pellet suspended in another 600 μl NEW WASH. This procedure was repeated 3-4x and the final pellet dissolved in TE buffer (pH 8.0). The dissolved sample was incubated for 2-3 min at 50°C and centrifuged for 30 secs at 13,000g. The supernatant containing

the DNA fragment was collected using a Pipetman and the pellet was suspended in TE buffer and the process repeated again. The total final volume of the extracted DNA fragment was 10-20 µl (from both times). The concentration and purity of the fragment were checked using 1 µl of the sample and known amounts of plasmid pBR322 on a 1.0% minigel. Samples were stored at 4°C. This method proved to be a very effective way of retrieving 80-90% of the DNA fragment from the agarose gel. This method allowed specific nucleic acid fragments from gels to be radioactively labelled for use as probes.

XI. Nucleic Acid Blotting

A. Southern blotting

DNA was transferred from agarose gels to nylon membranes (Nytran, Schleicher & Schuell) essentially as described by Southern, (1975). Following agarose gel electrophoresis, EtBr staining, visualization and photography, the DNA was denatured in 500 ml solution containing 0.5M NaOH, 1.5M NaCl for 1 hr with gentle agitation at RT. After 2-3 distilled water rinses, the gel was neutralized in 500 ml 0.5M Tris-Cl (pH 7.0), 3M NaCl for another hr at RT. After rinsing the gel 2-3x with distilled water, the gel was inverted and laid onto a soaked 3MM paper on a platform elevated in a tank containing 2 liters of 10X SSC. Air bubbles were removed between the gel and the 3MM paper by gently rolling and pressing a pipet on top of the gel. The excess gel was trimmed and a previously cut gel-sized piece of nylon membrane saturated with water was placed directly on top of the gel. A small piece of the right

corner of the membrane was cut for orientation and again the air bubbles were removed between the membrane and the gel. Three pieces of 3MM paper, paper towels and a textbook (that served as a weight) were added in order. The transfer was allowed to proceed for 18 hrs. The membrane with the bound DNA was baked for 90 min in a vacuum oven at 80°C and subjected to prehybridization and then, hybridization conditions. The membrane was placed in a heat-sealed plastic bag and prehybridized for 3-4 hrs at 42°C in a solution containing 50% formamide and 5X SSPE, 0.2% SDS, 2X Denhardt's and 100 µg/ml sheared salmon sperm DNA (Southern, 1975). After prehybridization, the radiolabelled probe was added, the bag was resealed and hybridization was carried out in a shaking water bath for 12-18 hrs at 42°C.

Following hybridization, the membrane was washed twice for 15 min in 500 ml 2X SSC, 0.5% SDS at RT, twice for 15 min in 500 ml 1X SSC at 37°C and once in 500 ml 0.1X SSC, 0.5% SDS for 30-45 min at 65°C in a shaking water bath with gentle agitation. The membrane was air dried completely, wrapped in plastic wrap and autoradiographed using XAR-5 film (Kodak) with an intensifying screen (Cronex Lightning Plus, Dupont) at -70°C. The X-ray films were exposed to the filters for appropriate times. They were developed for 1-5 min in Kodak GBX developer, immersed 1 min in a solution of 3.0% acetic acid to arrest development and then fixed in rapid fixer (Kodak) for 10 min. The films were rinsed in cold water for 15 min and air dried.

B. Northern blotting

RNA was transferred from formaldehyde gels (discussed previously) to Nytran nylon membrane in essentially the same way as the DNA gels. Following electrophoresis, one lane was cut from the gel and stained with 0.5 μg/ml EtBr for 15-20 min, while the rest of the gel was rinsed 2-3x with distilled water and denatured in 500 ml 50mM NaOH for 20 min with gentle agitation at RT. Following 2-3 distilled water rinses, the gel was immersed in 500 ml 10X SSC for 45 min with gentle agitation at RT. The gel was then transferred in the same exact way as previously discussed. The prehybridization, hybridization and washing conditions used were identical to those for Southern blots.

XII. Radioactive Labelling of DNA Probes

A. Random primer extension

This method was accomplished using a labelling kit and accompanying instructions (BMB) based upon the method of Feinberg and Vogelstein, (1984). To be successfully labelled, the DNA must be linear. Therefore, all circular DNAs were linearized using restriction enzyme digestion. In a single labelling reaction, 50-100 ng DNA was denatured by boiling for 10 min, then placed immediately on ice to prevent renaturation. The reaction components, dTTP, dATP, dGTP (25uM each), 50uCi (100 pmoles) ^{32}P-dCTP (3,000 Ci/mmole, NEN), 2 μl hexanucleotide reaction buffer (10X), 1 μl (5 units) DNA polymerase I (Klenow fragment) and distilled water were added to the denatured DNA to 20 μl final volume. The components were mixed and

incubated for 30-45 min at 37°C. Unincorporated nucleotides were removed by passing the mixture through a Quick Spin column containing Sephadex G-50 (BMB). The counts were determined by counting 1 µl of the labelled probe in 10 ml Ecoscint using the preset ^{32}P channel of the scintillation counter. The labelled probe was denatured by boiling for 10 min, cooled on ice (see above) and added to the hybridization solution. The specific activity (SA) of the probe was determined by multiplying the number of counts by the total volume of the probe, divided by the number of ng used.

B. Nick translation

Nick translation was achieved using a method outlined in Maniatis, *et al.*, (1982) and a labelling kit from BMB. Between 50-100 ng DNA was mixed with dTTP, dATP, dGTP (20uM each), 50uCi (100 pmoles) ^{32}P-dCTP (3,000 Ci/mmole, NEN), 2 µl 10X buffer, 2 µl DNA polymerase I and DNase I (in 50% v/v glycerol) and distilled water for a 20 µl final volume. The mixture was incubated for 45 min at 15°C. The unincorporated nucleotides were removed and the specific activity determined as previously discussed.

RESULTS

The results are divided into three parts. The first part describes the isolation of cloned rDNA from a cosmid library and their subsequent characterization. The second part describes the molecular analyses of the transfected cloned cells generated following the transfection of cloned rDNA into human fibroblasts. Finally, the third part describes the molecular analyses of nucleolar DNA of the transfected cells.

PART ONE: ISOLATION OF HUMAN rDNA FROM A COSMID LIBRARY

This study was initiated by isolating recombinant cosmid DNA containing rDNA from a human genomic cosmid library, kindly provided by Dr. Harvey Ozer. Subsequently, the isolated DNA was used to transfect human fibroblasts in order to determine which rDNA sequences are responsible for nucleolar organization and resulting satellite associations. The library was constructed so that the vectors contained between 25-45 kb human genomic DNA. The DNA was inserted at a *Bam*HI site flanked by two *Eco*RI sites, which allowed excision of the insert. The recombinant cosmid was designated pCV108 (Figure 4A and B) (Lau and Kan, 1983).

DNA of two human rDNA regions were used as probes for screening the library. These were the *Eco*RI A region, which includes two ITS regions, the 5.8S rDNA and 28S rDNA up to the last 500 bp and the *Eco*RI B region, which includes the transcription initiation region (encompasses promotor) and the 18S rRNA gene up to the last 200 bp, kindly provided by Dr. David Schlessinger (Figure 1). Ten colonies, from 30,000 colonies screened, were positive for rDNA content, as defined by hybridization

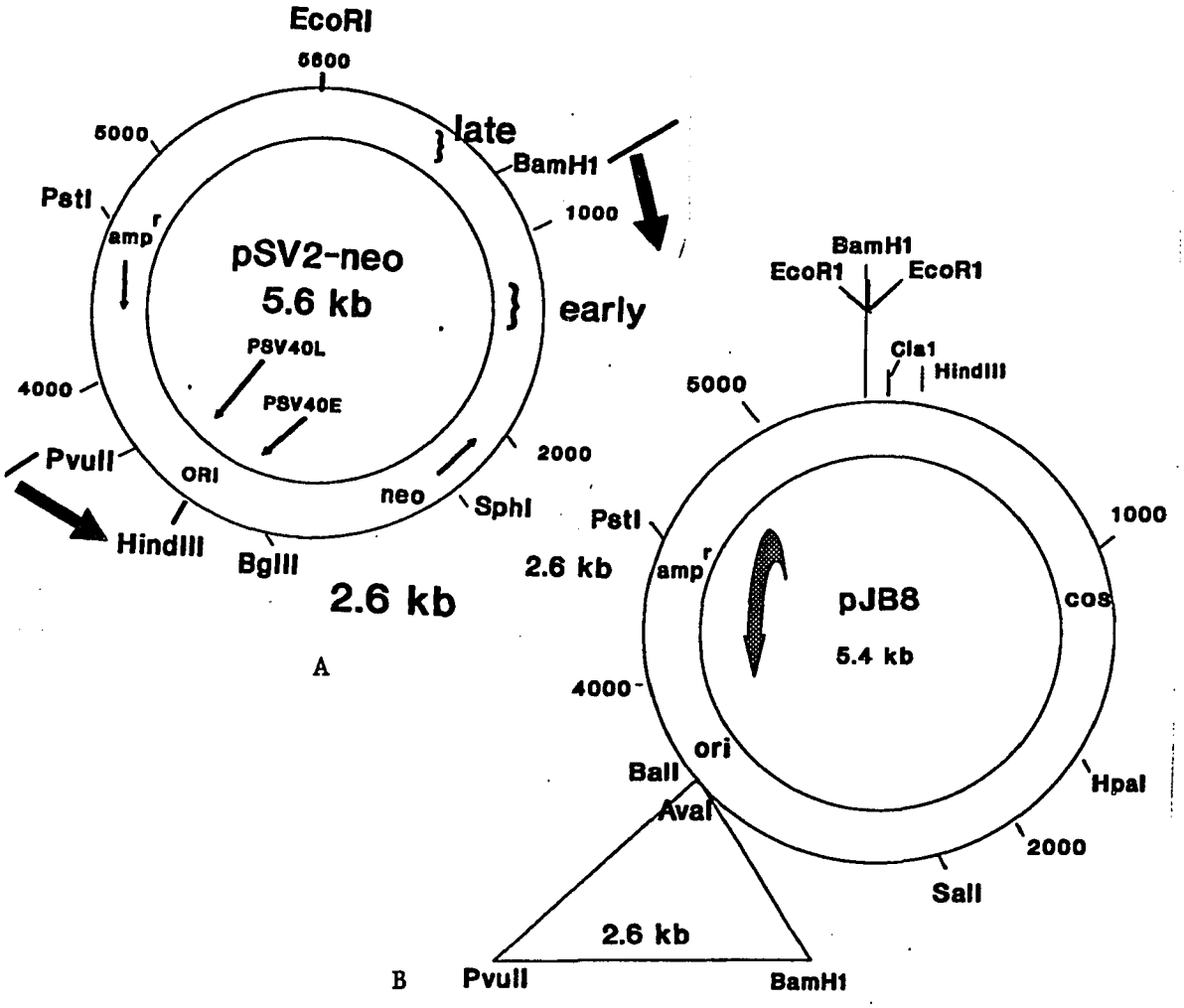


Figure 4. Schematic map of plasmid pSV2-neo and cosmid pJB8.

A. Restriction endonuclease map of plasmid pSV2-neo showing the 2.6 kb *Pvu*II-*Bam*HI region containing the SV40 regulatory signals (promotor, splice signals and polyadenylation signal) from the early region, the SV40 late promotor, including the tandemly repeated 72 bp segments and the selectable marker, the neo gene. The *Pvu*II-*Hind*III fragment of SV40 also contains the origin of replication (ori). The entire 2.6 kb region was inserted into cosmid pJB8 (see B). The resulting recombinant cosmid is designated pCV108.

B. Restriction endonuclease map of the 5.4 kb cosmid pJB8 showing the insertion of the 2.6 kb region from the pSV2-neo into the *Bal*I site making the overall size of the cosmid vector 8.0 kb. Note *Bam*HI site (where the genomic DNA was inserted) flanked by the two *Eco*RI sites, which allow excision of insert.

with both probes. These positive colonies were analyzed further.

Positive recombinant DNAs were designated as ML-1, ML-2,...ML-10. The DNAs were digested with *Eco*RI and analyzed on 0.8% gels. Four clones, ML-1, ML-3, ML-4 and ML-6, were selected for a large scale cosmid DNA isolation on the basis of analyses of insert sizes, restriction patterns and hybridization signals with *Eco*RI A and B rDNA as probes.

I. Overview of analyses of cloned ML-1, ML-3, ML-4 and ML-6 rDNA

The identity, size, orientation and site of insertion of cloned rDNA regions within the cosmid vector were determined for each cloned DNA using electrophoretic and hybridization analyses. On the basis of the analyses below, ML-1 and ML-4 DNA was selected for transfection because: a) ML-1 DNA contains the largest intact rDNA insert, including the intact transcribed spacer with the origin of replication, promotor, rRNA genes and termination sites and b) ML-4 DNA lacks the promotor and thus serves as a control in functional analyses. The means of selection is based on analyses of ML-1, ML-3, ML-4 and ML-6 DNA.

A diagrammatic representation of ML-1 DNA is given in Figure 5. ML-1 DNA contains: (a) the intact *Eco*RI A and B rDNA regions including the transcription initiation region and the three rRNA genes; (b) 14 kb of the D region, including the transcription termination sites, but lacking the internal *Alu* repeats and (c) 4.2 kb of the C region including a region known to contain *Alu* repeats. The rDNA regions were inserted in the correct 5'-3' orientation.

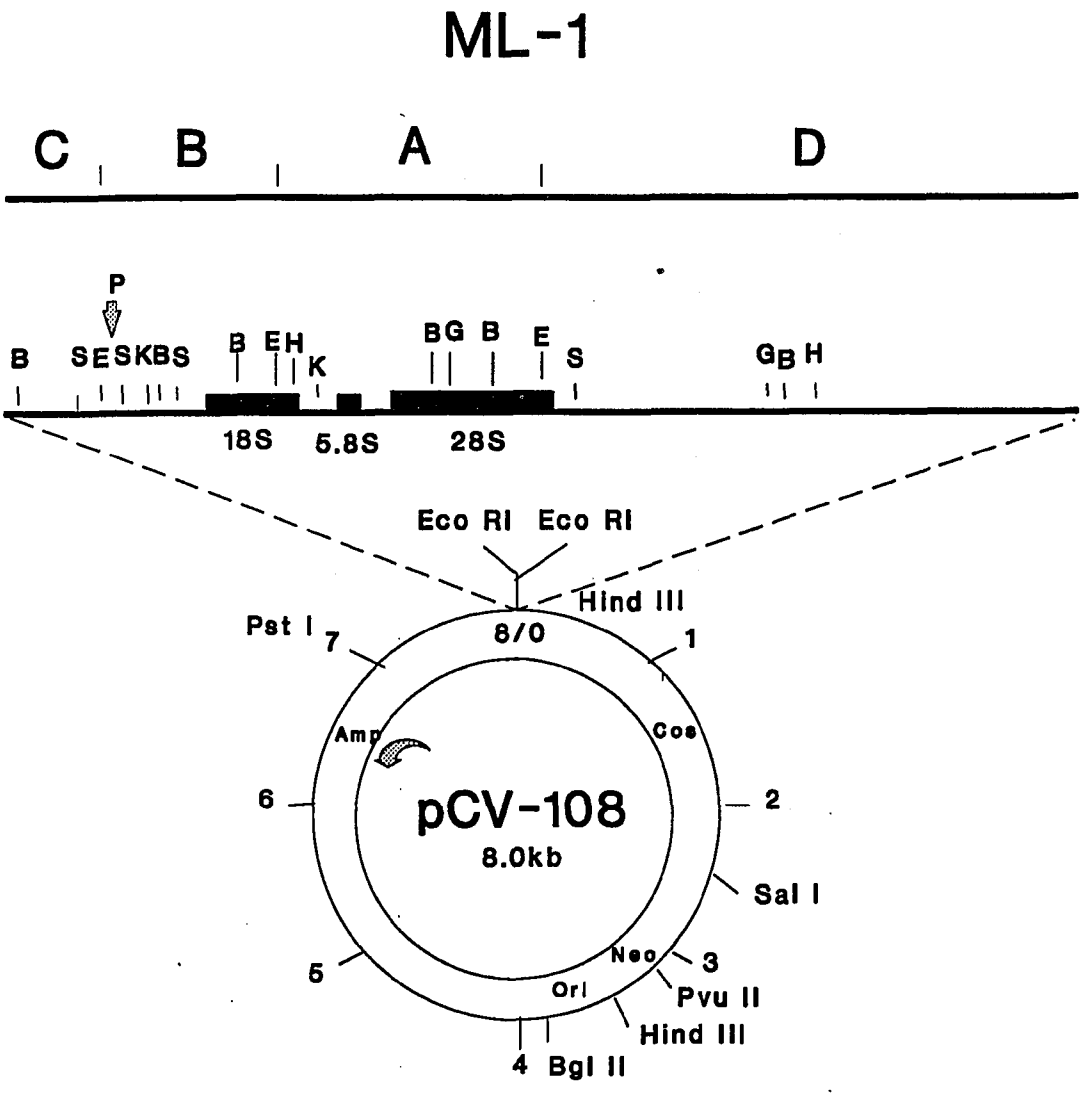


Figure 5. Schematic map of rDNA-containing cosmid, ML-1.

Schematic map of ML-1 DNA showing the cosmid vector, pCV108, with its rDNA insert. The four rDNA regions (A, B, C and D) as defined by *Eco*RI restriction sites, are shown on top. The arrow indicates the start site of rDNA transcription (P=promotor) which continues through the 18S, 5.8S and 28S sequences, shown shaded. These cloned rDNA regions are found in the correct 5'-3' orientation. Restriction endonuclease sites within the rDNA insert appear as *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Hind*III (H), *Kpn*I (K) and *Sal*I (S).

A diagram of ML-3 DNA is given on Figure 6. ML-3 DNA contains intact A and B regions and parts of the DNA of the C and D regions. There are some changes in C and D DNA relative to restriction sites previously determined. For example, there are additional *Eco*RI (3) and *Hind*III (3) sites. The presence of additional sites is probably caused by deletions, duplications, or rearrangements of DNA sequences during cloning. This has been previously observed following the cloning of human rDNA in lambda vectors (Erickson and Schmickel, 1985). The expected *Alu* repeats are present in the C, but are absent from the D region. The inserted rDNA regions of ML-3 DNA are found in an inverted 3'-5' orientation.

A diagram of ML-4 DNA is given in Figure 7. ML-4 DNA contains all of A and 4.2 kb of the 6.0 kb B region. The 1.8 kb DNA missing from the B region includes the transcription initiation region (origin of replication and promotor). DNA sequences flanking the promotor, normally part of the C region (including the *Alu* repeats), are missing. Other portions of the C and D DNA are not intact and there are some changes from the expected sequence. There are additional *Eco*RI (1), *Hind*III (3) and *Kpn*I (2) sites within the C and D regions at different locations as those seen in ML-3 DNA. The inserted rDNA is in an inverted 3'-5' orientation.

A diagram of ML-6 DNA is given in Figure 8. The cosmid vector contains the intact A and B rDNA regions. It also includes 7.1 kb of the C region, with *Alu* repeats and 8.3 kb of the rDNA D region. The *Alu* repeats in the D region are deleted. There are no additional or deleted restriction sites in the rDNA regions, but the orientation

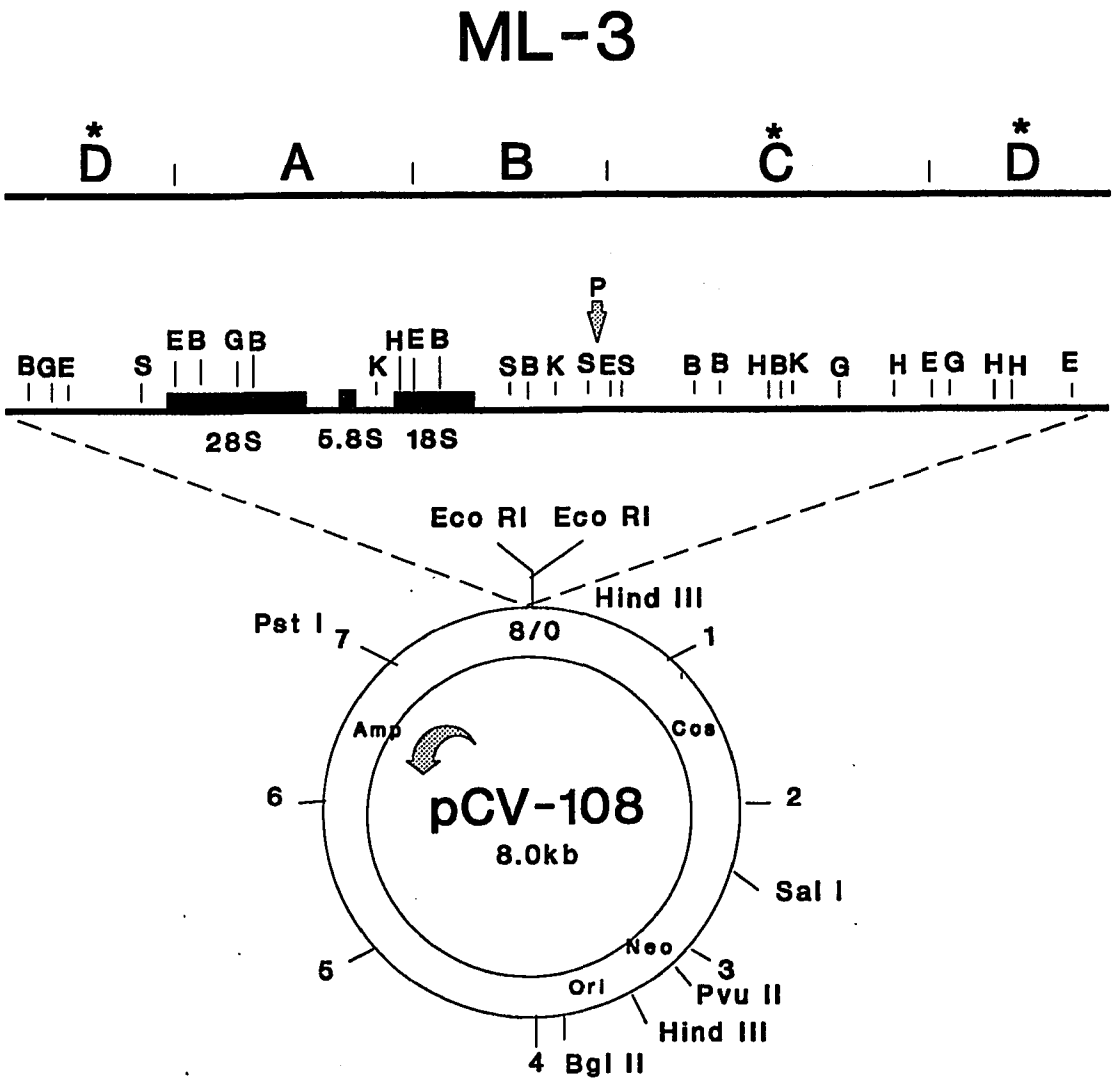


Figure 6. Schematic map of rDNA-containing cosmid, ML-3.

Schematic map of ML-3 DNA showing the cosmid vector, pCV108, with its rDNA insert. The four rDNA regions (A, B, C and D) as defined by *Eco*RI restriction sites, are shown on top. DNA sequence discrepancies in the *Eco*RI C and D regions are indicated with an *. The arrow indicates the start site of rDNA transcription (P=promotor) which continues through the 18S, 5.8S and 28S sequences, shown shaded. These cloned rDNA regions are found in an inverted 3'-5' orientation. Restriction endonuclease sites within the rDNA insert appear as *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Hind*III (H), *Kpn*I (K) and *Sal*I (S).

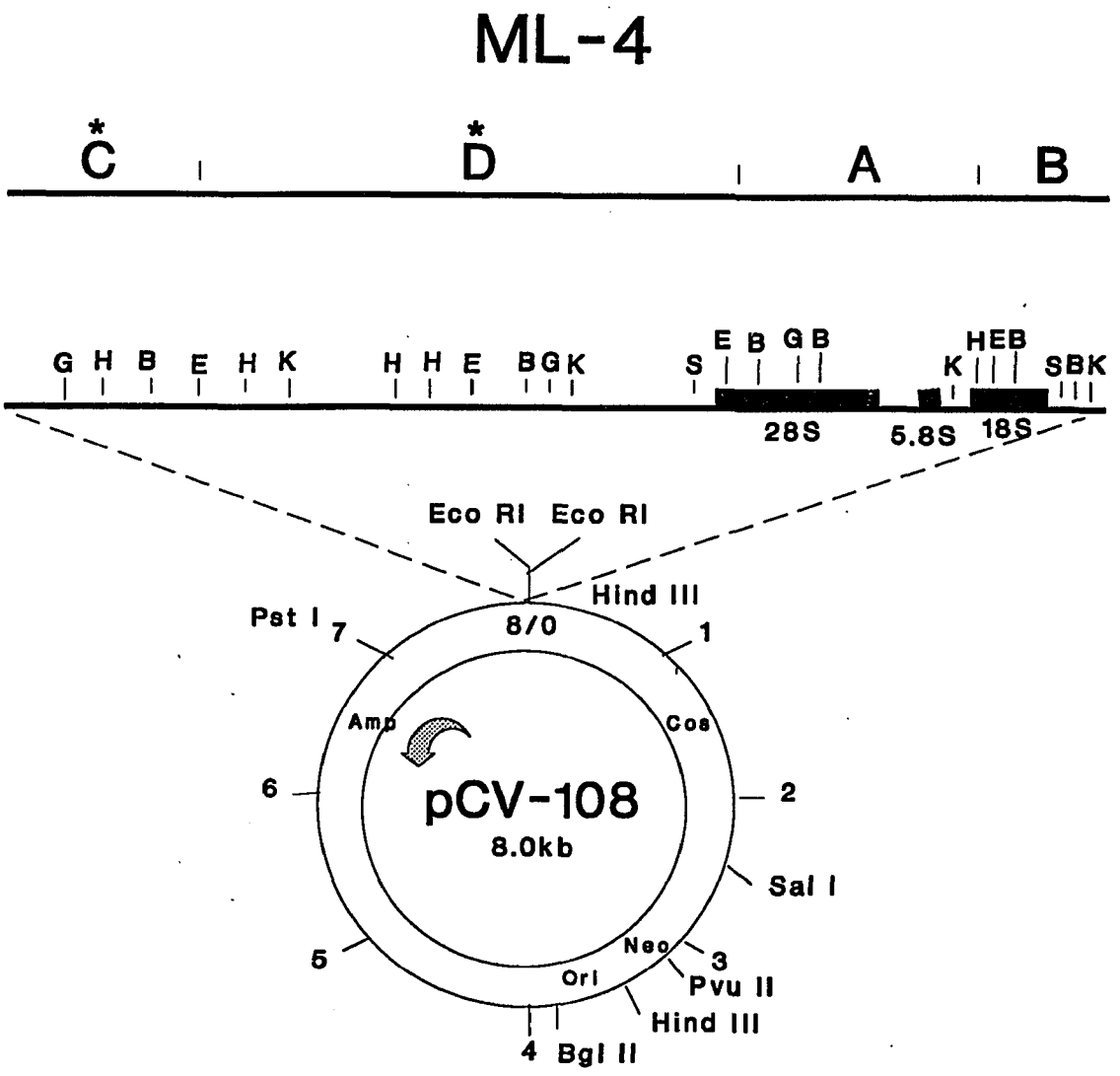


Figure 7. Schematic map of rDNA-containing cosmid, ML-4.

Schematic map of ML-4 DNA showing the cosmid vector, pCV108, with its rDNA insert. The four rDNA regions (A, B, C and D) as defined by *Eco*RI restriction sites, are shown on top. DNA sequence discrepancies in the *Eco*RI C and D regions are indicated with an *. The start site of rDNA transcription which includes the promotor is absent. These cloned rDNA regions are found in an inverted 3'-5' orientation. Restriction endonuclease sites within the rDNA insert appear as *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Hind*III (H), *Kpn*I (K) and *Sal*I (S).

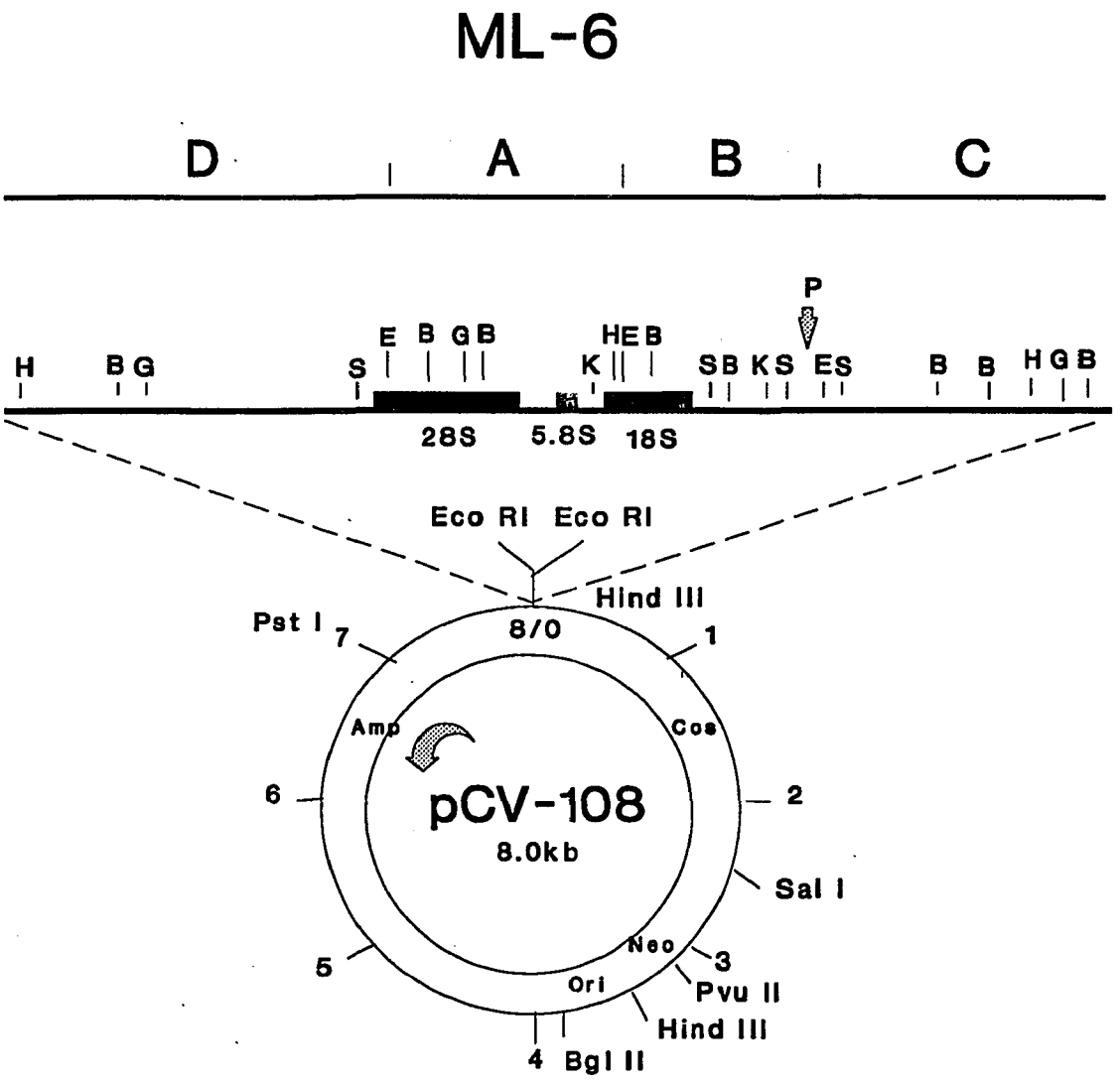


Figure 8. Schematic map of rDNA-containing cosmid, ML-6.

Schematic map of ML-6 DNA showing the cosmid vector, pCV108, with its rDNA insert. The four rDNA regions (A, B, C and D) as defined by *Eco*RI restriction sites, are shown on top. The arrow indicates the start site of rDNA transcription (P=promotor) which continues through the 18S, 5.8S and 28S sequences, shown shaded. These cloned rDNA regions are found in an inverted 3'-5' orientation. Restriction endonuclease sites within the rDNA insert appear as *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Hind*III (H), *Kpn*I (K) and *Sal*I (S).

within the vector is inverted.

II. Electrophoretic determination of insert size of rDNA-containing cosmid clones, ML-1, ML-3, ML-4 and ML-6 DNA

The insert size was used as one criteria to determine which of the four DNAs would be used for transfection. Restriction analysis was used to determine insert characteristics and size of each recombinant DNA. Each DNA was digested with restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I and *Sal*I. Restriction endonucleases were chosen on the basis of their known sites in the 44 kb human rDNA repeat unit (Arnheim and Southern, 1977, Wellauer and Dawid, 1979, Erickson *et al.*, 1981, Wilson *et al.*, 1982, Sylvester *et al.*, 1986, Maden *et al.*, 1987) and vector DNA (see Figure 4). A map of the rDNA repeat unit with restriction sites and fragment sizes is shown in Figure 9.

The length of each band was calculated using a computer program called Nucaln. ML-3 DNA contains the largest rDNA insert (45 kb); ML-4 rDNA insert is 36 kb, ML-1 rDNA insert is 32 kb and ML-6 rDNA insert is 29 kb. Restriction patterns of each cloned DNA on a 0.8% agarose gel are shown in Figure 10. *Eco*RI and *Hind*III digestion of each DNA generated restriction fragments that were comprised only of vector DNA. *Eco*RI digestion of each DNA excised the insert and resulted in an 8.0 kb fragment consisting entirely of vector DNA. This 8.0 kb fragment was obtained with each DNA (lanes 10-13). Other fragments represented inserted rDNA.

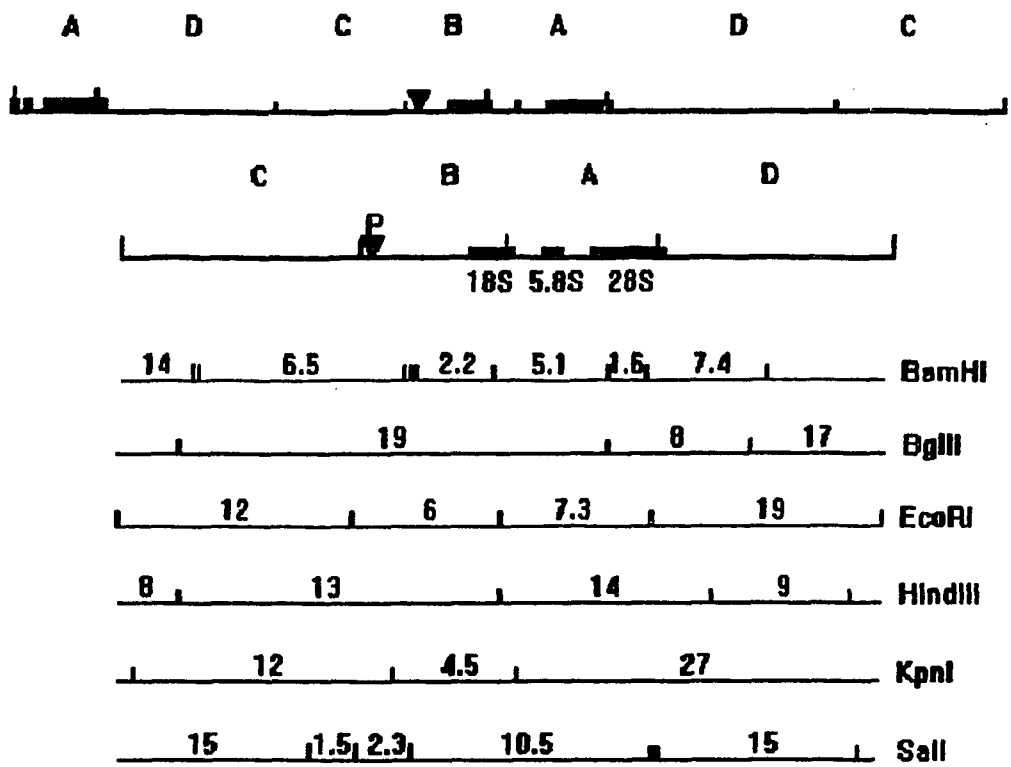
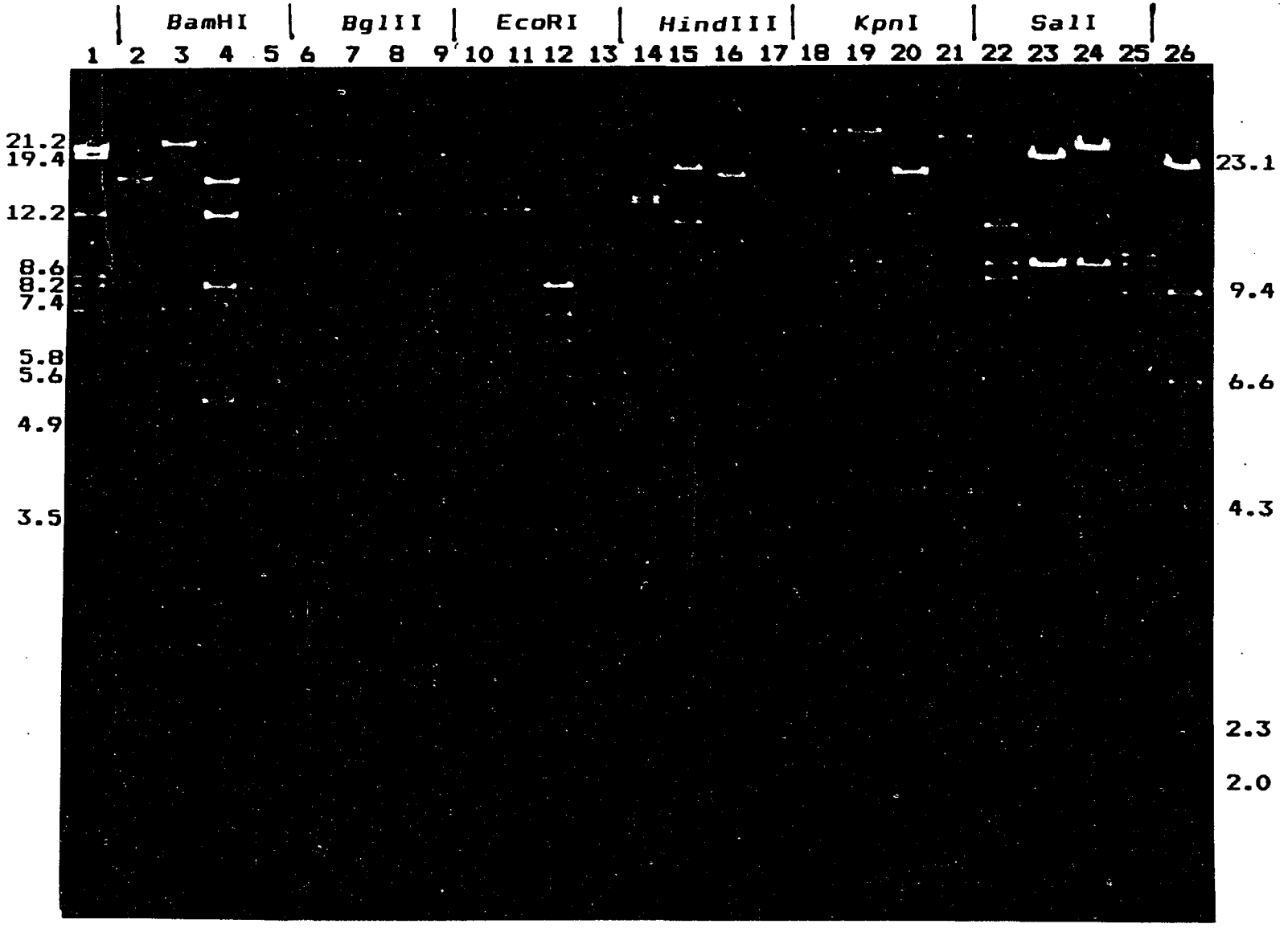


Figure 9. Restriction endonuclease map of the human rDNA repeat unit.

Restriction endonuclease map of a single human rDNA repeat unit, as defined by fragments produced by restriction endonuclease *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I and *Sal*I digestion.

Figure 10. Electrophoretic analysis of ML-1, ML-3, ML-4 and ML-6 DNA.

Size determination of recombinant ML-1, ML-3, ML-4 and ML-6 DNA using 0.8% agarose gel electrophoresis. Lane 1, 250 ng lambda DNA digested (separately) with *Eco*RI and *Sma*I, lanes 2-5, 250 ng ML-1, ML-3, ML-4 and ML-6 DNA digested with enzyme, *Bam*HI; lanes 6-9, 250 ng ML-1, ML-3, ML-4 and ML-6 DNA digested with *Bgl*II; lanes 10-13, 250 ng ML-1, ML-3, ML-4 and ML-6 DNA digested with *Eco*RI; lanes 14-17, 250 ng ML-1, ML-3, ML-4 and ML-6 DNA digested with *Hind*III; lanes 18-21, 250 ng ML-1, ML-3, ML-4 and ML-6 DNA digested with *Kpn*I; lanes 22-25, 250 ng ML-1, ML-3, ML-4 and ML-6 DNA digested with *Sal*I and lane 26, 250 ng lambda DNA digested with *Hind*III.


BamHI
BglII
EcoRI
HindIII
KpnI
SalI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
21.2
19.4
12.2
8.6
8.2
7.4
5.8
5.6
4.9
3.5
23.1
9.4
6.6
4.3
2.3
2.0

There are two *Hind*III sites within the vector. *Hind*III digestion generated two fragments as expected, one at 3.5 kb and the other, greater than 4.5 kb in all four DNAs (lanes 14-17). The 3.5 kb fragment was composed entirely of vector DNA. The other fragment contained the remaining 4.5 kb vector DNA and a variable amount of insert DNA extending to the next *Hind*III site in the insert (see Figure 4B). None of the fragments generated following *Bam*HI, *Bgl*II, *Kpn*I and *Sal*I digestions of ML-1, ML-3, ML-4 and ML-6 DNA were comprised entirely of vector DNA, as expected (Figure 9, lanes 1-4, 5-8, 17-20 and 21-24). The restriction pattern and size of insert fragments generated by *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I and *Sal*I digestion for ML-1 and ML-6 DNA are in agreement with previous studies describing cloned rDNA fragments.

The size and pattern of restriction fragments in ML-3 DNA corresponded to the *Eco*RI A and B rDNA regions. The expected restriction fragment size in ML-4 DNA was obtained for the A region, but not the B region. Instead, a smaller 4.2 kb region of B was obtained. There were additional *Eco*RI and *Hind*III sites in the C and D regions of ML-3 and ML-4 DNA, generating more restriction fragments than expected. For example, *Eco*RI digestion of ML-3 and ML-4 DNA should have produced four rDNA fragments and the vector DNA. Instead, ML-3 DNA and ML-4 DNA generated seven and five fragments, respectively (lane 10 and 11). *Kpn*I digestion of ML-4 DNA also revealed the presence of an extra *Kpn*I site in the C region. These discrepancies in fragment number were clarified in the hybridization analyses (section III).

The above analyses provided basic information on the size and characteristics of each of the four DNAs, but it was not sufficient for the selection of DNA to be used in transfections. This information was used in conjunction with results obtained from hybridization analyses in determining selection of the two DNAs.

III. Identification of rDNA regions in ML-1, ML-3, ML-4 and ML-6 DNA by hybridization

A. Northern blot hybridization

Northern analysis of ML-1, ML-3, ML-4 and ML-6 DNA was carried out to confirm the presence of rDNA genes within the inserts. Figure 11 shows that each cloned DNA has homology to the mature 5.8S, 18S, 28S rRNA genes, as well as to the 45S and some intermediates of rRNA gene processing.

B. Southern blot hybridization

1. Analysis using pBR322 DNA as a probe

Southern hybridization was carried out to further identify characteristics of the four cloned DNAs. Restriction fragments corresponding to vector DNA were identified using gels containing *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I and *Sal*I digested ML-1, ML-3, ML-4 and ML-6 DNA with hybridization against pBR322 DNA as the probe (Figure 12). The data is summarized in Tables I-VI. *Eco*RI digestion generated the expected 8.0 kb fragment in each DNA indicating the presence of the entire vector DNA (lanes 9-12, Table I).

*Hind*III digestion of ML-1, ML-3, ML-4 and ML-6 DNA generated two fragments

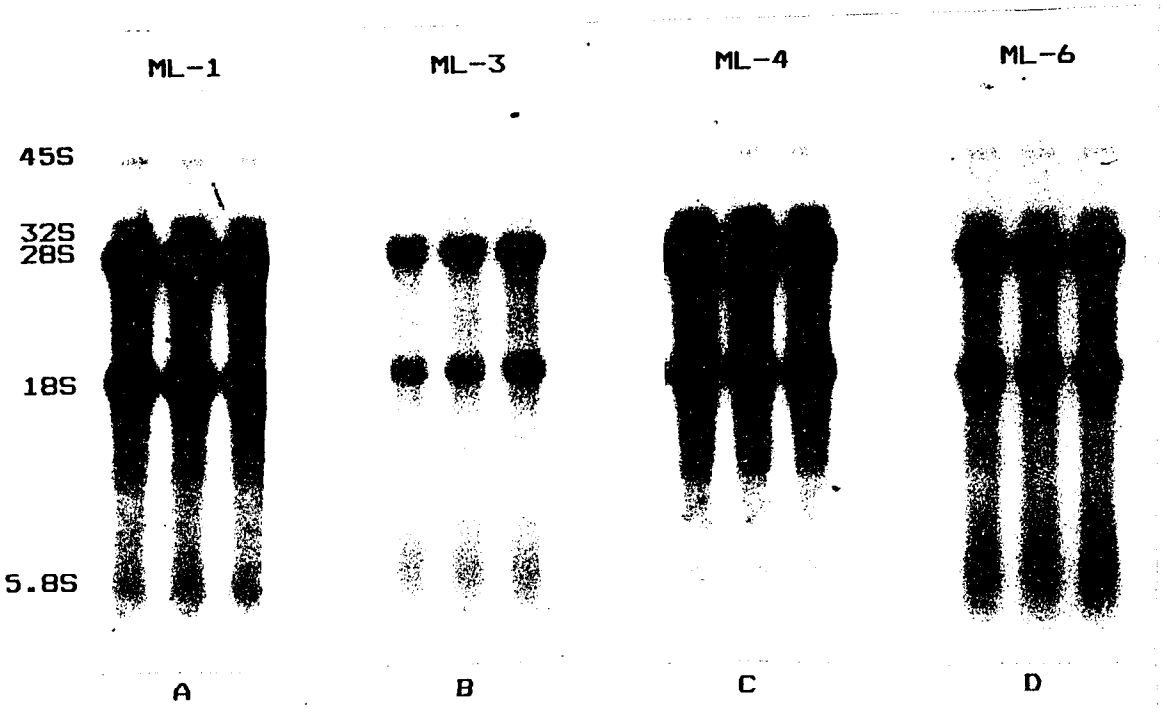


Figure 11. Northern analysis of cosmid clones, ML-1, ML-3, ML-4 and ML-6.

Total human RNA was hybridized with 100 ng of ^{32}P-labelled ML-1 [Specific Activity (SA)=4.8 x 10^8 cpm/ug], ML-3 (SA=7.4 x 10^8 cpm/ug), ML-4 (SA=4.8 x 10^8) and ML-6 (SA=9.6 x 10^8) DNA. The Northern blot used a 1.0% agarose-formaldehyde gel with each lane containing 5.0 ug of total human RNA. Three lanes were used for each hybridization conducted at 42°C as described in Materials and Methods. The autoradiograms were exposed for 16 hrs for ML-1 and ML-4 DNA (without an intensifying screen) and for 4 hrs for ML-3 and ML-6 DNA at -70°C with intensifying screens.

A. Northern blot of ML-1 DNA.
B. Northern blot of ML-3 DNA.
C. Northern blot of ML-4 DNA.
D. Northern blot of ML-6 DNA.

Figure 12. Southern analysis of ML-1, ML-3, ML-4 and ML-6 DNA with ^{32}P-pBR322 DNA.

*Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I and *Sal*I generated restriction fragments of cosmid clones ML-1, ML-3, ML-4 and ML-6 that hybridized to 100 ng ^{32}P-labelled pBR322 DNA. 250 ng DNA for each sample was digested with each enzyme and fractionated on a 0.8% agarose gel (see Figure 10). Lanes 1-4: *Bam*HI digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 5-8: *Bgl*II digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 9-12: *Eco*RI digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 13-16: *Hind*III digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 17-20: *Kpn*I digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 21-24: *Sal*I digested ML-1, ML-3, ML-4 and ML-6 DNA. *Eco*RI, *Sma*I and *Hind*III digested lambda DNA fragments were used for size markers. Hybridization and washings were conducted as described in Materials and Methods. The specific activity of the probe was 5.7 x 10^8 cpm/ug. The autoradiogram was exposed for 1 hours at -70°C with an intensifying screen.

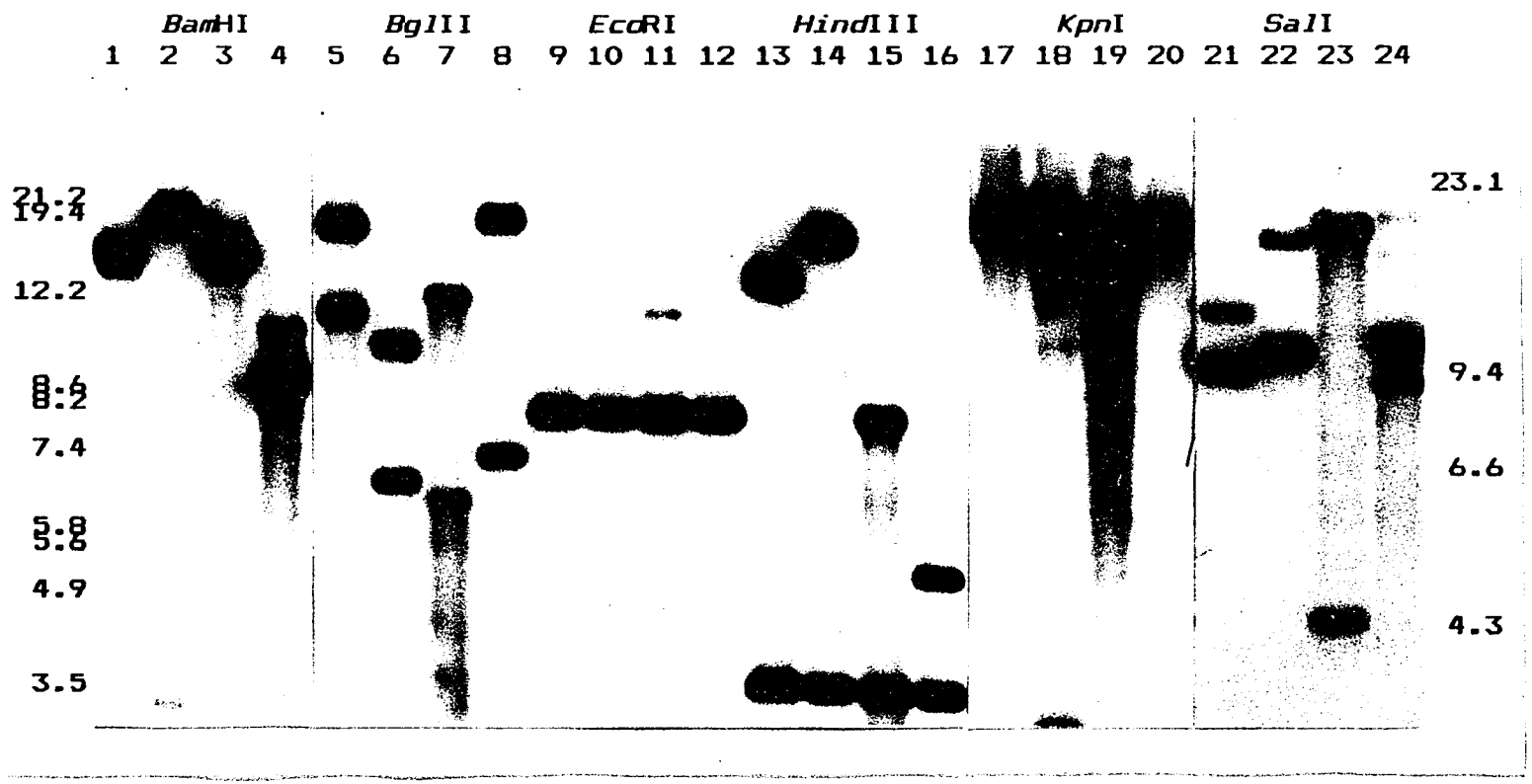

BamHI
BglII
EcoRI
HindIII
KpnI
SalI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
21.2
19.4
12.2
8.6
8.2
7.4
5.8
5.6
4.9
3.5
23.1
9.4
6.6
4.3

Table I

Comparison of *Eco*RI generated restriction fragments between ML-1, ML-3, ML-4 and ML-6 DNA and their hybridization patterns.

DNA CLONE	ENZ.	EXP. FRGM. rDNA Only	OBT. FRGM. Incl. Cosmid	PROBE pBR322	PROBE EcoRI A	PROBE EcoRI B	PROBE H2E4	PROBE Blur-8
ML-1	EcoRI	19.0 kb	14 .0 kb	-	-	-	+	-
		12.0 kb	8.0 kb	+	-	-	-	+
		7.3 kb	7.3 kb	-	+	-	-	-
		6.0 kb	6.0 kb	-	-	+	-	-
			4.2 kb	-	-	-	+	+
ML-3	EcoRI	Same as	14.0 kb	-	-	-	+	+
		above	8.0 kb	+	-	-	-	+
			7.8 kb	-	-	-	+	+
			7.3 kb	-	+	-	-	-
			6.0 kb	-	-	+	-	-
			5.2 kb	-	-	-	-	-
			3.1 kb	-	-	-	-	-
			1.5 kb	-	-	-	-	-
ML-4	EcoRI	Same as	9.2 kb	-	-	-	+	-
		above	9.2 kb	-	-	-	-	-
			8.0 kb	+	-	-	-	+
			7.3 kb	-	+	-	-	-
			6.3 kb	-	-	-	-	-
			4.2 kb	-	-	+	-	-
ML-6	EcoRI	Same as	8.3 kb	-	-	-	+	-
		above	8.0 kb	+	-	-	-	+
			7.3 kb	-	+	-	-	-
			7.1 kb	-	-	-	+	+
			6.0 kb	-	-	+	-	-

Table II

Comparison of *Hind*III generated restriction fragments between ML-1, ML-3, ML-4 and ML-6 DNA and their hybridization patterns.

DNA CLONE	ENZYME	EXP. FRGM. rDNA Only	OBT. FRGM. Incl. Cosmid	PROBE pBR322	PROBE EcoRI A	PROBE EcoRI B
ML-1	HindIII	14.0 kb	14.7 kb	-	+	-
		13.0 kb	14.7 kb	+	-	+
		9.0 kb	5.5 kb	-	-	-
		8.0 kb	3.5 kb	+	-	-
ML-3	HindIII	Same as	18.8 kb	+	+	-
		above	13.0 kb	-	-	+
			5.5 kb	-	-	-
			3.7 kb	-	-	-
			3.5 kb	+	-	-
			1.4 kb	-	-	-
ML-4	HindIII	Same as	17.5 kb	-	+	-
		above	7.9 kb	+	-	-
			5.4 kb	-	-	-
			4.3 kb	-	-	+
			4.1 kb	-	-	-
			3.5 kb	+	-	-
			1.5 kb	-	-	-
ML-6	HindIII	Same as	14.5 kb	-	+	-
		above	13.0 kb	-	-	+
			4.7 kb	+	-	-
			3.5 kb	+	-	-

Table III

Comparison of *Bgl*II generated restriction fragments between ML-1, ML-3, ML-4 and ML-6 DNA and their hybridization patterns.

DNA CLONE	ENZYME	EXP. FRGM. rDNA Only	OBT. FRGM. Incl. Cosmid	PROBE pBR322	PROBE EcoRI A	PROBE EcoRI B
ML-1	BglII	19.0 kb	20.4 kb	+	+	+
		17.0 kb	12.4 kb	+	-	-
		8.0 kb	8.0 kb	-	+	-
ML-3	BglII	same as	20.3 kb	-	+	+
		above	9.2 kb	+	-	-
			8.0 kb	-	+	-
			6.4 kb	+	-	-
			4.5 kb	-	-	-
ML-4	BglII	same as	14.0 kb	+	+	+
		above	8.7 kb	-	+	-
			6.6 kb	-	-	-
			6.1 kb	+	-	-
			3.6 kb	-	-	-
			2.6 kb	-	-	-
			2.3 kb	-	-	-
			2.1 kb	-	-	-
ML-6	BglII	same as	22.1 kb	+	+	+
		above	8.0 kb	-	+	-
			7.1 kb	+	-	-

Table IV

Comparison of *SalI* generated restriction fragments between ML-1, ML-3, ML-4 and ML-6 DNA and their hybridization patterns.

DNA CLONE	ENZYME	EXP. FRGM. rDNA Only	OBT. FRGM. Incl. Cosmid	PROBE pBR322	PROBE EcoRI A	PROBE EcoRI B
ML-1	SalI	15.0 kb	14.0 kb	+	-	-
		15.0 kb	10.5 kb	-	+	+
		10.5 kb	10.0 kb	+	-	-
		2.3 kb	2.3 kb	-	-	+
		1.5 kb	1.5 kb	-	-	+
ML-3	SalI	Same as	23.7 kb	+	-	-
		above	10.5 kb	+	-	-
			10.5 kb	-	+	+
			2.3 kb	-	-	+
			1.5 kb	-	-	+
ML-4	SalI	Same as	26.8 kb	+	-	-
		above	10.5 kb	-	+	+
			4.2 kb	+	-	+
			0.7 kb	-	-	-
ML-6	SalI	Same as	11.6 kb	+	-	-
		above	10.5 kb	-	+	+
			9.5 kb	+	-	-
			2.3 kb	-	-	+
			1.5 kb	-	-	+

Table V

Comparison of *Bam*HI generated restriction fragments between ML-1, ML-3, ML-4 and ML-6 DNA and their hybridization patterns.

DNA CLONE	ENZYME	EXP. FRGM. rDNA Only	OBT. FRGM. Incl. Cosmid	PROBE pBR322	PROBE EcoRI A	PROBE EcoRI B
ML-1	BamHI	14.0 kb	16.5 kb	+	-	-
		7.4 kb	7.4 kb	-	+	-
		6.5 kb	6.5 kb	-	-	+
		5.1 kb	5.1 kb	-	+	+
		2.2 kb	2.2 kb	-	-	+
		1.6 kb	1.6 kb	-	+	-
		Few < 1.0 kb				
ML-3	BamHI	Same as	22.0 kb	+	-	-
		above	7.4 kb	-	+	-
			6.5 kb	-	-	+
			5.1 kb	-	+	+
			2.2 kb	-	-	+
			2.1 kb	-	-	-
			1.6 kb	-	+	-
			1.3 kb	-	-	-
ML-4	BamHI	Same as	16.0 kb	+	-	+
		above	12.4 kb	-	-	-
			8.2 kb	-	+	-
			5.1 kb	-	+	+
			2.2 kb	-	-	+
			1.6 kb	-	+	-
ML-6	BamHI	Same as	8.1 kb	+	-	-
		above	7.4 kb	-	+	-
			6.3 kb	-	-	+
			5.1 kb	-	+	+
			2.4 kb	-	-	-
			2.2 kb	-	-	+
			1.6 kb	-	+	-
			1.3 kb	-	-	-

Table VI

Comparison of *Kpn*I generated restriction fragments between ML-1, ML-3, ML-4 and ML-6 DNA and their hybridization patterns.

DNA CLONE	ENZYME	EXP. FRGM. rDNA Only	OBT. FRGM. Incl. Cosmid	PROBE pBR322	PROBE EcoRI A	PROBE EcoRI B
ML-1	KpnI	27.0 kb	30.6 kb	+	+	+
		12.0 kb	4.5 kb	-	+	+
		4.5 kb				
ML-3	KpnI	Same as	30.6 kb	+	+	-
		above	12.0 kb	-	-	+
			4.5 kb	-	+	+
ML-4	KpnI	Same as	19.2 kb	+	+	-
		above	12.0 kb	-	-	-
			10.5 kb	-	-	-
			4.5 kb	-	+	+
ML-6	KpnI	Same as	29.6 kb	+	+	+
		above	4.5 kb	-	+	+

positive by hybridization to pBR322. One restriction fragment was 3.5 kb and a second was of variable size for each DNA, as expected (lanes 13-16, Table II). The presence of two *Hind*III sites in pCV108 gave rise to the 3.5 kb fragment. The remaining 4.5 kb vector DNA, along with the insert DNA, accounted for the variable size fragment in each sample. All other restriction fragments lacked vector sequences and therefore did not hybridize to pBR322.

Data obtained following *Bgl*II and *Sal*I digestion of ML-1, ML-3, ML-4 and ML-6 DNA showed the presence of vector DNA in the expected fragments. Two variable restriction fragments which hybridized to pBR322 were obtained for each DNA, since both endonucleases have a single site in the vector (lanes 5-8, 21-24, Tables III and IV). The variable length of the fragments depended on the location of the next *Bgl*II and *Sal*I site within the insert DNA whose size was unknown at this stage of the analysis.

One restriction fragment of variable length was expected to hybridize to pBR322 following *Bam*HI and *Kpn*I digestion of ML-1, ML-3, ML-4 and ML-6 DNA. A single restriction fragment was obtained in each DNA (lanes 1-4 and 17-20, Tables V and VI). Each positive restriction fragment was comprised of the entire vector and variable lengths of insert DNA which depended on the size DNA analyzed, since there are no *Bam*HI or *Kpn*I sites within the vector.

2. Analyses using *Eco*RI A and B rDNA regions as probes.

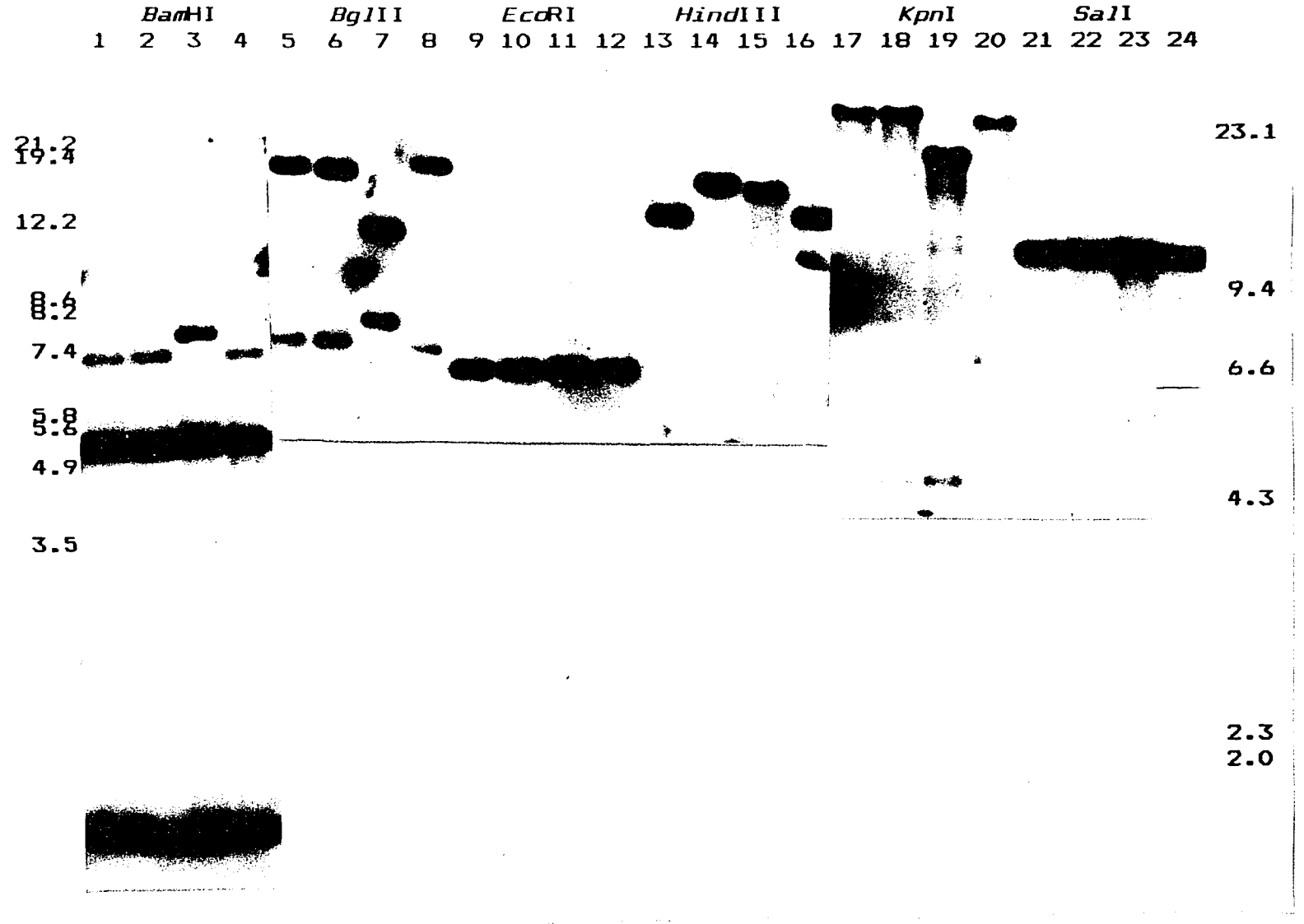
The purpose of the analyses was to further delineate the order of rDNA regions

within ML-1, ML-3, ML-4 and ML-6 DNA. Hybridizations with *Eco*RI A and B DNA was used to identify the rDNA regions corresponding to the promotor and the three rRNA genes. These analyses were critical in the selection of ML-1 and ML-4 DNA for transfections. *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I and *Sal*I digests were used in Southern blot analysis of ML-1, ML-3, ML-4 and ML-6 DNA. Results from the experiments are shown in Figures 13, 14 and 15 and are summarized in Table I-VI for each enzymatic digest.

The presence of the *Eco*RI A rDNA region and parts of the adjacent regions in all four DNAs was demonstrated using the A region as a probe (Figure 13; Table I-VI). Three restriction fragments were expected to hybridize to the probe following *Bam*HI digestion of ML-1, ML-3, ML-4 and ML-6 DNA: (a) a 7.4 kb DNA fragment, containing 5.5 kb of the D rDNA region; (b) a 5.1 kb DNA fragment, containing 1.2 kb of the B rDNA region and (c) a 1.6 kb DNA fragment generated from two *Bam*HI sites in the A region (see Figure 9). All three fragments were obtained with digestion of ML-1, ML-3 and ML-6 DNA (Figure 13, lanes 1, 2 and 4, Table V). The results clearly showed that the A and parts of the adjacent B and D rDNA regions were intact. In ML-4 DNA, the 5.1 kb and 1.6 kb restriction fragments were obtained indicating the intact presence of the A and part of its adjacent B rDNA region (lane 3). An 8.2 kb fragment was obtained, instead of the 7.4 kb, indicating that additional DNA was present in the D region where the other *Bam*HI site is located (see Figure 9).

Figure 13. Southern analysis of ML-1, ML-3, ML-4 and ML-6 DNA with ^{32}P-EcoRI A rDNA.

*Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I and *Sal*I generated restriction fragments of cosmid clones ML-1, ML-3, ML-4 and ML-6 that hybridized to 100 ng ^{32}P-labelled *Eco*RI A rDNA. 250 ng DNA for each sample was digested with each enzyme and fractionated on a 0.8% agarose gel (see Figure 10). Lanes 1-4: *Bam*HI digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 5-8: *Bgl*II digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 9-12: *Eco*RI digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 13-16: *Hind*III digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 17-20: *Kpn*I digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 21-24: *Sal*I digested ML-1, ML-3, ML-4 and ML-6 DNA. *Eco*RI, *Sma*I and *Hind*III digested lambda DNA fragments were used for size markers. Hybridization and washings were conducted as described in Materials and Methods. The specific activity of the probe was 4.2 x 10^8 cpm/ug. The autoradiogram was exposed for 20 hours at -70°C with an intensifying screen.


BamHI
BglII
EcoRI
HindIII
KpnI
SalI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
21.2
19.4
12.2
8.6
8.2
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6.6
4.3
2.3
2.0

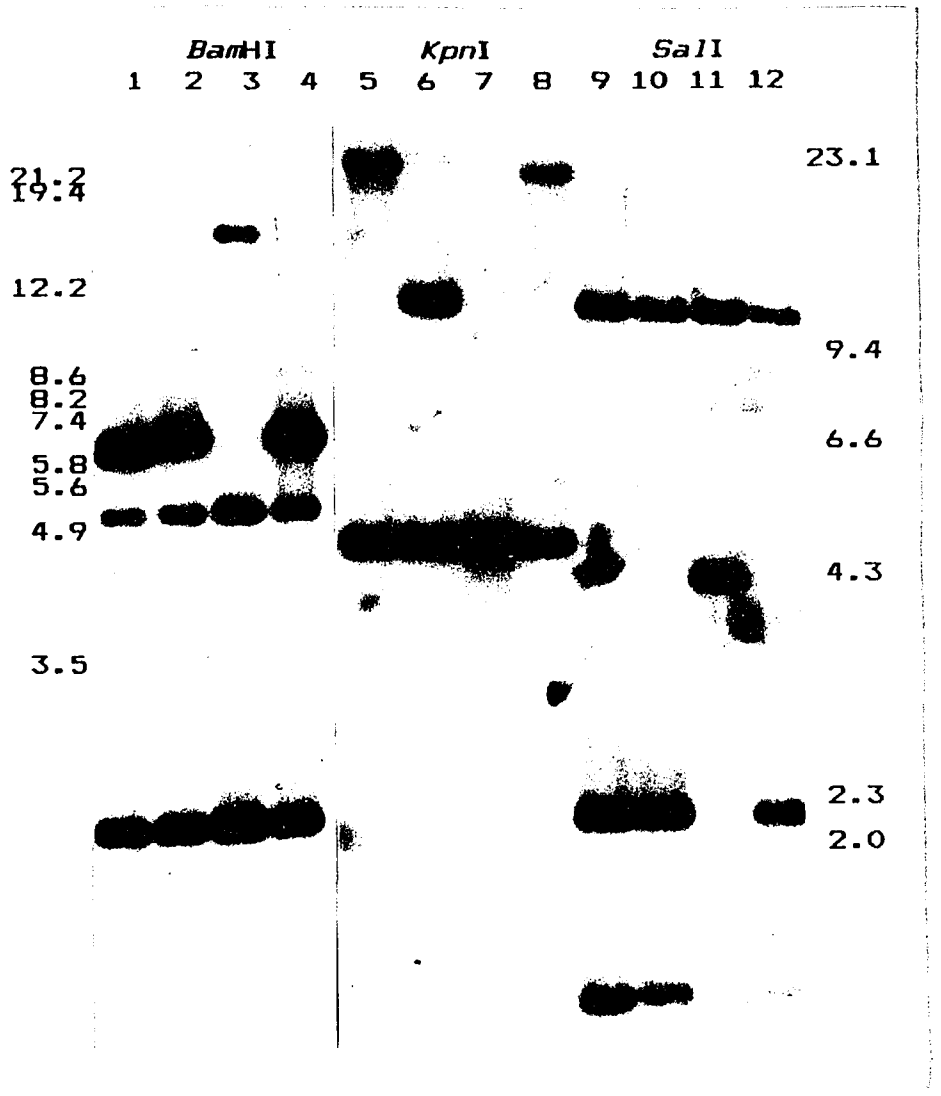

Figure 14. Southern analysis of ML-1, ML-3, ML-4 and ML-6 DNA with ^{32}P-*Eco*RI B rDNA.

*Bam*HI, *Kpn*I and *Sal*I generated restriction fragments of cosmid clones ML-1, ML-3, ML-4 and ML-6 that hybridized to 100 ng ^{32}P-labelled *Eco*RI B rDNA. 250 ng DNA for each sample was digested with each enzyme and fractionated on a 0.8% agarose gel (see Figure 10). Lanes 1-4: *Bam*HI digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 5-8: *Kpn*I digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 9-12: *Sal*I digested ML-1, ML-3, ML-4 and ML-6 DNA. *Eco*RI, *Sma*I and *Hind*III digested lambda DNA fragments were used for size markers. Hybridization and washings were conducted as described in Materials and Methods. The specific activity of the probe was 3.1 x 10^8 cpm/ug. The autoradiogram was exposed for 1 hour at -70°C with an intensifying screen.

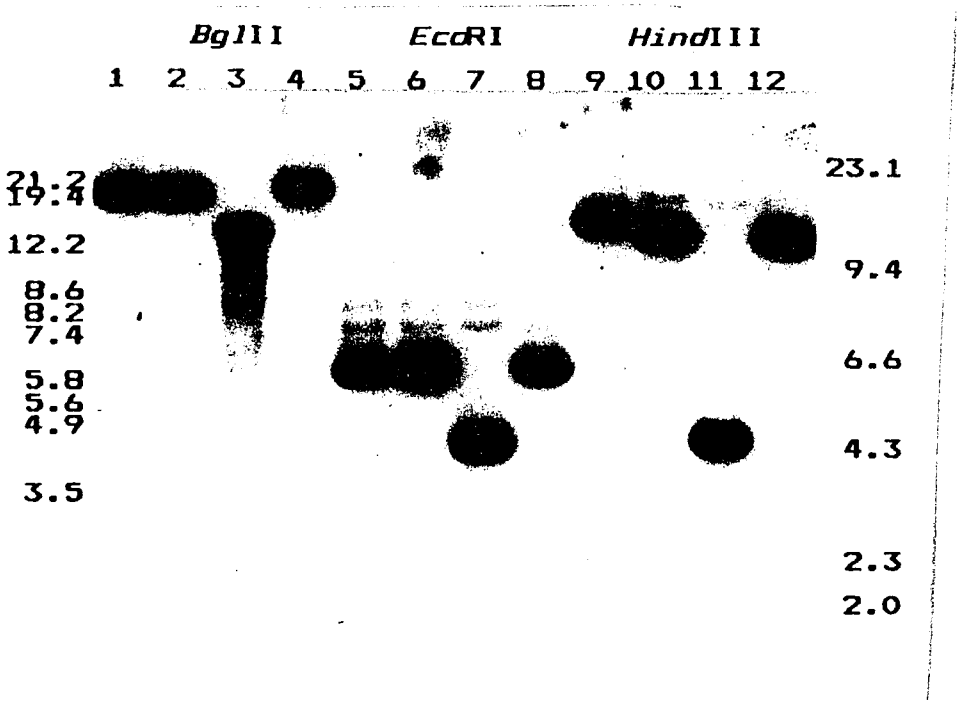

Figure 15. Southern analysis of ML-1, ML-3, ML-4 and ML-6 DNA with ^{32}P-*Eco*RI B rDNA.

*Bgl*II, *Eco*RI and *Hind*III generated restriction fragments of cosmid clones ML-1, ML-3, ML-4 and ML-6 that hybridized to 100 ng ^{32}P-labelled EcoRI B rDNA. 250 ng DNA for each sample was digested with each enzyme and fractionated on a 0.8% agarose gel (see Figure 10). Lanes 1-4: *Bgl*II digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 5-8: *Eco*RI digested ML-1, ML-3, ML-4 and ML-6 DNA; lanes 9-12: *Hind*III digested ML-1, ML-3, ML-4 and ML-6 DNA. *Eco*RI, *Sma*I and *Hind*III digested lambda DNA fragments were used for size markers. Hybridization and washings were conducted as described in Materials and Methods. The specific activity of the probe was 3.4 x 10^8 cpm/ug. The autoradiogram was exposed for 1 hour at -70°C with an intensifying screen.

Two restriction fragments were expected to hybridize to the A probe following *Bgl*II digestion of ML-1, ML-3, ML-4 and ML-6 DNA. One at 19 kb contained 14.4 kb of the adjacent B and C rDNA regions and the second at 8 kb, contained 5.3 kb of the D region (see Figure 9). The 8 kb restriction fragment in ML-1, ML-3 and ML-6 DNA was positive showing the intact presence of the corresponding A and D rDNA regions. A second fragment of variable length, 20.4 kb, 20.3 kb and 22.1 kb, respectively, was obtained for each of the three DNAs (Figure 13, lanes 5, 6 and 8, Table III). In ML-4 DNA, 6.1 kb and 13.9 kb restriction fragments were obtained (lane 7). The presence of a 6.1 kb restriction fragment in ML-4 DNA confirmed changes in the D region. The length variation of the large restriction fragment in all DNAs was due to the presence of vector DNA as confirmed by hybridization to pBR322 DNA (see Figure 12, lanes 5-8, Table III).

*Eco*RI digestion of ML-1, ML-3, ML-4 and ML-6 DNA, generated a 7.3 kb fragment that hybridized to the A probe in the four DNAs. The results showed the intact presence of the A rDNA region in all DNAs (Figure 13, lanes 9-12, Table I).

A single 14 kb restriction fragment containing 6.7 kb of the D region, was expected to hybridize to the A probe following *Hind*III digestion of ML-1, ML-3, ML-4 and ML-6 DNA (see Figure 9). ML-1 and ML-6 DNA generated the expected 14 kb fragment indicating the presence of the entire A and 6.7 kb of the flanking D rDNA region (Figure 13, lanes 13 and 16, Table II). A 18.8 kb restriction fragment containing rDNA and vector DNA was generated in ML-3 DNA (Figures 12, 13 lane

15). This demonstrated that a portion of the D region was deleted which represents the site of insertion into the vector in ML-3 DNA (Figure 6). *Hind*III digestion of ML-4 DNA generated a 17.5 kb instead of the expected 14 kb restriction fragment, showing the presence of additional DNA sequences within the D region (Figure 13, lane 15 and Table II).

Two restriction fragments, one at 4.5 kb, containing 4.3 kb of the B region, the second, at 27 kb, containing 19 kb of the D region, were expected to hybridize to the A probe following *Kpn*I digestion of ML-1, ML-3, ML-4 and ML-6 DNA (Figure 9). The 4.5 kb fragment was obtained with all four DNAs (Figure 13, lanes 17-20, Table VI). A greater than 23 kb fragment following *Kpn*I digestion of ML-1, ML-3 and ML-6 DNA was obtained (lanes 17, 18 and 20). A smaller, 19.2 kb restriction fragment, was obtained following *Kpn*I digestion of ML-4 DNA (lane 19). The large restriction fragments in ML-1, ML-3 and ML-6, as well as the 19.2 kb of ML-4 DNA, hybridized to pBR322 DNA (Figure 12, lanes 17-20, Table VI). The results showed that the vector DNA replaced a portion of the C region at a *Kpn*I site. The deleted portion of the C and its adjacent D region represent the site of insertion within the vector in ML-1, ML-3, ML-4 and ML-6 DNA (see Figures 5, 6, 7 and 8).

A single 10.5 kb restriction fragment containing 3.0 kb and 200 bp of the B and D regions, respectively, was expected to hybridize to the A probe following *Sal*I digestion of ML-1, ML-3, ML-4 and ML-6 DNA (see Figure 9). A 10.5 kb fragment was obtained following *Sal*I digestion of all four DNAs and confirmed the presence

of the entire A, 3.5 kb of B and 200 bp of D region (Figure 13, lanes 21-24, Table IV).

The presence of the B rDNA region in ML-1, ML-3, ML-4 and ML-6 DNA was directly confirmed by using B DNA as a probe (Figures 14 and 15; Tables I-VI)). Three restriction fragments were expected following *Bam*HI digestion of ML-1, ML-3, ML-4 and ML-6 DNA: a 6.4 kb DNA fragment containing 1.2 kb of the promotor and 3.9 kb of C region, a 5.1 kb fragment containing 4.3 kb of A region and a third restriction fragment at 2.2 kb comprised of the 18S rDNA (see Figure 9). *Bam*HI digestion of ML-1, ML-3 and ML-6 DNA generated the three expected fragments (Figure 14, lanes 1, 2 and 4, Table V). *Bam*HI digestion of ML-4 DNA generated the 5.1 kb and 2.2 kb fragments, but not the 6.4 kb fragment (lane 3). Instead, a 16 kb fragment that hybridized with pBR322 DNA, as well, was obtained (see Figure 12, lane 3). The results showed that the next *Bam*HI site, located within the C region of ML-4 DNA, was absent and that this rDNA region is the site of insertion within the vector (see Figure 7).

Two restriction fragments, a 4.5 kb fragment containing 200 bp of the A region and a 12 kb fragment containing 10.3 kb of the C region, were expected to hybridize to the B probe following *Kpn*I digestion of ML-1, ML-3, ML-4 and ML-6 DNA (see Figure 9). All four DNAs generated the expected positive 4.5 kb fragment and showed the intact presence of these rDNA regions (Figure 14, lanes 4-8, Table VI). The results also confirmed data obtained with those using the *Eco*RI A probe (see Figure 13, lanes

17-20, Table VI). It was already established that the portion of the C region containing a *Kpn*I site which would normally generate a 12 kb fragment, was absent in ML-1 and ML-6 DNA. As a result, a larger fragment was expected to hybridize to the B and pBR322 probes. This was obtained in ML-1 and ML-6 DNA and confirmed the insert orientation and site of insertion within the vector (Figure 12, lanes 17 and 20 and Figure 14, lanes 4 and 8, Table VI). On the other hand, *Kpn*I digestion of ML-3 and ML-4 DNA, generated the expected 12 kb fragment (Figure 14, lanes 5 and 6). The 12 kb fragment of ML-3 DNA hybridized to the B probe, as expected (lane 6) but that of ML-4 DNA did not (lane 7). The results also confirmed DNA deletions in the C and D regions of ML-4 DNA.

*Sal*I proved to be the most useful endonuclease in confirming the absence of the promotor in ML-4 DNA. Three restriction fragments were expected to hybridize to the B probe following *Sal*I digestion of ML-1, ML-3, ML-4 and ML-6 DNA: (a) a 10.5 kb fragment comprised of 7.3 kb and 0.5 kb portions of the A and D regions, respectively; (b) a 2.3 kb restriction fragment and (c) a 1.5 kb fragment in which 1.2 kb define the rDNA promotor and 300 bp are part of the C region (see Figure 9). In the four DNAs, the 2.3 kb fragment hybridized to B DNA (Figure 14, lanes 9-12, Table IV). A 10.5 kb restriction fragment in ML-1, ML-3 and ML-6 DNA also hybridized to B DNA showing the intact corresponding rDNA regions (lanes 9, 10 and 12). In ML-1, ML-3 and ML-6 DNA, the 1.5 kb fragment also hybridized to the B probe and showed the presence of the promotor region (lanes 9, 10 and 12). In

contrast, *Sal*I digestion of ML-4 DNA did not generate the expected 2.3 kb and 1.5 kb restriction fragments and clearly showed the absence of the promotor and portions of the adjacent downstream and upstream sequences (lane 11). Instead, in ML-4 DNA a positive 4.2 kb fragment was generated that hybridized to pBR322 DNA (Figure 12, lane 23 and Figure 14, lane 11, Table IV). The results demonstrated that DNA prior to the *Sal*I site upstream of the *Kpn*I site (which includes the promotor) in the B region is absent and is also the site of insertion into the vector (see Figure 7 and 9).

A single 20 kb restriction fragment, containing 5.1 kb and 14 kb of the A and C regions, respectively, was expected to hybridize to the B DNA following *Bgl*II digestion of ML-1, ML-3, ML-4 and ML-6 DNA (see Figure 9). *Bgl*II digestion ML-1, ML-3 and ML-6 DNA generated the 20 kb fragment (Figure 15, lanes 1, 2 and 4, Table III). The fragment also hybridized to the A probe following *Bgl*II digestion of ML-1, ML-3 and ML-6 DNA (Figure 13, lanes 5, 6 and 8, Table III) and with pBR322 in ML-1 and ML-6 DNA (Figure 12, lanes 5 and 8, Table III). Taken together, the findings demonstrated that the D region of ML-1, ML-3 and ML-6 DNA is the site of insertion in vector DNA (Figure 5, 6 and 8). *Bgl*II digestion of ML-4 DNA generated a 14 kb restriction fragment that hybridized to the B probe (lane 7) and pBR322 and A probes (see Figure 12, lane 7 and Figure 13, lane 7). These results showed that the B region of ML-4 DNA is the site of insertion within the vector (see Figure 7).

A single 6 kb fragment corresponding to the B region, was expected to

hybridize to the B probe following *Eco*RI digestion of the four DNAs (Figure 9). *Eco*RI digestion of ML-1, ML-3 and ML-6 DNA generated the intact 6.0 kb fragment (Figure 15, lanes 5, 6 and 8, Table I). On the other hand, *Eco*RI digestion of ML-4 DNA generated a smaller 4.2 kb fragment (lane 7). The results showed that the upstream sequences of the B region in ML-4 DNA are deleted. This information was substantiated by *Bam*HI and *Sal*I digestion of ML-4 DNA which clearly showed that the region upstream of the *Bam*HI and *Sal*I site was absent (see Figure 9). Taken together, the results indicate that the adjacent upstream portion next to the *Bam*HI and *Sal*I site (which includes the rDNA promotor) is the site of insertion in the vector (see Figure 7).

A single 13 kb restriction fragment, containing 8 kb of the C region was expected to hybridize to the B probe following *Hind*III digestion of ML-1, ML-3, ML-4 and ML-6 DNA (see Figure 9). ML-1 DNA generated a 14.7 fragment, containing 4.2 kb of the C region (Figure 15, lane 9, Table II) and 3.5 kb of vector DNA (see Figure 12, lane 13). The results further substantiated that the C region in ML-1 DNA was the site of insertion into vector DNA (see Figure 5). *Hind*III digestion of ML-3 or ML-6 DNA generated the expected 13 kb fragment showing the intact presence of the corresponding rDNA regions (lanes 10 and 12). Finally, *Hind*III digestion of ML-4 DNA generated a 4.3 kb fragment that hybridized to the B probe (lane 11). The results provided more evidence showing the absence of the remaining B and the adjacent C regions in ML-4 DNA. This finding, along with the presence of the *Hind*III site 90 bp

from the beginning of vector (see Figure 4B), substantiates that the B region in ML-4 DNA is the site of insertion in the vector which deleted the promotor (see Figure 7).

3. Analysis using *Eco*RI D rDNA region as a probe

The *Eco*RI D 19 kb rDNA region (cloned in lambda DASH and designated H2E4) was used to confirm the presence of D rDNA region in ML-1, ML-3, ML-4 and ML-6 DNA. The D region contains several *Alu* repeats between two internal *Hind*III sites (see Figure 3). Previous studies have reported the presence of these *Alu* repeats within the C and D regions of the human ribosomal repeat unit (Higuchi *et al.*, 1981, Sylvester *et al.*, 1986, Dickson *et al.*, 1989, Sylvester *et al.*, 1989, Safrany and Hidgevi, 1989).

*Eco*RI digestion of ML-1, ML-3 and ML-6 DNA generated the two expected fragments: a) at 14 kb and 4.2 kb in ML-1 DNA; b) at 14 kb and 7.8 kb in ML-3 DNA and c) at 8.3 kb and 7.1 kb in ML-6 DNA (Figure 16A, lanes 1, 2 and 4, Table 1). The 14 kb, 14 kb and 8.3 restriction fragments of ML-1, ML-3 and ML-6 DNA, respectively, corresponded to the D region, while the 4.2 kb, 7.8 kb and 7.1 kb fragments of ML-1, ML-3 and ML-6 DNA, respectively, corresponded to the C region. Finally, a single 9.2 kb fragment of ML-4 DNA hybridized to the D probe (lane 3). This fragment corresponded to the D region since it was previously shown that the C region containing the *Alu* repeats was deleted from ML-4 DNA.

4. Analysis with an *Alu* repeat as a probe

The presence of *Alu* repeats within ML-1, ML-3, ML-4, and ML-6 DNA was

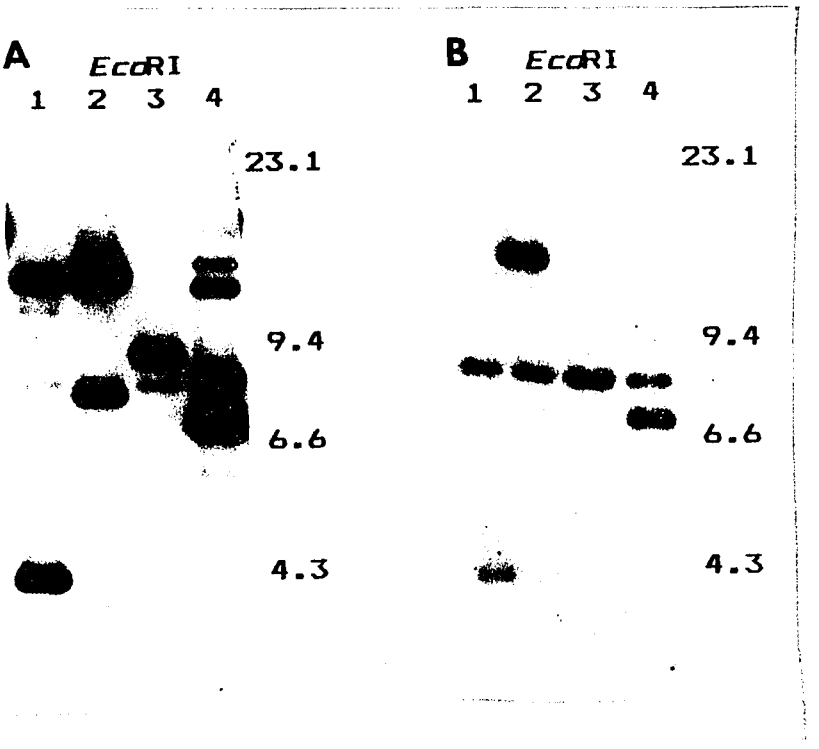

Figure 16. Southern analyses of ML-1, ML-3, ML-4 and ML-6 DNA with ^{32}P-*Eco*RI D rDNA and ^{32}P-*Alu* repeat.

A. *Eco*RI generated restriction fragments of cosmids clones ML-1, ML-3, ML-4 and ML-6 that hybridized to 100 ng ^{32}P-labelled *Eco*RI D rDNA. 250 ng DNA of each DNA sample was digested with *Eco*RI and fractionated on a 0.8% agarose gel. Lanes 1-4: *Eco*RI digested ML-1, ML-3, ML-4 and ML-6 DNA, respectively.

B. *Eco*RI generated restriction fragments of cosmids clones ML-1, ML-3, ML-4 and ML-6 that hybridized to 100 ng ^{32}P-labelled *Alu* repeat. 250 ng DNA of each DNA sample was digested with *Eco*RI and fractionated on a 0.8% agarose gel (see Figure 10). Lanes 1-4: *Eco*RI digested ML-1, ML-3, ML-4 and ML-6 DNA, respectively.

*Hind*III digested lambda DNA fragments were used for size markers. Hybridization and washings were conducted as described in Materials and Methods. The specific activity of the *Eco*RI D rDNA probe was 1.1 x 10^8 cpm/ug. The autoradiogram was exposed for 20 hours at -70°C with an intensifying screen. The specific activity of the *Alu* probe was 1.3 x 10^8 cpm/ug. The autoradiogram was exposed for 20 hours at -70°C with an intensifying screen.

confirmed using recombinant plasmid BLUR-8 containing an Alu SINES element (Rubin *et al.*, 1980) as a probe in a Southern hybridization. One restriction fragment, corresponding to the C region, was expected to hybridize to the probe following EcoRI digestion of all four DNAs since *Alu*-containing regions are deleted in the D region. The expected fragments, 4.2 kb of ML-1 DNA, 14 kb of ML-3 DNA and 7.1 kb of ML-6 DNA were obtained (Figure 16B, lanes 1, 2 and 4, Table I). No fragment hybridized in ML-4 DNA, as expected (lane 3). The results clearly showed that in ML-1, ML-3 and ML-6 DNA, the portion of the C region containing the *Alu* repeats located adjacent to the promotor is present, while in ML-4 DNA, it is absent. The uniform 8.0 kb fragment present in all lanes corresponded to the vector and not the *Alu* DNA, since there is homology between plasmid BLUR-8 (it is plasmid pBR322) and pCV108.

IV. Summary of analyses

ML-1 and ML-4 DNA were selected for transfection of human cells on the basis of results obtained from the electrophoretic and hybridization analyses. More specifically, the large intact insert rDNA of ML-1 DNA and the absence of the promotor in ML-4 DNA were the deciding factors for their selection and further analyses. It was assumed that: a) ML-1 rDNA would be functional since the transcribed spacer is intact and b) ML-4 DNA would serve as a control since it is missing the promotor. On the other hand, the DNA modifications in the nontranscribed spacer of the ML-3 rDNA and the small insert size of ML-6 DNA were the deciding

factors for their rejection.

PART TWO: MOLECULAR ANALYSES OF rDNA TRANSFECTED CLONED HUMAN CELLS

This part of the study was undertaken to determine the presence, copy number and site of recombination within the transfected DNA of the transfected ML-1 and ML-4 DNA in human fibroblasts.

I. Overview of analyses of transfected DNAs

One to two copies of ML-1 DNA are present in transfected HAL cells, called 1K1 (see Part Three, sections II). The site of recombination within the transfected ML-1 DNA in 1K1 cells occurred between the second *Hind*III site and the end of the vector (see Figure 5). One copy of ML-4 DNA was found in transfected HAL cells, called 4K2. The site of recombination within the transfected ML-4 DNA in 4K2 cells occurred distal to the neo gene found within the 3.5 kb region defined by the two *Hind*III sites in the vector (see Figure 7).

II. Generation of 1K1 and 4K2 transfected cloned HAL cells

The successful transfer of ML-1 and ML-4 DNA into human cells was necessary to elucidate the role of rDNA in satellite associations and nucleolar organization. ML-1 DNA is expected to be involved in nucleolar formation and satellite association. ML-4 DNA is not expected to be involved in functional processes of rDNA, because it is lacking the promotor. These expectations are based on the hypothesis that nucleolar formation and resultant satellite associations cannot occur

without rDNA transcription.

The transfection of ML-1 and ML-4 DNA into human HAL cells was successfully completed (by Kathi McDowell) using lipofection (Felgner *et al.*, 1987). Cloned cells containing ML-1 DNA and ML-4 DNA were selected for molecular and cytological analyses following G418 selection.

Southern analysis was carried out to detect the presence and site of recombination within the transfected ML-1 and ML-4 DNA in 1K1 and 4K2 cells. The analysis showed that the transfected rDNA was intact; transcription would produce rRNA transcripts that are indistinguishable from the endogenous rRNA transcripts.

III. Genomic DNA Analysis of 1K1 and 4K2 Cells

Restriction digests of 1K1 and 4K2 genomic DNA were used to identify the presence, copy number and site of recombination within the transfected ML-1 and ML-4 DNA in the genome. Southern analysis of total genomic DNA from non-transfected HAL cells and transfected 1K1 and 4K2 cells was carried out using restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI and *Hind*III on the basis of their known sites in ML-1 and ML-4 DNA (see Figures 5 and 7). Cosmid pJB8 was used as a probe because vector pCV108 was originally constructed using pJB8 (see Figure 4B). Results from the analysis are shown in Figure 17 and are summarized in Table VII.

Some restriction fragments in the control HAL DNA were expected to hybridize to pJB8 DNA, since HAL cells were immortalized by transfection of *ori*$^-$ SV40 sequences cloned in plasmid pMK16. There is a 1.8 kb homology between pJB8

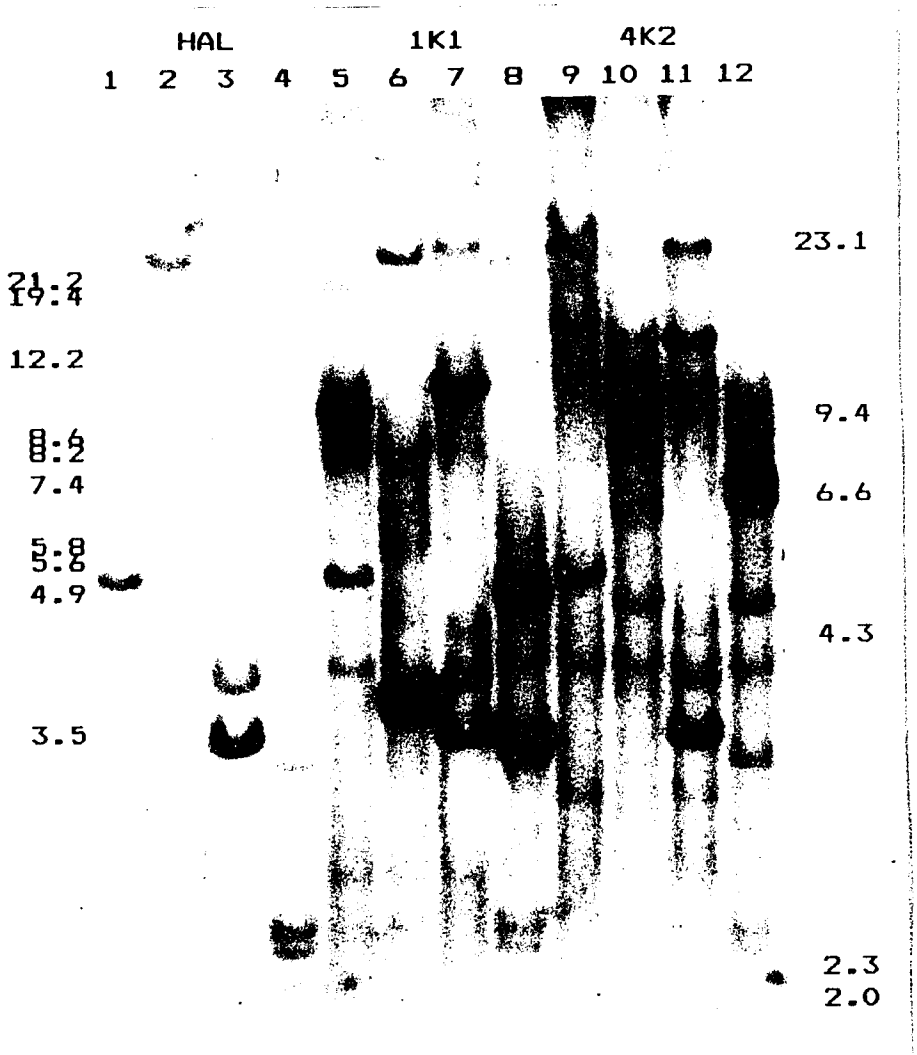

Figure 17. Southern analysis of HAL, 1K1 and 4K2 genomic DNA with ^{32}P-pJB8 DNA.

*Bam*HI, *Bgl*II, *Eco*RI and *Hind*III generated restriction fragments of HAL, 1K1 and 4K2 genomic DNA that hybridized to 200 ng ^{32}P-labelled pJB8. 10 ug DNA of each sample was digested with each enzyme and fractionated on a 0.8% agarose gel. Lane 1: *Bam*HI digested HAL DNA; lane 2: *Bgl*II digested HAL DNA; lane 3: *Eco*RI digested HAL DNA; lane 4: *Hind*III digested HAL DNA; lane 5: *Bam*HI digested 1K1 DNA; lane 6: *Bgl*II digested 1K1 DNA; lane 7: *Eco*RI digested 1K1 DNA; lane 8: *Hind*III digested 1K1 DNA; lane 9: *Bam*HI digested 4K2 DNA; lane 10: *Bgl*II digested 4K2 DNA; lane 11: *Eco*RI digested 4K2 DNA; lane 12: *Hind*III digested 4K2 DNA. *Eco*RI, *Sma*I and *Hind*III digested lambda DNA fragments were used for size markers. Hybridization and washings were conducted as described in Materials and Methods. The specific activity of the probe was 1.4 x 10^9 cpm/ug. The autoradiogram was exposed for 40 hours at -70°C with an intensifying screen.

Table VII

Comparison of restriction fragments generated following endonuclease digestions of HAL, 1K1 and 4K2 genomic DNA with ^{32}P-pJB8.

CELL LINE DNA	ENZYME	FRGMS. EXP.	COSMID DNA FRAGMENTS OBTAINED	FRGMS. OBT. PROBE pJB8	pMK16-SV40 INS. SITE	pCV108-rDNA INS. SITE
HAL	BamHI	N.A.	N.A.	>20.0 kb	+	-
		N.A.	N.A.	5.1 kb	+	-
	BglII	N.A.	N.A.	>20.0 kb	+	-
	EcoRI	N.A.	N.A.	3.9 kb	+	-
		N.A.	N.A.	3.4 kb	+	-
	HindIII	N.A.	N.A.	3.3 kb	+	-
				2.5 kb	+	-
				2.4 kb	+	-
1K1	BamHI	>20.0 kb		>20.0 kb	+	-
		5.1 kb		5.1 kb	+	-
			16.5 kb	9.5 kb	-	+
				8.4 kb	-	+
	BglII	>20.0 kb		>20.0 kb	+	-
			20.4 kb	7.9 kb	-	+
			12.4 kb	3.7 kb	-	+
	EcoRI	3.9 kb		3.9 kb	+	-
		3.4 kb		3.4 kb	+	-
			8.0 kb	>20.0 kb	-	+
				10.8 kb	-	+
	HindIII	3.3 kb		3.3 kb	+	-
		2.5 kb		2.5 kb	+	-
		2.4 kb		2.4 kb	+	-
			14.7 kb	8.0 kb	-	+
			3.5 kb	3.5 kb	-	+
4K2	BamHI	>20.0 kb		>20.0 kb	+	-
		5.1 kb		5.1 kb	+	-
			16.0 kb	16.9 kb	-	+
				3.1 kb	-	+
	BglII	>20.0 kb		>20.0 kb	+	-
			14.0 kb	16.4 kb	-	+
			6.1 kb	10.0 kb	-	+
				4.8 kb	-	+
	EcoRI	3.9 kb		3.9 kb	+	-
		3.4 kb		3.4 kb	+	-
			8.0 kb	>20.0 kb	-	+
				14.2 kb	-	+
	HindIII	3.3 kb		3.3 kb	+	-
		2.5 kb		2.5 kb	+	-
		2.4 kb		2.4 kb	+	-
			7.9 kb	7.5 kb	-	+
			3.5 kb	6.6 kb	-	+
				4.7 kb	-	+

and pMK16. *Bam*HI digestion of HAL DNA generated a 24.3 kb and a 5.1 kb fragment (lane 1). *Bgl*II digestion of HAL DNA generated a single high molecular weight fragment (lane 2). *Eco*RI digestion of HAL DNA generated a 3.9 kb and a 3.4 kb fragment (lane 3). Finally, *Hind*III digestion of HAL DNA generated three fragments, 3.3 kb, 2.5 kb and 2.4 kb (lane 4).

Additional positive restriction fragments were obtained in 1K1 and 4K2 genomic DNA following *Bam*HI, *Bgl*II, *Eco*RI and *Hind*III digestion. These correspond to ML-1 and ML-4 vector DNA and indirectly to the rDNA insert (discussed below). The site of recombination within ML-1 and ML-4 DNA in the genome was determined on the basis of the absence of the 8.0 kb fragment following *Eco*RI digestion of 1K1 and 4K2 DNA. The absence of the 8.0 kb fragment indicated that the site of recombination in the transfected ML-1 and ML-4 DNA into the genome occurred within the vector. Two restriction fragments, one at high molecular weight and the other at 10.8 kb, hybridized to pJB8 DNA following *Eco*RI digestion of 1K1 DNA (lane 7). Two restriction fragments, one at high molecular weight and the other at 14.2 kb, were obtained following *Eco*RI digestion of 4K2 DNA (lane 11).

Two restriction fragments, at 3.5 kb and at 8.0 kb, were expected to hybridized to pJB8 DNA following *Hind*III digestion of 1K1 DNA. Results show the presence of the 3.5 kb and a 4.9 kb fragment (instead of the 8.0 kb fragment, lane 8). The results demonstrated that the region of the vector between the two *Hind*III sites was intact. The region between the second *Hind*III site and the end of the vector was not

intact and represents the site of recombination in transfected ML-1 DNA into genomic DNA (see Figure 5).

Two restriction fragments, at 3.5 kb and at 7.5 kb were expected to hybridize to pJB8 DNA following *Hind*III digestion of 4K2 DNA. Three restriction fragments, at 7.5 kb, at 6.6 kb and at 4.7 kb were obtained (lane 12). Absence of the 3.5 kb fragment indicated that the site of recombination between ML-4 DNA and the genomic DNA occurred between the two *Hind*III sites distal to the neo gene of the vector (Figure 7). G418 selection could not be possible if integration took place within the neo gene since this event would render it nonfunctional. The third positive fragment was obtained as a result of the integration event.

The results were confirmed from data obtained with *Bam*HI digestion of 1K1 and 4K2 DNA. Two restriction fragments of variable length were expected to hybridize to pJB8 DNA, since no *Bam*HI sites are present within the vector. These were obtained in both 1K1 and 4K2 DNA (lanes 5 and 9). The results confirmed that vector DNA has been split and is the site of recombination in both transfected ML-1 and ML-4 DNA.

Three fragments were expected to hybridized to pJB8 DNA following *Bgl*II digestion of 1K1 and 4K2 DNA since there is a single *Bgl*II site present within the vector. *Bgl*II digestion of generated two fragments, a 7.9 kb and a 3.7 kb with 1K1 DNA and three fragments, a 16.4 kb, a 10 kb and 4.8 kb with 4K2 DNA (lane 6 and 10). Failure to observe one of the expected fragments for 1K1 DNA is probably due

to its smaller size thus resulting in low homology to the probe.

Comparison of band signal intensity and number of bands obtained by various digests of pMK16, ML-1 and ML-4 DNA was used to estimate their copy number present in HAL, 1K1 and 4K2 cells. Previous results estimated that there is a single copy of pMK16 present in HAL cells (Radna *et al.*, 1989). The present analyses estimate that there are one to two copies of ML-1 DNA present within 1K1 cells and one copy of ML-4 DNA present within 4K2 cells.

PART THREE: MOLECULAR ANALYSIS OF NUCLEOLAR DNA OF TRANSFECTED CLONED 1K1 AND 4K2 CELLS

Critical to the original hypothesis is whether or not transfected rDNA participates in nucleolar formation. One criterion for testing the hypothesis is the presence of vector sequences in the nucleolar DNA. Analysis of genomic, extra-nucleolar chromatin (ENC or DNA that is not present in the nucleolar fraction) and nucleolar DNA was conducted in the transfected cloned cell lines 1K1 and 4K2.

I. Overview of analysis of transfected DNAs

ML-1 DNA with homology to pJB8 was found in nucleolar DNA of 1K1 cells. This suggests that the rDNA insert participates in formation of the nucleolus and is transcriptionally active. ML-4 DNA was not present in nucleolar DNA of 4K2 cells, indicating that the rDNA is not transcriptionally active and did not participate in nucleolar formation. The results confirm our hypothesis that participation in nucleolar activity is a function of transcriptional activity.

II. Nucleolar DNA analysis of 1K1 and 4K2 cells

Southern analysis was used to identify the presence of the transfected ML-1 and ML-4 vector DNA and indirectly the rDNA in ENC and nucleolar DNA from HAL, 1K1 and 4K2 cells and to confirm their participation in nucleolar organization. Each DNA sample from HAL, 1K1 and 4K2 cells was digested with *Eco*RI and screened with pJB8 (Figure 18). Results are also summarized in Table VIII. Genomic DNA for each cell line was used as a control in this experiment.

It is crucial to mention that in addition to the expected fragments, other smaller positive fragments were obtained. These fragments are generated from the breakdown of DNA during sonication of the nuclei. During sonication of nuclei (Beebee, 1986), the DNA can acquire extensive single strand breaks. This technical problem was overcome by comparative analysis with sonicated control DNA (ENC).

*Eco*RI digestion generated a 3.9 kb and a 3.4 kb fragment with homology to pMK16 in HAL genomic DNA, as expected (lane 1). The fragments were also present in HAL ENC, 1K1 genomic, 1K1 ENC, 4K2 genomic and 4K2 ENC DNA (lanes 2, 4, 5, 7, 8). The high MW and 10.8 kb fragment of ML-1 and the high MW and 14.2 kb fragment of ML-4 vector DNA was also obtained with *Eco*RI digestion of 1K1 and 4K2 genomic DNA (lane 4 and 7). No fragment hybridized to pJB8 DNA in HAL nucleolar DNA (lane 3).

The transfected ML-1 DNA was indicated to be absent from 1K1 ENC since pJB8 DNA did not hybridize with any fragment following *Eco*RI digestion (lane 5).

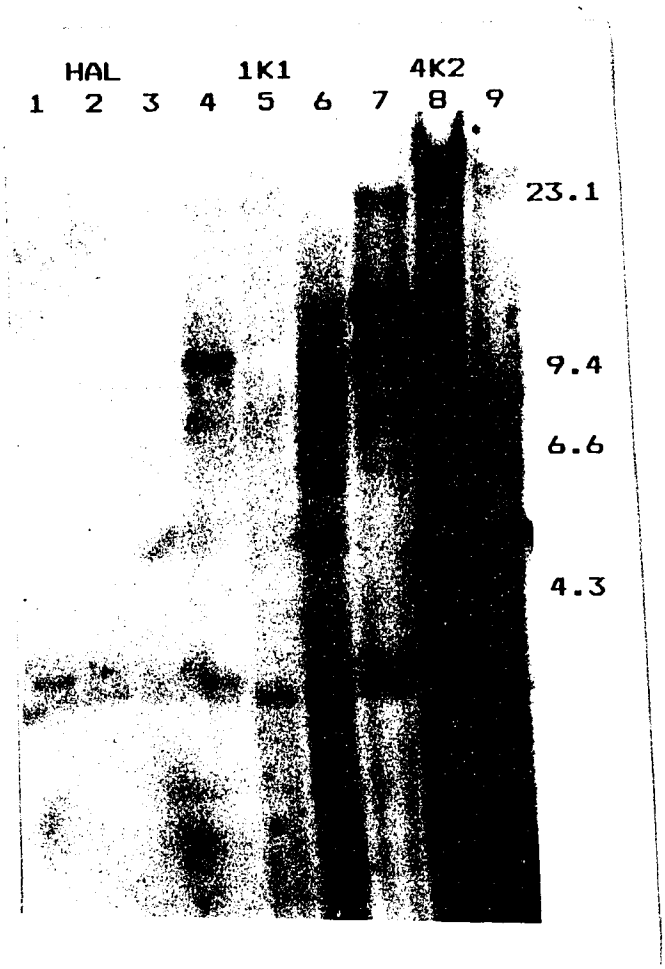

Figure 18. Southern analysis of HAL, 1K1 and 4K2 nucleolar DNA with ^{32}P-pJB8 DNA. *Eco*RI generated restriction fragments of HAL, 1K1 and 4K2 genomic, extra-nucleolar (ENC) and nucleolar DNA that hybridized to 100 ng ^{32}P-labelled pJB8. 5 ng DNA of each sample was digested with EcoRI and fractionated on a 0.8% agarose gel. Lane 1: HAL genomic DNA; lane 2: HAL ENC; lane 3: HAL nucleolar DNA; lane 4: 1K1 genomic DNA; lane 5: 1K1 ENC; lane 6: 1K1 nucleolar DNA; lane 7: 4K2 genomic DNA; lane 8: 4K2 ENC; lane 9: 4K2 nucleolar DNA. *Hind*III digested lambda DNA fragments were used for size markers. Hybridization and washings were conducted as described in Materials and Methods. The specific activity of the probe was 1.1 x 10^8 cpm/ug. The autoradiogram was exposed for 24 hours at -70°C with an intensifying screen.

Table VIII

Comparison of *Eco*RI generated restriction fragments between genomic DNA, ENC and nucleolar DNA of cell lines HAL, 1K1 and 4K2 with ^{32}P-pJB8.

CELL LINE	ENZYME	DNA	FRAGMENTS EXPECTED	FRAGMENTS OBTAINED	PROBE pJB8
HAL	EcoRI	Genomic	3.9 kb	3.9 kb	+
			3.4 kb	3.4 kb	+
	EcoRI	Extra-nucleolar	3.9 kb	3.9 kb	+
			3.4 kb	3.4 kb	+
	EcoRI	Nucleolar	None	None	-
1K1	EcoRI	Genomic	3.9 kb	3.9 kb	+
			3.4 kb	3.4 kb	+
			>20.0 kb	>20.0 kb	+
			10.8 kb	10.8 kb	+
	EcoRI	Extra-nucleolar	3.9 kb	Not obtained	-
			3.4 kb	3.4 kb	+
	EcoRI	Nucleolar	>20.0 kb	>20.0 kb	+
			10.8 kb	10.8 kb	+
				Five smaller	All +
4K2	EcoRI	Genomic	3.9 kb	3.9 kb	+
			3.4 kb	3.4 kb	+
			>20.0 kb	>20.0 kb	+
			14.2 kb	14.2 kb	+
	EcoRI	Extra-nucleolar	3.9 kb	3.9 kb	+
			3.4 kb	3.4 kb	+
			>20.0 kb	>20.0 kb	+
			14.2 kb	Not obtained	-
				Four smaller	All +
	EcoRI	Nucleolar	None	None	-

On the other hand, the probe hybridized with a number of fragments following *Eco*RI digested 1K1 nucleolar DNA, including those obtained with 1K1 genomic DNA (lane 6). This finding revealed the presence of the ML-1 vector DNA and indirectly the rDNA insert in the nucleolar fraction of 1K1 DNA. The data indicated that the transfected ML-1 rDNA insert established a transcriptionally active NOR within the human genome. This significant finding provides evidence that a single rDNA repeat unit, has the ability to form an active NOR which participates in nucleolar formation.

In contrast, 4K2 DNA showed the opposite results. The ML-4 vector DNA and indirectly the rDNA insert were present in the ENC and not in the nucleolar DNA (lane 8 and 9). Results are summarized in Table VIII. The results indicated the transfected ML-4 rDNA did not form a transcriptionally active NOR in 4K2 cells. Cytological analyses of nucleolar organization processes will prove useful in substantiating the results of this study.

DISCUSSION

I. Overview

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 crux of the problem addressed was what component or components of the rDNA complex contribute to nucleolar organization and resultant satellite associations.

A functional comparison of human rDNA following transfection into human fibroblasts was made. Two cloned human rDNA fragments were compared, one of which included a single complete transcribed spacer (including the promotor) and large segments of the nontranscribed spacer region (ML-1). The other included a single transcribed spacer (lacking the promotor) and portions of the nontranscribed spacer (ML-4). The results demonstrated that ML-1, which contains the promotor region, is transcribed and also displays the ability to function in the formation of the nucleolus. On the other hand, the absence of the promotor in ML-4 DNA deterred it from participating in nucleolar formation. This is a direct demonstration which supports the hypothesis that transcriptional capacity is necessary for participation in nucleolar organizing activity.

Examination of genomic DNA from transfected cells, 1K1 and 4K2, indicated the presence of one to two copies of the transfected ML-1 and ML-4 DNA into the

human genome. Subsequent cytological analyses demonstrated that the region of chromosome 3 (containing ML-1 DNA) participated in satellite associations with other rDNA-bearing acrocentric chromosomes and was silver stained, a test showing transcriptionally active rRNA gene clusters (Goodpasture and Bloom 1975). In contrast, the abnormal 7 chromosome containing the insertion site for ML-4 DNA failed to participate in satellite associations with other acrocentric chromosomes and was not silver stained. These findings clearly indicate that the DNA critical to associations is rDNA. Taken together, the results also provide evidence that tandem repetition is not an absolute requirement for rDNA function and that a single rDNA transcribed spacer has the ability to be transcribed and includes the rDNA sequences necessary for participation in nucleolar organization and satellite associations.

Transfection using intraspecies DNA allowed us to address the question of which rDNA sequences, normally contributing to rRNA gene expression, participate in satellite associations and nucleolar organization. Transcriptional activity was observed in transfected ML-1 DNA, but this DNA lacked portions of the *Eco*RI D (including the *Alu* repeat) and C region. This demonstrates that this *Alu*-containing D region and adjacent sequences of the rDNA NTS are not necessary for rDNA function.

II. rDNA content within cosmid vectors

Ten clones were isolated from the Lau and Kan cosmid library, four of which were further analyzed during the course of this investigation in order to select appropriate DNA for transfection. Three of the cloned DNAs, designated ML-1, ML-3

and ML-6, contained the intact 13 kb transcribed region, that includes the transcription initiation region (encompassing the origin of transcription and promotor), the three rRNA genes and the 3' downstream termination sites. The fourth, ML-4 DNA, contained the transcribed region without the transcription initiation region. Specific regions of the upstream EcoRI C and downstream *Eco*RI D nontranscribed spacer, were also present in ML-1, ML-3 and ML-6 DNA. The upstream C region, found directly adjacent to the missing promotor, as well as part of the downstream D region of the nontranscribed spacer, was absent in ML-4 DNA.

Isolation of cloned rDNA from this library has been reported previously (Renalier *et al.*, 1989). Five cosmid clones were isolated, characterized and found to contain variant forms of complete human ribosomal genes and intact rDNA transcribed regions. One other study reported the first isolation of intact clones containing at least one full copy of the entire 44 kb repeat unit of rDNA from a yeast artificial chromosome (YAC) library (Labella and Schlessinger, 1989). This study showed that some of the YAC clones had all features characteristic of the normal rDNA repeat unit. The authors also reported that a number of other clones contained a large amount of other "non-rDNA" sequences at either one or both ends of the repeat unit. The authors suggested that the presence of this "non-rDNA" sequences could have arisen from co-cloning events that occurred during ligation to produce the YACs.

In the present study, some DNA rearrangements and deletions were found within the C and D regions of ML-4 (and ML-3) nontranscribed spacer DNA. These

were manifested in the form of deleted sequences and extra *Eco*RI, *Hind*III and *Kpn*I sites. It is assumed that these DNA changes resulted from rearrangements, deletions or insertions of DNA during cloning.

Previous studies have also reported sequence rearrangements within cloned rDNA fragments in lambda vectors (Erickson *et al.*, 1981, Respess *et al.*, 1983, Erickson and Schmickel, 1985). The exact cause of these rearrangements and why they occur is still unknown. The size of the rDNA fragment could lead to instability within the vector (Erickson *et al.*, 1981). Since some deletions have been linked to the heterogeneity of the rDNA nontranscribed spacer (Schmickel *et al.*, 1980), it has also been proposed that these deletions might be the product of normally occurring unequal homologous exchange among ribosomal genes (prior to cloning) (Erickson and Schmickel, 1985).

Further analyses of the C and D regions of the NTS are needed in order to determine the significance of these changes and whether they play a role in any of the proposed functions of the NTS. They could correspond to variable regions of discrete size heterogeneity that have been previously described (Erickson and Schmickel, 1985, Sylvester *et al.*, 1985, La Volpe *et al.*, 1985). In addition, these changes might also correspond to minor forms of rDNA units, such as the 3.7 kb portion of the C region 3' upstream from the promotor. This region represents the site where the ribosomal gene cluster begins in the first member of the tandem series and forms a junction with non-rDNA (Worton *et al.*, 1988).

The presence of repetitive DNAs (*e.g.*, *Alu* repeats) is a general feature of the nontranscribed spacer and an important characteristic of the identity of the rDNA repeat unit. Usually, members of the human *Alu*I family contain a region of homology to the origin of replication of some DNA viruses and have been proposed as origins of human DNA replication (reviewed in Doolitle, 1985). One study suggests that the repetitive DNA repeats may function both as the origin of replication of the rDNA and as target sites for the homologous recombination of the rRNA genes (Safrany and Hidgevi, 1989).

The present study shows that *Alu* repeats and surrounding DNA found in the D region of the rDNA NTS are not necessary for rDNA function. *Alu* sequences present in the internal *Hind*III fragment of the D region in the NTS were not detected in either ML-1, ML-4 DNA. The data indicate that a 4.3 kb (Dickson *et al.*, 1989) and 4.6 kb XbaI element (Safrany and Hidgevi, 1989) in the D region of the NTS containing the *Alu* repeats was missing.

The presence of *Alu* repeats in ML-1 (and ML-3 and ML-6) DNA, however, was shown in the C region just upstream of the promotor, as expected (Higuchi *et al.*, 1981, Sylvester *et al.*, 1986, Dickson *et al.*, 1989, Sylvester *et al.*, 1989, Safrany and Hidgevi, 1989). The presence of *Alu* repeats was not detected in ML-4 DNA since this region was deleted. Further research is necessary to determine if this region containing *Alu* sequences is critical for function.

III. Analyses and functions of the transfected human rDNA in human fibroblasts

Molecular analyses of transfected human rDNA in human cells is complicated due to the presence of abundant and identical amounts of endogenous rDNA. Although this presented a technical disadvantage, it was possible to overcome this problem by the use of indirect methods such as identification of vector DNA coupled with restriction patterns of the transfected DNA.

There are very few reported studies where the question of rDNA function has been addressed. In four previous studies, analyses of mammalian rDNA function was simplified by transfection of rDNA genes into cells of a related mammalian species [mouse and hamster (Dhar *et al.*, 1985), mouse and rat (Vance *et al.*, 1985), human and hamster (Dhar *et al.*, 1987) and mouse and hamster (Raziuddin *et al.*, 1989)]. These studies employed probes unique to spacer sequences in the transfected heterologous rDNA to analyze the transfected rDNA gene constructs without cross hybridizing to the endogenous rDNA.

In the above studies, a major question addressed was whether a particular rDNA gene in one species functions normally when transfected in cultured cells of a different, but related species. However, none of these studies used an entire rDNA transcribed region. Rather, they transfected smaller portions of mammalian cloned rDNA. In one study, using a 3.2 kb segment of mouse rDNA containing the promotor, the transcription initiation site and part of the external transcribed spacer that lacked 18S and 28S transcription units were integrated into hamster chromosomes (Dhar *et*

al., 1985). In a second study, 8.2 kb of the 5' mouse NTS and 5.8 kb of the mouse transcribed spacer, including parts of the 18S rDNA, were used to transfect rat cells (Vance *et al.*, 1985). Another study used the 1.2 kb human transcription initiation region (Dhar *et al.*, 1987). Finally, various plasmids containing the mouse promotor and different amounts of downstream DNA, as well as rDNA units containing deletions in the region upstream of the start site have also been tested in hamster cells (Raziuddin *et al.*, 1989).

All studies showed that the small cloned rDNA fragments were transcribed. Two of these studies also reported processing of the rRNA transcripts generated by transcription of the chromosomally integrated rDNA gene constructs (Vance *et al.*, 1985, Raziuddin *et al.*, 1989). In addition, two studies also investigated silver staining, as a means of detecting transcription of the transfected rDNA. One study showed that the chromosomal region where a transfected mouse rDNA fragment had inserted and amplified, was silver stained, indicating nucleolar organizing activity (Dhar *et al.*, 1985). In contrast, the second study reported the failure of silver staining of a chromosome region where the 1.2 kb human transcription initiation region inserted, even though it was transcribed by RNA pol II (Dhar *et al.*, 1987). The authors of the second study concluded that silver staining is restricted to rDNA that is transcribed only by RNA pol I.

Taken together, all studies, including the current one, showed that transcription of cloned mammalian rDNA occurs *in vivo* and that tandem repetition is not an

absolute requirement for rRNA gene expression. Nevertheless, complete function of full rRNA transcripts in nucleolar organization or satellite associations was not determined in the four previous studies. Only the present study examined the relationship between rDNA and these normal cellular processes.

The approach used here for determining the presence of inserted rDNA in human chromosomes was indirect. Evidence for the presence of the exogenous rDNA constructs, ML-1 and ML-4, within genomic DNA of transfected HAL cells (1K2 and 4K2) was provided by using the vector DNA as a probe. Ribosomal DNA was not a successful probe due to 100% homology to the endogenous rDNA regions. A previous study reported some cross hybridization even when a fragment of the mouse ETS was used as a probe with its counterpart in the rat, despite the advantage of using a unique spacer sequence as probe (Vance *et al.*, 1985). This finding clearly shows the difficulty encountered in working with DNA from close evolutionary relatives.

Our results showed that the site of genomic recombination in ML-1 and ML-4 DNA occurred within the vector, leaving the length of the transfected rDNA regions (especially the transcribed spacer) intact. On the basis of restriction patterns, it was then confirmed that inserted rDNA is present in the genome of 1K1 and 4K2 transfected cells.

The presence of a transcriptionally active region at the insertion site was determined on the basis of cytological tests. Other analyses, via Northerns, were also done (by Kathi McDowell) to determine if rRNA transcripts of a different size could

be recognized in the transfected 1K1 and 4K2 cells and would correspond to the transfected rDNA. This was not the case. In a second series of experiments, the vector DNA was also utilized as a probe for Northern analysis on the basis that RNA pol I could continue transcribing the vector, somehow bypassing the termination sites. 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. A second possibility is that the level of transcript from the insertion site was so low as to be undetectable (or obscured) in a Northern blot where huge amounts of the normal transcripts were observed.

Previous analyses in *Drosophila* (Tautz and Dover, 1986) and *Xenopus* (Labhardt and Reeder, 1986) have demonstrated that RNA pol I transcribes the rDNA unit up to the 5' regulatory region of the next downstream gene and beyond the 3' end of the 28S rRNA sequences where termination sites are found. A recent study reported that in mouse constructs where the rDNA inserts contained less than the full 18S rRNA sequences, longer transcripts containing vector sequences could be detected in Northern blots (Raziuddin *et al.*, 1989). The authors suggest that in other constructs where the intact 18S rDNA sequence is present, longer transcripts (into vector sequences) may be generated, but processing rapidly degrades the adjacent vector sequences. This is consistent with our results in the failure to detect altered size rRNA transcripts in either 1K1 nor 4K2 cells when vector DNA was used as a probe.

In the absence of positive results using Northerns, a cytological test, silver staining, was employed to determine transcription of ML-1 and ML-4 rDNA. Silver staining utilizes a method that identifies the presence of argyrophilic proteins in metaphase chromosomes. This technique detects rRNA clusters (NORs) that have been transcriptionally active by staining these acidic proteins involved in rDNA transcription (Goodpasture and Bloom, 1975). The silver stained chromosomal regions can be equated with the location of 18S and 28S rDNA genes (Goessens *et al.*, 1987). One study even implicates the presence of RNA pol I molecules as an attribute of silver staining (Williams *et al.*, 1982). A remnant of the nucleolus remaining attached to the NORs was suggested as the most likely interpretation of silver staining of NORs in metaphase (Schwarzacher and Wachtler, 1987).

Preliminary results from 1K1 metaphase chromosomes shows an accumulation of silver grains on the middle of the p-arm of the 3 chromosome containing the insertion site of ML-1 DNA. No significant silver grains accumulated on the q-arm of the abnormal 7 chromosome, which contains the site of ML-4 DNA integration. The findings show that the inserted rDNA is being transcribed in 1K1 cells but is not being transcribed in 4K2 cells.

Determination of transcriptional activity was also based on analysis of nucleolar DNA. The involvement of transcriptionally active rDNA in the formation of the nucleolus has been previously suggested (reviewed in Schwarzacher and Wachtler, 1987). Supporting evidence for transcriptional activity in nucleolar DNA comes from

several studies. A recent study on the functional and dynamic aspects of the mammalian nucleolus clearly demonstrated the intrinsic relationship between rDNA transcription and the formation of the nucleolus (Scheer and Benavente, 1990). The authors concluded that the primary determinants of nucleolus development and maintenance appear to be the growing transcripts, be it the pre-rRNA transcript molecules themselves or specific proteins bound to their 5' termini. Their conclusions were based on: a) microinjection of antibodies to RNA pol I, which suppressed rDNA transcription resulting in the failure of the early steps of nucleolar reconstitution; b) the presence of nascent rRNA transcripts which interacted with a nucleolar protein (fibrillarin) which is involved in nucleologenesis and maintenance of interphase nucleoli and c) the presence of micronucleated cells lacking NORs which were unable to organize nucleoli.

Another study showed directly that a single rDNA repeat unit is sufficient to organize a nucleolus in *Drosophila* (Karpen *et al.*, 1988). The authors demonstrated, by using P-element-mediated transformation of a single rDNA repeat unit, that it can be transcribed at a high rate when inserted into chromosomal sites other than the NOR. It is noteworthy to mention that the authors exploited the spatial resolution allowed by polytene chromosomes which enabled them to analyze insert function without marking the gene with foreign (vector) sequences as well as visualizing directly nucleolar material. Thus, they were able to make a test for nucleolar function possible and to overcome the technical difficulties encountered in previous studies that

used interspecies DNA. In addition, results showed that structures resembling the morphology of endogenous nucleoli, "mininucleoli" were associated with the chromosomal regions of the exogenous rDNA insertion. The mininucleoli were shown to contained both rRNA and an antigen specific to nucleoli by molecular analyses. The authors concluded that the two conserved features of rDNA organization in eukaryotes, tandem repetition and heterochromatic localization, are not required for rRNA gene function and NOR activity is an intrinsic property of the rDNA or its RNA products.

The findings obtained from the current investigation concur fully with results obtained from the *Drosophila* study (Karpen *et al.*, 1988). Transfected ML-1 rDNA formed an NOR that is transcriptionally active. In contrast, ML-4 rDNA which lacked a promotor was not present in the nucleolus. More importantly, this study as well as the *Drosophila* study (Karpen *et al.*, 1988), clearly demonstrate that tandem repetition is not an absolute requirement for rRNA gene expression since transfected rDNA was transcribed and functioned in processes normally involving hundreds of copies of endogenous tandem rDNA repeats. It has been previously suggested that tandem repetition of rRNA genes may play a role in optimizing rDNA transcription (rather then participating in rRNA gene expression) and thus could contribute to the regulation of rDNA function in cells subject to environmental and developmental stresses (reviewed in Baker and Platt, 1986; Reeder *et al.*, 1987).

A final aspect of the rDNA-bearing acrocentric chromosomes of many species is their participation in satellite associations in metaphase. These associations could

lead to abnormal segregation or exchange by failure to dissociate resulting in chromosomal anomalies. Satellite associations were suggested as one possible causative factor in both nondisjunction and Robertsonian translocation (reviewed in Jacobs *et al.*, 1976). The 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 *et al.*, 1976).

The exogenous ML-1 rDNA-bearing chromosome 3 was shown to participate in satellite associations with other acrocentric chromosomes at a level of 9% above background when compared to other non-acrocentric chromosomes of 1K1 cells. In contrast, the ML-4 rDNA-bearing abnormal 7 chromosome did not participate in satellite associations with other acrocentric chromosomes of 4K2 cells. The results definitively show that rDNA presence in the nucleolus is the causative factor of satellite associations among rDNA-bearing chromosomes. It is concluded that the presence of the promotor together with the entire transcribed spacer which includes the remainder of the transcription initiation region and the three rRNA genes and their transcription, represent the main factors in the process of satellite associations.

IV. Perspectives

The detailed and complex mechanisms involved in the relationship between NORs and organization and maintenance of the nucleolus and resultant satellite association still remain partly unknown. A future detailed analytical continuation of the 1K1 and 4K2 transfected cell lines is necessary to elucidate more specifically the

processes and specific sequences responsible for rDNA function with respect to nucleolar formation, satellite association and chromosomal anomalies. The availability of large intact regions of the human rDNA repeat unit provides the opportunity to further identify various aspects of rDNA function in view of special sequences using *in vitro* and even additional *in vivo* studies. Further molecular and cytological analysis can be used to analyze data obtain from the creation of point mutations within the transcribed region, especially the promotor, in order to determine whether any special segment of the transcribed 13 kb sequences (or other sequences identified) are solely responsible for rDNA function in nucleolar formation and satellite associations.

APPENDIX

Other experiments have been carried out. One approach was to test whether the promotor alone with the 18S rRNA gene can be transcribed and participate in nucleolar formation and satellite associations. The 6.0 kb rDNA *Eco*RI B region was subcloned into vector pSV2-neo. This region contains the entire 1.2 kb transcription initiation region (encompassing the origin of replication and promotor) and the 18S rRNA gene up to the last 200 bp. Two clones were selected for further analyses and were designated T-1 and T-4. Their schematic representation is shown in Figure 19 and 20.

Restriction mapping and Southern and Northern analyses were used to determine the presence and orientation of the insert (data not shown). Southern analysis confirmed the results obtained from the restriction analysis indicating the presence and orientation of each insert. Northern analysis also showed that both T-1 and T-4 hybridized very strongly to 18S rRNA and some intermediates of rRNA processing. It was found that both contain the entire intact insert, one in an inverted 3'-5' orientation (T-1), the other in the correct 5'-3' orientation (T-4).

Both T-1 and T-2 were transfected in human fibroblasts using lipofection (by Kathi McDowell). One clone from each transfection was selected for further analysis following selection in the presence of G418. The transfected cloned cells were designated 1T2 and 4T2, respectively. Restriction and Southern analyses of genomic DNA digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III and *Pst*I from these transfected cells

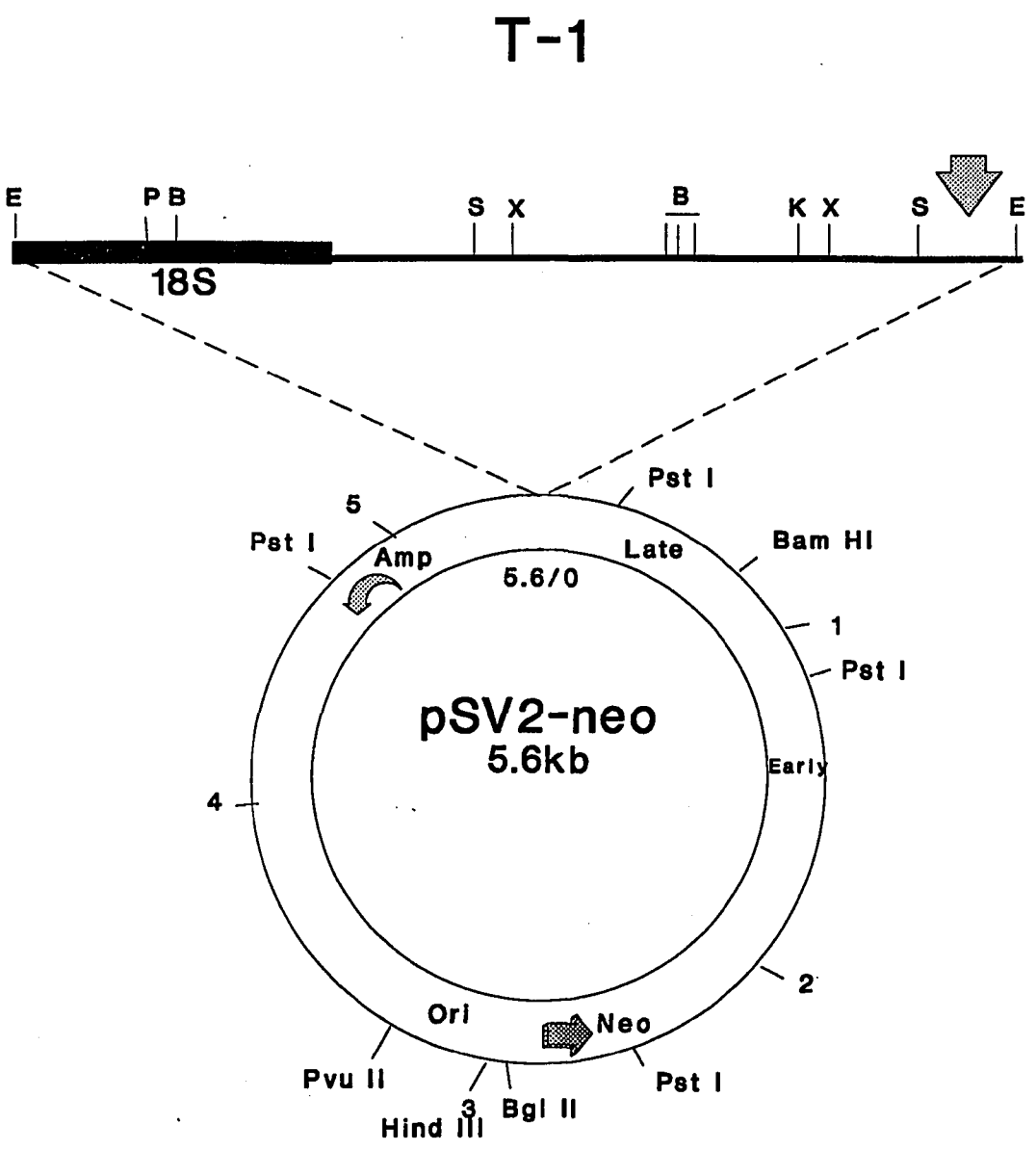

Figure 19. Schematic map of *Eco*RI B rDNA-containing plasmid, T-1.

Schematic map of T-1 DNA showing the plasmid vector, pSV2-neo, with its *Eco*RI B rDNA insert. The insert region defined by the downward arrow indicates the start site of rDNA transcription (promotor) which continues through the 18S rRNA sequences, shown shaded. The cloned rDNA fragment is found in an inverted 3'-5' orientation. Restriction endonuclease sites within the rDNA insert appear as *Bam*HI (B), *Eco*RI (E), *Kpn*I (K), *Pst*I (P), *Sal*I (S) and *Xho*I (X).

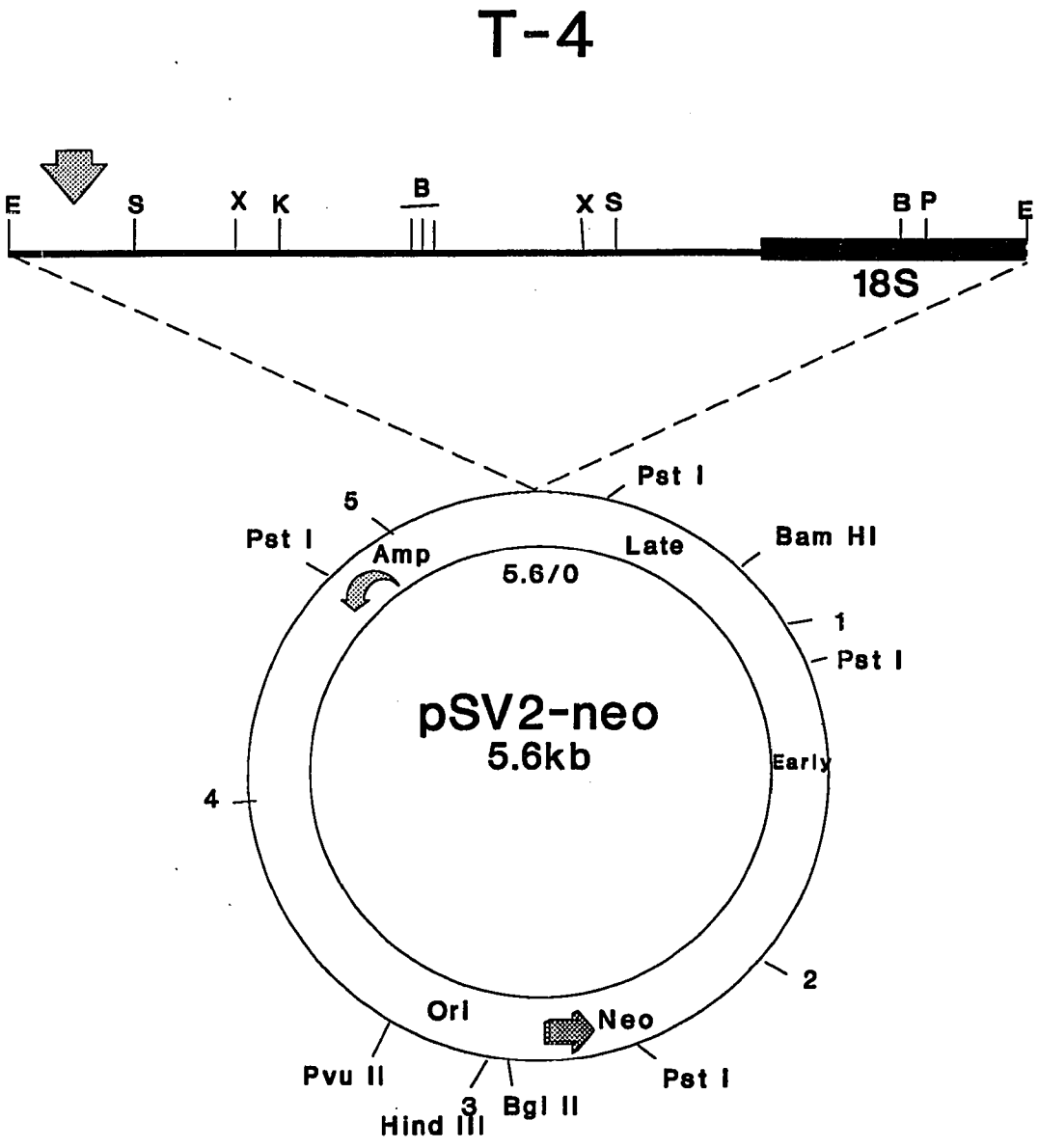

Figure 20. Schematic map of *Eco*RI B rDNA-containing plasmid, T-4.

Schematic map of T-4 DNA showing the plasmid vector, pSV2-neo, with its *Eco*RI B rDNA insert. The insert region defined by the downward arrow indicates the start site of rDNA transcription (promotor) which continues through the 18S rRNA sequences, shown shaded. The cloned rDNA fragment is found in the correct 5'-3' orientation. Restriction endonuclease sites within the rDNA insert appear as *Bam*HI (B), *Eco*RI (E), *Kpn*I (K), *Pst*I (P), *Sal*I (S) and *Xho*I (X).

determined the presence, copy number and site of recombination within the transfected DNA (data not shown).

Based on the signal intensity and restriction patterns obtained by results, it was shown that two copies of T-1 DNA integrated in 1T2 cells. The site of recombination within the transfected T-1 DNA in the genome occurred between the *Pst*I site present in the 18S rRNA gene and the end of the B region in the first copy and between the *Hind*III and fourth *Pst*I site within the vector in the second copy (Figure 19). One copy T-4 was found in 4T2 cells. The site of recombination within the transfected T-4 DNA occurred between the *Bam*HI site present in the 18S rRNA gene and the end of the B region (Figure 20).

Critical to the original hypothesis is whether or not the *Eco*RI B region is participating in nucleolar formation. As mentioned previously, one criterion for testing the hypothesis is the presence of vector DNA in the nucleolar DNA. Results showed this to be the case, but further research is necessary for confirmation. T-1 and T-4 vector DNA was present in same amounts in both non-nucleolar and nucleolar DNA. There is a high possibility that some contamination of nucleolar DNA with non-nucleolar DNA occurred during the extraction procedure, thus resulting in the ambiguous results. Additional experiments should be done to determine whether the transcribed rDNA displays any NOR activity.

It will also be very interesting to map the chromosomal locations of the transfected T-1 and T-4 *Eco*RI B region and to determine whether it is transcribed (by

Northern blots and silver staining), and/or participates in satellite associations. In addition, further molecular analyses in the context of mutations within the B region followed by transfection into human cells should shed more light on the intrinsic relationship between rDNA promotor function and NOR activity.

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